COMBINING BACTERIAL PATHOGENS WITH *Beauveria bassiana* TO IMPROVE HOUSE FLY (*Musca domestica*) MANAGEMENT

By

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To my boyfriend and committee for their unending support

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The common house fly (*Musca domestica* L.) is a widely distributed, non-biting pest that poses a public health and agricultural concern. Their high fecundity and short development time can rapidly lead to overwhelming population sizes, especially in animal production units. Unfortunately, house flies are resistant to most insecticides used today, making them a difficult synanthropic pest to control. The use of biological control has been increasingly popular due to low environmental impact and deserves more investigation, especially entomopathogenic fungi like, *Beauveria bassiana*. As *B. bassiana* can take >7 days to kill house flies, this study aimed to reduce the lethal time (LT) by combining gram-negative bacteria with *B. bassiana* to enhance virulence. The three bacterial strains chosen for investigation were *Photorhabdus temperata* NC19, *Serratia marcescens* Db11, and *Pseudomonas protegens* pf5. The overall objective of this study was to apply compatible bacteria with *B. bassiana* to LT_{90} in adult house flies. Preliminary compatibility studies were performed, as well as selection of an adequate pathogen surfactant. Topical treatments of combined pathogens were applied to the house fly thorax. No significant reduction in LT_{90} was recorded in *B. bassiana* combined with *S. marcescens* or *P. temperata*, however, significant reduction in LT_{90} was observed when *P. protegens* and *B.*

bassiana were combined, indicating a complementary action. These findings suggest that the addition of *P. protegens* can enhance effectiveness of *B. bassiana*. Improvements in house fly management can increase animal health and welfare, and improve human comfort in residential areas neighboring animal facilities.

CHAPTER 1
LITERATURE REVIEW OF COMPLIMENTARY INTERACTION BETWEEN A BACTERIAL AND A FUNGAL PATHOGEN TO IMPROVE HOUSE FLY (*Musca domestica*) MANAGEMENT

The common house fly, *Musca domestica* L., is a well-known worldwide pest belonging to the order Diptera (Family: Muscidae). Because of the fly's close frequent association with animals and humans, they also are referred to as "filth flies", along with several other filth breeding fly species, and commonly are seen around manure, decaying plant material and other substrates where microorganisms are abundant.

Importance to Livestock and Public Health

Since these flies have a close relationship with humans, domestic animals, and livestock, they pose a public health and agricultural concern (West, 1951). House flies can develop and breed in a variety of substrates associated with animals. Intensive animal production facilities are a prime location for fly breeding because of the large quantities of manure, soiled bedding, decaying feeds, and other substrates common on such facilities. Flies can be managed through an integrated pest management (IPM) system with a focus on sanitation, but this is often poorly implemented and so not always successful. House flies are prolific breeders and even modest amounts of breeding material can quickly lead to overwhelming populations. High fly populations can irritate the animals and the people who work with them. Axtell (1986, 1999) examined fly populations on poultry farms and discussed the decrease in egg production in hens due to increased stress, associated with nuisance fly behaviors. Similarly, a high density of *M. domestica* on dairy farms has been shown to reduce milk production in dairy cows because of house fly annoyance (reviewed by Malik et al. 2007).

In addition to being an irritant, *M. domestica* has been shown to be a mechanical vector for many pathogenic organisms including the causal agents of bacterial, viral, protozoan, and helminthic infections to humans and other animals (Sasaki et al. 2000, Shono et al. 2003). Past studies demonstrated that flies could transmit pathogens to mucous membranes (Kieding 1986). One study found an isolated *Campylobacter fetus* subsp. *jejuni* strain being carried by *M. domestica* from porcine and poultry farms to surfaces touched by humans, inferring that *Campylobacter,* which causes human enteritis, could be transferred from livestock facilities to human food by house flies (Rosef and Kapperud 1983). Graczyk et al. (2001) observed the transport of a pathogen, *Cryptosporidium parvum,* which causes human gastroenteritis, in bovine feces onto surfaces by *M. domestica.* Macovei et al. (2008) reported that flies from cattle feedlots transmitted antibiotic-resistant enterococci to ready-to-eat human food. In a recent review, Zurek and Ghosh (2014) conclude that flies are important in the movement of antibiotic-resistant pathogens of many kinds between animal production units and nearby human populations. Flies have been implicated as carriers of *Salmonella enterica* and enterohemorrhagic strains of *Escherichia coli* to fresh produce such as spinach and lettuce (Wasala et al. 2013, Pace et al. 2017). A recent special issue of the Annals of the Entomological Society of America summarizes the status of the role of filth flies in disease transmission (Nayduch and Burrus 2017).

Life Cycle

House flies are holometabolous and prolific breeders that can utilize a wide variety of foods to their advantage including human and animal waste. Adults emerge from puparia and begin mating one day after emergence. Mating can be facilitated by the pheromone Z-9-tricosene (Carlson et al. 1971), and also involves a detailed courtship

ritual that prevents copulation with other related species (Tobin and Stoffolano 1973a,b). Fletcher et al. (1990) stated that female house flies can produce 75-200 eggs during their lifetime, but estimates of lifelong fecundity have been reported to be as high as ~1000 eggs (LaBrecque et al. 1972). The larvae feed on microorganisms present in a wide variety of substrates including manure, silage, wet feed, and decomposing vegetation (Nayduch and Burrus 2017). Mature larvae generally move out of the habitat and seek dry and protected sites to pupate. The life cycle, behavior and density is dependent on environmental resources and temperature. When conditions are favorable and the temperature is around 30° C, house flies can complete their life cycle in approximately ten days, and the cooler the temperature the longer the life cycle takes to complete (Stafford 2008). Adults can fly a significant distance searching for food and oviposition substrates (Sacca 1963), which is why it is common for residential areas that are near animal rearing facilities or any other favorable breeding resources to experience fly problems (Winpisinger et al. 2005). Under field conditions, average longevity is generally 5-10 days, but in the laboratory, females can survive upwards of two months (West 1951).

Adult Morphology

House flies are roughly 6-8 mm in length, with females usually larger than the males, and have four dark stripes on a grey thorax (West 1951, Chapman 1998). The abdomen of the female house fly has nine segments, with the first five segments noticeable and the last four retracted until the female oviposits (West 1951, Chapman 1998). In contrast, male abdomens contain only eight segments, with the eighth segment having a darkened end (West 1951). Adult house flies are non-biting and have modified sponging mouthparts that allow them to regurgitate crop and salivary gland secretions

onto solid food to liquefy it so that it can be ingested (West 1951, Graczyk et al. 2001). The tarsi have setae (i.e. sensory hairs) for assessing food quality and a sack-like pad, the pulvillus, which produces a tacky residue that enables house flies to climb on vertical and smooth surfaces.

Current Management Techniques

The use of chemical insecticides to control *M. domestica* has long been the standard management tactic. However, house flies have developed insecticide resistance over time, a phenomenon that was first documented after extensive use of dichlorodiphenyltrichloroethane (DDT) for fly control (Varzandeh et al. 1954). During the ensuing years, the house fly has shown a remarkable ability to develop resistance to new classes of insecticides, often within a few years of their introduction to the market (Boxler & Campbell 1983, Plapp 1984, Scott & Georghiou 1986, Scott et al. 1989, Kaufman et al. 2001b, Butler et al. 2007, Kozaki et al. 2009, Kaufman et al. 2010, Memmi 2010, Scott et al. 2013, Shah et al. 2015). A neonicotinoid, Imidacloprid, was used on a Florida strain of house flies, FDm, to evaluate the level of resistance. After five selections, they observed a 331-fold increased resistance to imidacloprid compared to the parental FDm population (Kaufman et al. 2010). Insecticide resistance can take a variety of forms and result from behavioral resistance, reduced penetration of the cuticle, target site insensitivity, neurotransmitter inhibition, or a number of metabolic detoxification mechanisms (Hemingway and Ranson 1990). Resistance of house flies to synthetic pyrethroid insecticides has been particularly well studied. When permethrin was first introduced for fly management in ca. 1980 it was widely welcomed for its efficacy, long residual life, and low toxicity compared to the organophosphates that had previously been the mainstay of fly control (Scott 2017). Widespread use of and reliance

on permethrin products led to the rapid development of resistance throughout the U.S. (Scott et al. 1989, Kaufman et al. 2001b, 2010, Scott et al. 2013). Metabolic resistance to permethrin is complex and involves multiple alleles affecting voltage-sensitive sodium channel (*Vssc*) mutations and enhanced oxidative detoxification via cytochrome P450 (Scott 2017).

Insecticide resistance has driven the search for alternative methods to control persistent populations of house flies. These alternatives include manure management, sticky traps, light traps, oviposition traps, baited traps, biological control (discussed below) and other technologies that do not incorporate conventional insecticides. An example of new tools for fly management are insect growth regulators (IGR's). IGR's are typically hormones or analogs of hormones that when applied prevent the insect from developing successfully to the adult reproducing stage. However, despite being somewhat "natural" these compounds are not immune to the house fly's ability to develop resistance. A juvenile hormone mimic, pyriproxyfen, was utilized on a poultry farm strain (PYR) of larval *M. domestica* that were already 25.70-fold resistant to the active ingredient. After 22 generations of laboratory selection by repeated exposure to pyriproxyfen, resistance in the PYR strain developed 130-fold compared to a susceptible house fly strain (Shah et al. 2015). Similarly, flies developed very high levels of resistance to the IGR cyromazine shortly after it became widely used as a feed-through product for fly control on caged-lager (egg producing) poultry farms in the 1980s, and there was evidence of cross-resistance to another IGR (diflubenzuron) (Sheppard et al. 1989, Shen and Plapp 1990).

Biological Control of House Flies

In addition to concerns about low efficacy due to insecticide resistance, public concern about food and worker safety because of insecticides has caused controversy and is the driving force behind the growth in the organic segment of modern agriculture. These concerns have spurred interest in the use of biological control methods for some time, as biological control is perceived by the public as a "safer" approach that can minimize potential harm to the environment and human health. Researchers have investigated many avenues for biological control of house flies, although because of the biology of *M. domestica*, most literature focuses on the immature life stages, where predators attack egg and larval stages and parasitoids prey upon pupae. Adult flies are susceptible to microbial infections but this approach has received comparatively less attention. The use of predators, parasitoids, and botanicals, as well as fungal, bacterial, and viral pathogens have all shown efficacy in controlling this pest under certain situations (reviewed by Malik et al. 2007). Although all of these agents have shown some promise in controlling *M. domestica*, each has certain limitations that hinder their use for successful management in the field. Overviews of the principal biological control candidates for house flies are presented below.

Predators

The most important natural enemies of eggs and young fly larvae are beetle and mite predators, especially the histerid beetle *Carcinops pumilio* (Erichson; Coleoptera: Histeridae), and the mite *Macrocheles muscaedomesticae* (Scopoli; Mesostigmata: Macrochelidae). *Macrocheles muscaedomesticae* live in poultry, pig, sheep and cattle manure, which are typical house fly breeding sites, and the female adults prey on fly eggs and newly emerged larvae (reviewed by Geden 1990, 2006). This mite has a 2-3 day

development time and population sizes can fluctuate extensively based on environmental conditions and prey availability. At this time, *M. muscaedomesticae* are not available commercially, perhaps because rearing in the laboratory is difficult due to the need for nematode prey (reviewed by Geden 2006). *Carcinops pumilio* is the most important and common coleopteran predator of filth flies (reviewed by Geden 1990). They reside mostly in poultry manure, but also commonly are found in calf bedding. *Carcinops pumilio* prey on fly eggs starting in their second larval instar and continue to do so throughout their motile life stages. Development time is approximately three weeks, with an adult life span of as long as two years. *Carninops pumilio* can be reared on house flies in laboratory conditions, however, it is difficult and expensive due to cannibalistic behavior and lengthy development time (Geden 1990, 2006). Conservation of natural *C. pumilio* populations is possible with cultural manure management practices (Geden and Stoffolano 1988, Kaufman et al. 2002, Hinton and Moon 2003).

Parasitoids

The use of pupal parasitoids, especially those in the Pteromalidae (Order: Hymenoptera), has been considered a viable biological control option since they were used to successfully manage flies under field conditions 40 years ago (Morgan et al. 1975, 1976). The literature on fly parasitoids is voluminous and has been reviewed several times (Patterson et al. 1981, Patterson and Rutz 1986, Rutz and Patterson 1990, Legner 1995, Geden and Hogsette 2001, Geden 2006, Machtinger et al. 2015, Machtinger and Geden 2017). There are about a dozen common parasitoid species, several of which are available to end-users through commercial insectaries. Parasitoid releases have been effective in many instances (Rutz and Axtell 1979, Morgan and Patterson 1990, Geden et al. 1992, Petersen et al. 1992, Petersen and Cawthra 1995, Crespo et al. 1998, Geden and

Hogsette 1996) and unsuccessful in others (Meyer et al. 1990, Andress and Campbell 1994, Weinzierl and Jones 1998, McKay and Galloway 1999, Kaufman et al. 2001a). Selection of the appropriate parasitoids species for fly management remains an inexact science. The different parasitoid species differ in their development times, foraging behavior, attack rates, host preferences and distances traveled to locate pupae (Machtinger et al. 2015). The two most commonly used species are *Muscidifurax raptor* (Girault and Sanders) and *Spalangia cameroni* (Perkins). *Muscidifurax raptor* is known for its high attack rate, relatively short (~14 day) development time and success with parasitizing pupae at the surface. However, *M. raptor* struggles with locating buried pupae and is sensitive to insecticides (Geden 1996, 1997, 1999, 2002). In comparison, *S. cameroni* has a lower attack rate, longer development time, less sensitivity to pesticides, and is capable of locating buried pupae (Geden 1996, 1997, 1999, 2002). Releases of multiple species can expand the niche breadth covered by biological control efforts, but competitive interactions among species can limit the effectiveness of releasing multiple species (Geden et al. 2014).

Pathogens

Entomopathogenic nematodes (EPN's) in the families Steinernematidae and Heterorhabditiae (Order: Rhabditida) have a broad host range and have been studied extensively for management of a wide variety of arthropod pests (Gaugler and Kaya 1990, Gaugler 2002, Georgis et al. 2006, Poinar and Grewal 2012). An interesting aspect of their biology is that EPN's carry symbiotic bacteria that are largely responsible for the host death (Akhurst and Boemare 1990, Forst et al. 1997, Boemare 2002). EPN's have shown mixed results with *M. domestica* control. Laboratory studies have revealed that *M. domestica* larvae had a high susceptibility to most EPN's, but when experiments were

performed on natural fly breeding substrates, such as manure efficacy was disappointing (reviewed in Georgis et al. 2006, Geden 2012). These nematodes survive better in sandy soils, which allow better movement and oxygen quality, and perhaps cannot survive well in poultry and pig manure. Unfortunately, manure and moistened substrates are common and favorable for *M. domestica* oviposition and breeding sites, thus making nematodes not an ideal house fly biological control method. Adult flies are less susceptible to nematodes than larvae, perhaps because of their reluctance to feed on solutions containing motile parasites. For the purpose of this study, we are focusing on the control of the adult house fly, and as commercial nematodes target the larvae they would not be suitable. However, the bacterial symbionts of EPNs could be used against adult flies if a method can be developed to deliver them into the fly hemocoel. One of the objectives of this research is to evaluate delivery methods of entomopathogens into the fly hemocoel. This could be key to the use of EPN symbionts for fly control as the symbiotic bacteria lack the means to gain entry to the fly by themselves.

Salivary gland hypertrophy virus (SGHV) is a double stranded DNA virus that is commonly found on dairy farms in Florida (Coler et al. 1993). Lietze et al. (2011) discovered that SGHV is detrimental to females by causing hypertrophy of the salivary glands leading to undeveloped ovaries (reviewed by Lietze et al. 2011). Infected flies release millions of virus particles whenever they feed, suggesting that the most likely transmission route is oral (Lietze et al. 2009). However, per os (i.e. by mouth) infection rates are low because of the barrier presented by the peritrophic matrix (PM) (Boucias et al. 2015). This has limited the development of SGHV into a bait for house fly management.

Entomopathogenic fungi research has shown promising results in recent years. *Entomophthora muscae* (Cohn) Fresen. (Entomophthoraceae) is known to kill flies in 4-6 days once the flies have been exposed to spores (Pinnock and Mullens 2007, Geden 2012). This fungus spreads due to uninfected fly activity on and around infected cadavers; flies are exposed during conidial expulsion (Geden et al. 1993). The success of this fungus is relative to the density of the fly population and the fly's ability to spread conidia to one another (Geden et al. 1993). *Entomophthora muscae* is difficult to propagate outside of live *M. domestica*, and currently the conidia cannot be stockpiled under normal storage conditions for use in management. Other options for entomopathogenic fungi are *Metarhizium anisopliae* (Metschn.) Sorokin (Clavicipitaceae) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Cordycipitaceae). Both species have a wide host range and have long been the subject of intense research for management of a myriad of pest species (Roberts and Hajek 1992, Shah and Pell 2002, Lacey et al. 2015).

Although some strains of *M. anisopliae* have been tested with some success against house flies (Renn et al. 1999, Mishra et al. 2011, Acharya et al. 2015), to the author's knowledge this species has never been recovered from field-collected house flies, whereas *B. bassiana* has frequently been found naturally infecting flies (Steinkraus et al. 1990, Geden et al. 1995, Skovgärd and Steenberg 2002). For an entomopathogenic fungus to be effective in the field it needs to have a high germination rate, cause high mortality and result in high sporulation to transmit the infection. The possession of these desirable qualities can vary a lot by species and isolate. One study found that *B. bassiana* strain BNBCRC had the highest germination and caused the highest mortality (80%) on

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) (72%) but the lowest conidial yield compared to other strains of *B. bassiana* and *M. anisopliae* (Petlamul and Prasertsan 2012). Although, in that study, all strains were found to be virulent, the problem lies, as with other entomopathogenic fungi, with the time to 100% mortality. The average time to death of *S. litura* larvae was six days for *B. bassiana* and longer for *M. anisopliae* (Petlamul and Prasertsan 2012). In the case of adult house flies, the average time to death is also ~ six days, although mortality rates are affected by isolate and method of delivery (Geden et al. 1995, Lecuona et al. 2005, Mwanburi et al. 2010).

Beauveria bassiana is an anamorphic entomopathogenic fungi that is ubiquitous. It is commonly found in soil and has been reported to infect hundreds of insect species in nature (Lipa 1963). Similar to conventional insecticides, the mode of action of *B. bassiana* is first through contact with the insect pest, however, in the case of fungi-host, contact occurs with spores or conidia (Barbarin et al. 2012). Conidia adhere to the insect cuticle due to their surface hydrophobic proteins (Hyd1 and Hyd2) that contribute to *B. bassiana* virulence (Ortiz-Urquiza and Keyhani 2013). Once attached, germination occurs, where the appressorium is driven into the cuticle assisted by secreted hydrolytic enzymes, e.g. chitinases, proteases, and lipases (Ortiz-Urquiza and Keyhani 2013). Eventually, *B. bassiana* reaches the hemocoel of the insect causing mortality by a combination of actions including invading organs, circulating poisonous metabolites, and depletion of vital nutrients (Chamley 1989, Ferron 1981, Inglis et al. 2001).

Barriers to Fungal Infection

House flies are attracted to putrefying environments, and they have evolved an efficient immune system to ward off the many microbes to which they are constantly exposed (Malik et al. 2007, Nayduch and Joyner 2013). The hard exoskeleton protects

flies from microorganisms penetrating the cuticle and reaching the hemocoel. Insect cuticle contains different components that aid in its strength, e.g. waxes, hydrocarbons, lipids, chitin, and sclerotized proteins (Ortiz-Urquiza and Keyhani 2013). The cuticle may also contain antifungal compounds such as benzoquinone oxidoreductase that inhibit germination (Pedrini et al. 2013). Insect-fungal metabolic interactions are complex and have been described as an "arms race" between insects and the pathogen that try to overcome them (Singh et al. 2016). In addition, flies have grooming behaviors to remove foreign substances from their body (Ortiz-Urquiza and Keyhani 2013, Jacques et al. 2017). If a pathogen enters a fly via ingestion, it must endure the rigors of digestion, the fly immune system, as well as physical barriers. When a pathogen is ingested, it is first stored in the cuticle-lined crop, then regurgitated before being transferred to the midgut (Nayduch and Joyner 2013). The midgut is lined with a PM that ends at the midgut-hindgut juncture, where the hindgut is again protected by cuticle. The PM is impermeable to most ingested bacteria and it secretes lytic digestive enzymes and antimicrobial agents that limit pathogen entrance (Joyner et al. 2013, Nayduch and Joyner 2013). House flies have an immune system consisting of cellular and humoral innate factors that contribute to the identification of microorganisms by utilizing pattern recognition receptors (PRR's), as well as killing microorganisms by encapsulation, phagocytosis, and/or the production of antimicrobials (Mishra et al. 2012, Nayduch and Burrus 2017).

The immune system of the house fly is the main reason why biological control techniques, such as entomopathogenic fungi do not work as desired, and the time taken to kill is extended. Given that *B. bassiana* is effective at breaching the fly's defenses, it is

reasonable to wonder whether it could be combined with other entomopathogens that are faster in action but without the means to penetrate the fly cuticle, which would be carried into the hemocoel by the fungus potentially causing a shortened time to death.

Research Objectives

As mentioned above, *B. bassiana* typically takes approximately six days to kill house fly hosts. However, there is indirect evidence that the time to death is shorter in microbe-rich environments. Kaufman et al. (2005) noted that although *B. bassiana* seemed to cause fly population reductions in poultry houses, very few cadavers from the treated houses produced conidia because fly cadavers were overwhelmed by bacteria in this highly septic environment. A company that produces a commercial *B. bassiana* product targeted at fly pests has long maintained that efficacy is improved in "dirty" environments, because the fungus provides a point of entry for opportunistic bacterial pathogens in the environment (Jim Arends, personal communication). The objectives of this research explored avenues for a multi-dimensional method of *M. domestica* biological control. These objectives were:

1. To investigate the compatibility of *Beauveria bassiana* with three bacteria species (i.e. *Serratia marcescens* Db11, *Photorhabdus temperata* NC19, and *Pseudomonas protegens* Pf-5)
2. To determine a suitable carrier for topical application of both fungi and bacteria to *M. domestica*
3. To quantify the effectiveness of topical applications of *M. domestica* with pathogens that are individual, combined, and sequentially applied

CHAPTER 2
COMPATIBILITY OF BACTERIA WITH *Beauveria bassiana*

Introduction

A study in 2014 discovered that the combination of *Bacillus subtilis* and *B. bassiana* reduced fruit borer attack on tomato plants in an additive manner compared with either pathogen alone (nukarthikeyan et al. 2014). Similarly, Lednev et al. (2008) reported an additive effect of *B. bassiana* with *Pseudomonas* sp. against migratory locusts. *Beauveria bassiana* and *Pseudomonas* sp. also were observed to act synergistically against leafminers, and the activity was further promoted by incorporating chitin into the formulation (Senthilraja et al. 2010). Wraight and Ramos (2005) observed additive effects of *B. bassiana* and *Bacillus thuringiensis tenebrionis* against the Colorado potato beetle, and Mwamburi et al. (2009) found evidence for additivity, if not synergy, between *B. bassiana* and *B. thuringiensis* var. *israelensis* against house fly larvae. They found that combining these two pathogens had an additive effect and significantly reduced adult house fly emergence in a poultry facility. These results suggest that combination products of *B. bassiana* with bacterial pathogens warrant closer study. The overall objective of this master's research was to examine the combination of *B. bassiana* with three gram-negative entomopathogens with the goal of reducing the time to death of treated house flies beyond that which occurs due to *B. bassiana* infection alone. In developing a combination product with bacterial entomopathogens, the three bacteria evaluated, which were all in the class Gammaproteobacteria, were *Serratia marcescens*, *Photorhabdus temperata*, and *Pseudomonas protegens.*

Serratia marcescens (Bizio) was discovered in 1819 and was originally thought to be a stemless fungus (Mahlen 2011). It is reported to contain flagella and believed to be motile with cells that divide longitudinally by fission (Mahlen 2011). *Serratia* is a ubiquitous genus of gram-negative bacteria belonging to the family Enterobacteriaceae, with roughly 14 species including two subspecies (Mahlen 2011). *Serratia marcescens* is a common laboratory contaminant and has been found to be a natural bioinsecticide (Pineda-Castellanos et al. 2015). This bacterium inhabits soil and water and some strains are pathogenic to insects, whereas others are pathogenic to humans and other mammals. It displays virulence in approximately 70 species of insects including wasp, termite, grasshopper and fly species (Grimont et al. 1979). In addition, it has been isolated from more than 30 different insect species and is a common contaminant in insect colonies (Grimont et al. 1979, Flyg et al. 1980).

The use of *S. marcescens* has been shown to be a potential alternative to chemical insecticides in some instances (Pineda-Castellanos et al. 2015). *Serratia marcescens* is well known for its mobility and its capacity to secrete a multitude of virulence factors, such as proteases and chitinases, making it a promising pathogen for biological control of targeted insects (Grimont et al. 2006). The bacterium is documented to suppress insect host immunity by manipulating immunosurveillance cells (Ishii et al. 2014, Stout 2015). A recent study described the protease serralysin as one of the causes of high fatality in larvae of *Phyllophaga blanchardi* (Arrow; Coleoptera: Melolonthidae) when orally inoculated with *S. marcescens* (Pineda-Castellanos et al. 2015). Similarly, an endochitinase gene isolated from *S. marcescens* and expressed in *Escherichia coli* enhanced the activity of recombinant *Bacillus thuringiensis* δ-endotoxin CryIC against

larvae of *Spodoptera littoralis* (Boisduval; Lepidoptera: Noctuidae) by significantly decreasing the lethal dose to 50% mortality (LD_{50}) values (Regev et al. 1996, Sampson and Gooday 1998). In preliminary studies, *S. marcescens* was injected into adult female house flies in a range of doses and the lethal time to 50% (LT_{50}) was less than 12 hours, a lethal dose to 50% (LD_{50}) could not be calculated because a single cell appeared to be sufficient to kill the fly (Johnson, unpublished data).

Photorhabdus temperata Fischer-Le Saux et al., is a gram-negative, bioluminescent, motile, entomopathogenic bacterium belonging to the family Enterobacteriaceae (Wollenberg et al. 2016). This bacterium has a symbiotic relationship with entomopathogenic heterorhabditid nematodes. The nematode actively seeks out insect hosts while the bacterium resides in the gut of the nematode protected from environmental harm (Hurst et al. 2015). Once the EPN releases the bacterium into the hemocoel of the insect host, bacterial virulence factors such as proteases, chitinases, and toxins act quickly to cause host death (Duchaud et al. 2003). This bacterium has two phases, one where it is pathogenic to insects and the other where it is symbiotic with EPNs (Forst et al. 1997). Together, the nematode and its symbiotic bacteria are pathogenic to a wide range of insects (Duchaud et al. 2003).

Although EPNs and their associated symbiotic bacteria have shown success together as a bioinsecticide, the bacteria are the direct cause of insect mortality. It has been documented that *Photorhabdus* spp. cause immunosuppression in *Spodoptera exigura* Hübner (Lepidoptera: Noctuidae) by inhibiting phospholipase, one of the enzymes responsible for modulating insect immune function (Jung and Kim 2006). Other studies have found *P. temperata* in the absence of its host EPN to have potential

for future bioinsecticidal use. For instance, Shrestha and Kim (2010) found 100% mortality in larvae of *Tribolium castaneum* (Herbst, Coleoptera: Tenebionidae) 48 hours post injection of *P. temperata*. In addition, some studies have discovered combining *P. temperata* with another microbial agent having potential as an alternative biological control method. For example, Jung and Kim (2006) found a synergistic interaction between *P. temperata* and *Bacillus thuringiensis aizawai* when the two pathogens were fed to *S. exigua* larvae, causing high mortality.

Pseudomonas spp. are gram-negative bacteria in the family Pseudomonadaceae with more than 100 species distinguished (Palleroni 2003). They have a highly diverse metabolism and include plant and human pathogens, remediation agents for chemical pollutants, and plant-protecting species with antifungal properties. One member, *P. protegens* Ramette et al., is capable of producing a range of plant defending products such as antibiotic metabolites, chitinases, exoproteases, and an insect toxin, FitD, that contributes to the deterrence of insect and fungal plant invaders (Cronin et al. 1997, Ellis et al. 2000, Loper et al. 2016). According to Kupferschmied et al. (2014), *P. protegens* switches between two modalities, one that is virulent to insects and another that is plant-benefiting. Kupferschmied et al. (2014) provided evidence that a sensor, histidine kinase, FitF, regulates the production of insecticidal toxins in *P. protegens*. Inhibition of FitF when *P. protegens* colonizes plant roots prevents the differentiation between an insect host and a plant, thereby explaining how the bacterium knows when to switch modes (Kupferschmied et al. 2014). Another study determined that the components contributing to oral toxicity of *P. protegens* in *Drosophila melanogaster* Macquart (Diptea: Drosophilidae) were a macrolide, rhizoxin, and a secreted chitinase, chiC (Loper et al.

2016). The first objective of my study aimed to determine whether the three bacterial species described above have inhibitory effects on the growth of *B. bassiana*.

Materials and Methods

Entomopathogens

All strains discussed below are biosafety level one (BSL1), that is, they are well-characterized agents that do not cause disease in healthy humans.

Beauveria bassiana

For this study, the *B. bassiana* strain used was L90, which was isolated from house flies in upstate New York, and is known to be virulent to flies (Geden et al. 1995). *Beauveria bassiana* was preserved in 10% glycerol at -80° C at the University of Florida Institute of Food and Agricultural Sciences (UF/IFAS) Entomology and Nematology Department prior to culturing for use in these experiments.

Serratia marcescens

The strain Db11 is a streptomycin-resistant mutant of strain Db10 that was isolated from a dying Drosophila melanogaster (Iguchi et al. 2014). This strain was received from the Caenorhabditis Genetics Center (CGC) of the University of Minnesota (Saint Paul, MN) to be used in this study.

Photorhabdus temperata

The strain NC19 has been genetically sequenced and is predicted to contain many encoded insecticidal toxins (Duchaud et al. 2003). This strain was provided for this research by Byron Adams at Brigham Young University (Provo, UT).

Pseudomonas protegens

The strain Pf-5 was formally classified as P. fluorescens Pf-5 but was reclassified as *P. protegens* along with strain CHA0 as it was found to cluster away from the fluorescent pseudomonads (Takeuchi et al. 2014). This strain was purchased through the American Type Culture Collection (ATCC; Manassas, VA) for use in this study.

Bacterial and Fungal Cultivation

Bacterial strains were cultured on Luria-Bertani (LB) agar plates (LB broth, Fisher BioReagents, Pittsburgh, PA) and incubated at 28 °C, overnight and then placed at 4 °C. For preparation of bioassays, whichever bacteria needed was set up a day prior in an "overnight culture", consisting of 3 mL LB broth in a 10 mL conical tube with the respective bacterial colony picked with a sterile loop from the refrigerated agar plates, then incubated in a controlled environment shaker (New Brunswick Scientific, Edison, NJ) at 28 °C, overnight. The following morning, the overnight culture was transferred into a 50 mL glass flask in a 1:20 dilution with LB broth. Initially, each bacterium had to be plated at differing concentrations to first establish the colony forming units (cfu) at optical density (OD) of 0.5 measured using a spectrophotometer (Biochrom LKB Ultrospec II; Cambridge, UK) at absorbance set to 600 nm. From this, bacterial counts could be estimated for future bioassays. Utilizing knowledge from bacterial enumeration described above, I was able to perform simple calculations involving ratios to determine bacterial concentrations once each bacteria was cultured until it reached an OD of 0.5.

Beauveria bassiana was cultured on Sabouraud's dextrose agar with yeast extract (SDY) (2% glucose, 1% peptone, 0.5% yeast extract; pH 7.0) at room temperature (RT) for 7 days to obtain heavily sporulating cultures. After 1 week, the plates were dried in a sterile hood for an additional week. After drying, conidia were scraped from each plate

with a sterile small metal spatula and stored at -4° C in a sterile glass vial. For conidial counts, 10 mg of dried harvested conidia were suspended in 0.1% Tween® 20 (Sigmal-Aldrich, Saint Louis, MO) and distilled water. After mixing well, 10 µL of this stock was counted using an Automatic Cell Counter (Cellometer® Vision HSL; Nexcelom Bioscience LLC, Lawrence, MA) to determine the conidial concentration.

Central Disc Test

A heavily modified Kirby-Bauer method was used to determine the compatibility of each bacterial strain and *B. bassiana*. The conidial stock was diluted to $1x10^6$ cfu/mL. An aliquot of 100 µL of this dilution was spread evenly onto an SDY plate to create a fungal lawn. The plate was allowed to dry for 30 minutes under a laminar flow hood, then one blank 6 mm filter disc (Becton Dickinson and Company, Washington, DC) was placed in the center of each SDY plate. The appropriate overnight culture of a bacterial strain was then inoculated onto the center of the blank disc. Three separate plates for each bacterial strain were carried out in the following combinations: *S. marcescens* on the disc with a *B. bassiana* lawn, *P. temperata* on the disc with a *B. bassiana* lawn, and *P. protegens* on the disc with a *B. bassiana* lawn. To assess inhibition in a positive control, amphotericin B (an antifungal) was used on the center disc with a *B. bassiana* lawn, and the negative control was a blank disc with a *B. bassiana* lawn. Zone of inhibition diameter was measured with a standard ruler in millimeters after 72 hours. If there was no inhibition of fungal growth then the measurement is 6 mm, the diameter of the disc. The zone of inhibition is measured from the edge of the clear area on either side of the disc.

Statistical Analysis

Data appeared normal on a plot of quantiles (Q-Q plot) and a one-way analysis of variance (ANOVA) was carried out in R 0.99.491 (RStudio, Inc. Boston, MA) as well as Tukey's honest significant difference multiple comparison tests.

Results

Since *S. marcescens* and *P. protegens* are motile, the bacteria grew past the center disc creating a halo effect (Figure 2-2 and 2-3). To compensate for this motility in the bacteria halo was measured as an extension of the center disc and the data measurements were adjusted by subtracting the halo from the overall diameter. Comparing each bacterial strain to the blank control (Figure 2-3), *P. temperata* ($p<0.001$) and *P. protegens* ($p<0.001$) inhibited the growth of *B. bassiana*, while *S. marcescens* did not ($p>0.05$). Similarly, when bacteria strains were compared with the positive control, amphotericin B, *P. temperata* ($p = 0.808$) and *P. protegens* ($p = 0.808$) showed no significant difference, whereas *S. marcescens* did ($p<0.001$). If no zone of inhibition was observed, the measurement was the same as the blank disc, i.e. 6 mm (Figure 2-6). *Photorhabdus temperata* had a distinct orange color on the disc (Figure 2-4) and also inhibited *B. bassiana* growth. Amphotericin B, the antifungal control, produced the widest zone of inhibition against *B. bassiana* (Figure 2-5).

Discussion

Two out of three bacterial pathogens inhibited the growth of *B. bassiana* in the center disc test using the modified Kirby-Bauer method. Because prokaryotic and eukaryotic organisms are fundamentally different, combining them can be a delicate task because of possible negative interactions such as competition and antagonism. The goal

of this objective was to see how these pathogens interacted on simple growth media while recognizing that these interactions could change substantially when the combinations were tested on house fly cuticle. *Serratia marcescens* was the only bacterial pathogen that showed no inhibition towards the growth of *B. bassiana*. One possible explanation for lack of inhibition is that the strain used in this study lacks the red pigment prodigiosin, a well-studied toxin that has some antifungal properties (Darshan and Manonmani. 2015). The test substrate may have played a role as well. Pathogens can live in a wide range of changing environments; it could be that *S. marcescens* is more inhibitory towards invading fungi at a different pH than occurs in SDY agar. Additionally, SDY contains glucose to aid in fungal growth, however, bacteria media (e.g. LB) typically lack glucose. This indicates that bacteria do not need the extra carbon source and the addition of it could be changing gene expression, which is difficult to determine if virulent factors are being expressed (Hua et al. 2004).

Pseudomonas protegens inhibited *B. bassiana* growth similarly to the amphotericin B positive control. This bacterium is known for its antifungal properties while residing in the rhizosphere of plants and for its ability to ward off a variety of plant invaders. My observations were consistent with the plant-protecting properties that this species is known to possess. However, the inhibition of vegetative fungal growth on agar plates does not necessarily rule out the use of *P. protegens* in combination with *B. bassiana* as a means to kill the target insect. If *B. bassiana* were able to facilitate bacterial entry into the fly hemocoel then the FitD toxin associated with *P. protegens* would be likely to kill the host rapidly (Ruffner et al. 2015). As stated above, the behavior of these organisms on SDY plates may differ from what occurs on/in a living

host. For example, local competition for nutrients on growth media may affect the outcome of the interaction differently than would occur on insect cuticle or in the hemocoel.

Photorhabdus temperata also inhibited *B. bassiana* growth similarly to the antifungal positive control. *Photorhabdus temperata* has two phenotypic phases, the primary phase (which has an orange appearance on LB plates) that is symbiotic to the entomopathogenic nematode, and the secondary phase (which grows white) that is conducive to life outside the nematode, i.e. inside the insect host (Hurst et al. 2015). Although both phases are virulent to insects, the secondary phase impedes nematode reproduction and sustenance, suggesting more pathogenicity in secondary phase (Hurst et al. 2015). For the duration of this research, *P. temperata* remained in the primary phase as indicated by colony color.

SDY media was chosen for these assays because it contains minimal nutrients that allowed adequate growth of all four microorganisms and thus allowed me to examine interactions under controlled conditions. However, the environment on and in a living fly is quite different and this could have an impact on the outcome of the interactions. One way to approximate host conditions would be to incorporate chitin or fly homogenates into the growth media, as this could affect the growth, metabolism and interactions of all of the test organisms. Another concern is that these bioaasays did not allow an evaluation of the effect of the bacteria on germination of. *B. bassiana* conidia, as successful germination would be critical to the prospect of using fungal penetration to deliver bacteria to the hemocoel. These topics are addressed in Chapter 4, where species combinations were tested in vivo by topical applications to the thoraces of living flies.

Overall, more work needs to be done to modify the Kirby-Bauer method for this use in order to mimic the biological application more closely. Potential entomopathogens are plentiful and well deserving of our attention in the study of their efficacy to control the insect pest of choice. Moreover, the more knowledge gained about the combinations of different pathogens will aid in the development of a better biological control tool. The use of microbial control has been increasingly a target of study due to its variety and appeal of less harmful biological methods of control. Recently, removing certain toxins that these microbial agents acquire has been another realm of research worth mentioning. For instance, *Bacillus thuringiensis* contains a protein called Bt toxin that has been isolated and used as a successful bioinsecticide for many years (Roh et al. 2007). More importantly, the use of insecticides has long provided a way to reduce pest numbers rapidly during outbreaks when rapid action is needed. Fast-acting microbial biocontrol agents would provide an attractive alternative to both chemical toxicants and the slow response that conventional biological control agents typically deliver.

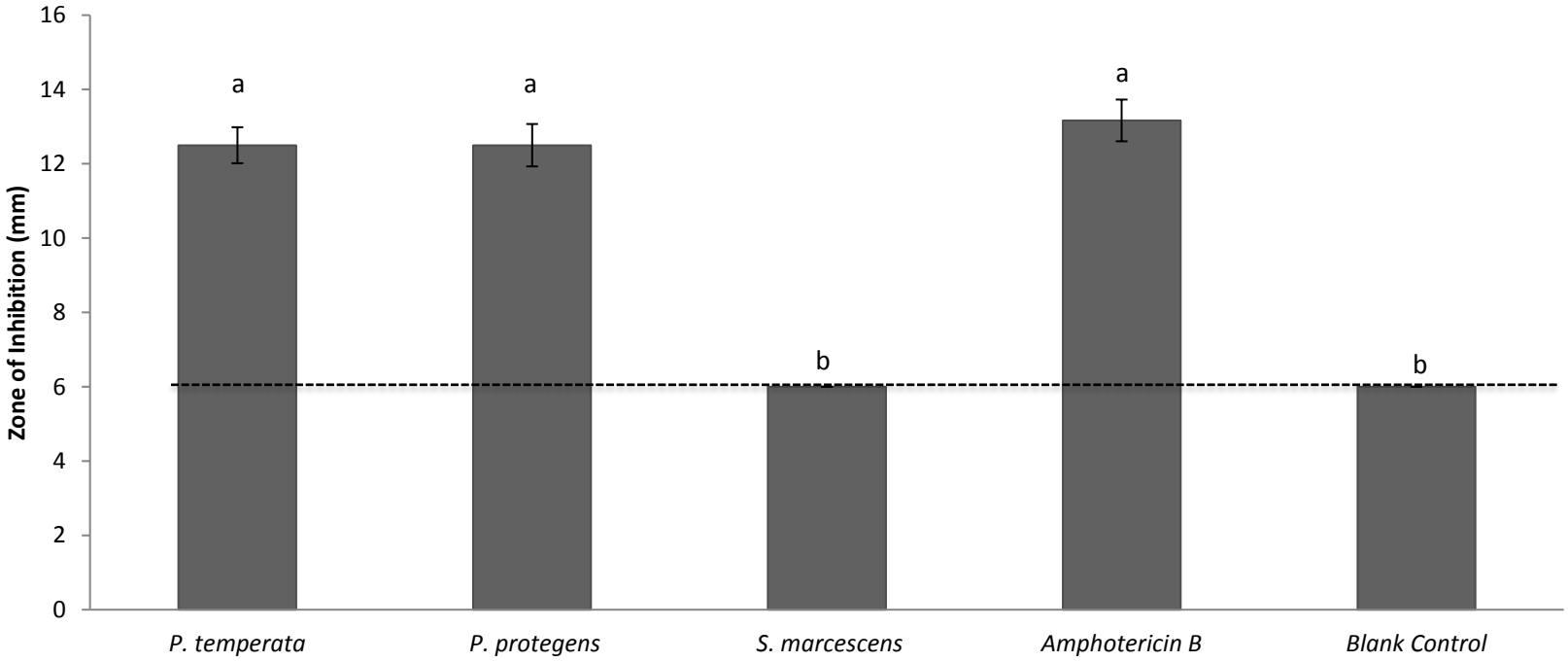

Figure 2-1. Zone of inhibition (mm) of an entomopathogenic fungus, *Beauveria bassiana* by various bacteria species using a modified Kirby-Bauer method known as the center disc test. The three pathogens, *Photorhabdus temperata*, *Pseudomonas protegens*, and *Serratia marcescens*, were compared to a positive control of amphotericin B and a negative control, which was a blank disc. Zone of inhibition diameter was measured and 6 mm was equivalent to no inhibition (dotted line). Bars are means ± standard errors, different letters indicate significant differences ($P<0.05$)

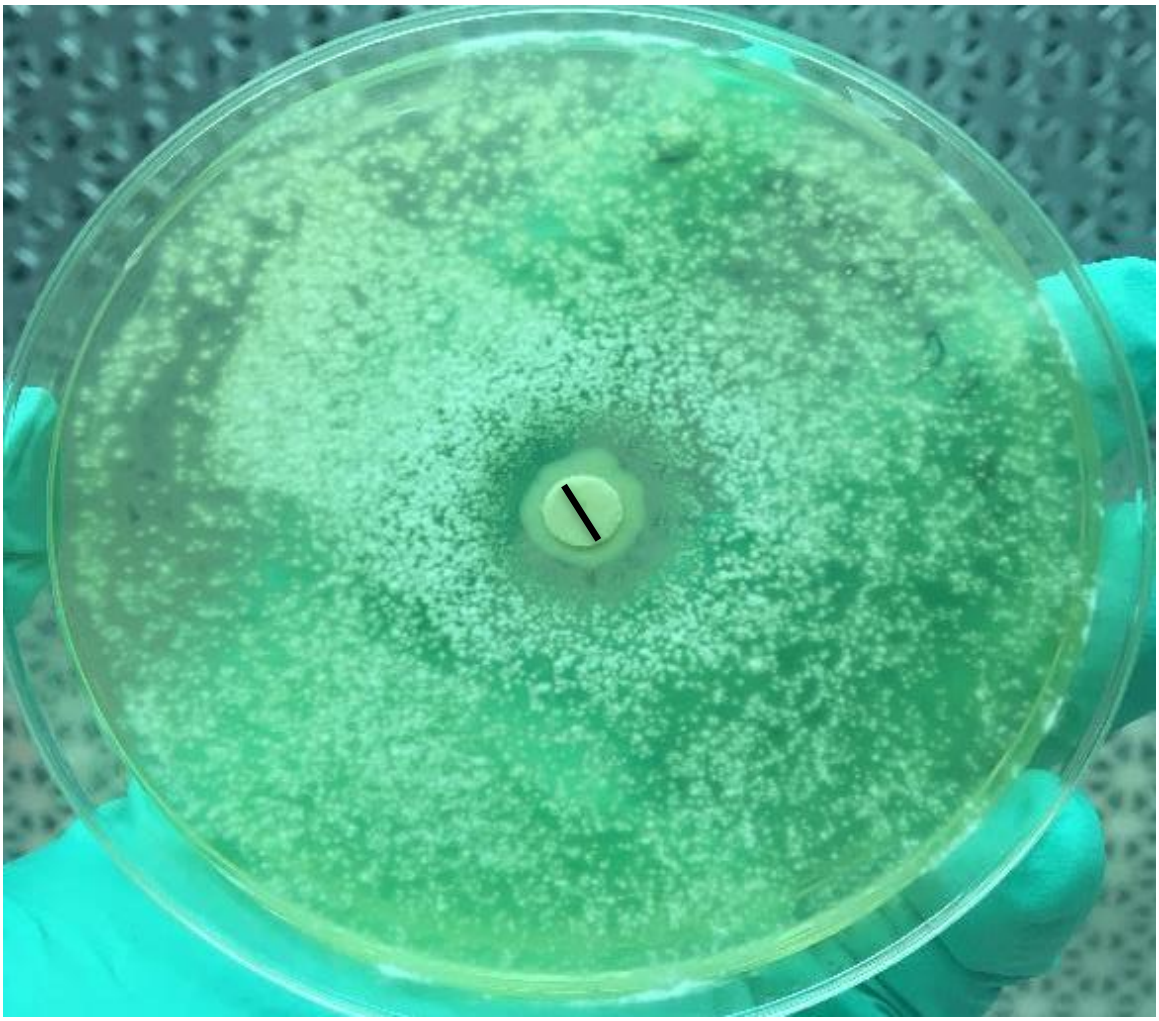

Figure 2-2. A Luria Bertani agar plate with *Pseudomonas protegens* on the center disc showing zone of inhibition (solid black line) against *Beauveria bassiana* lawn, as well as a visibly observable bacterial halo that grew past the disc.

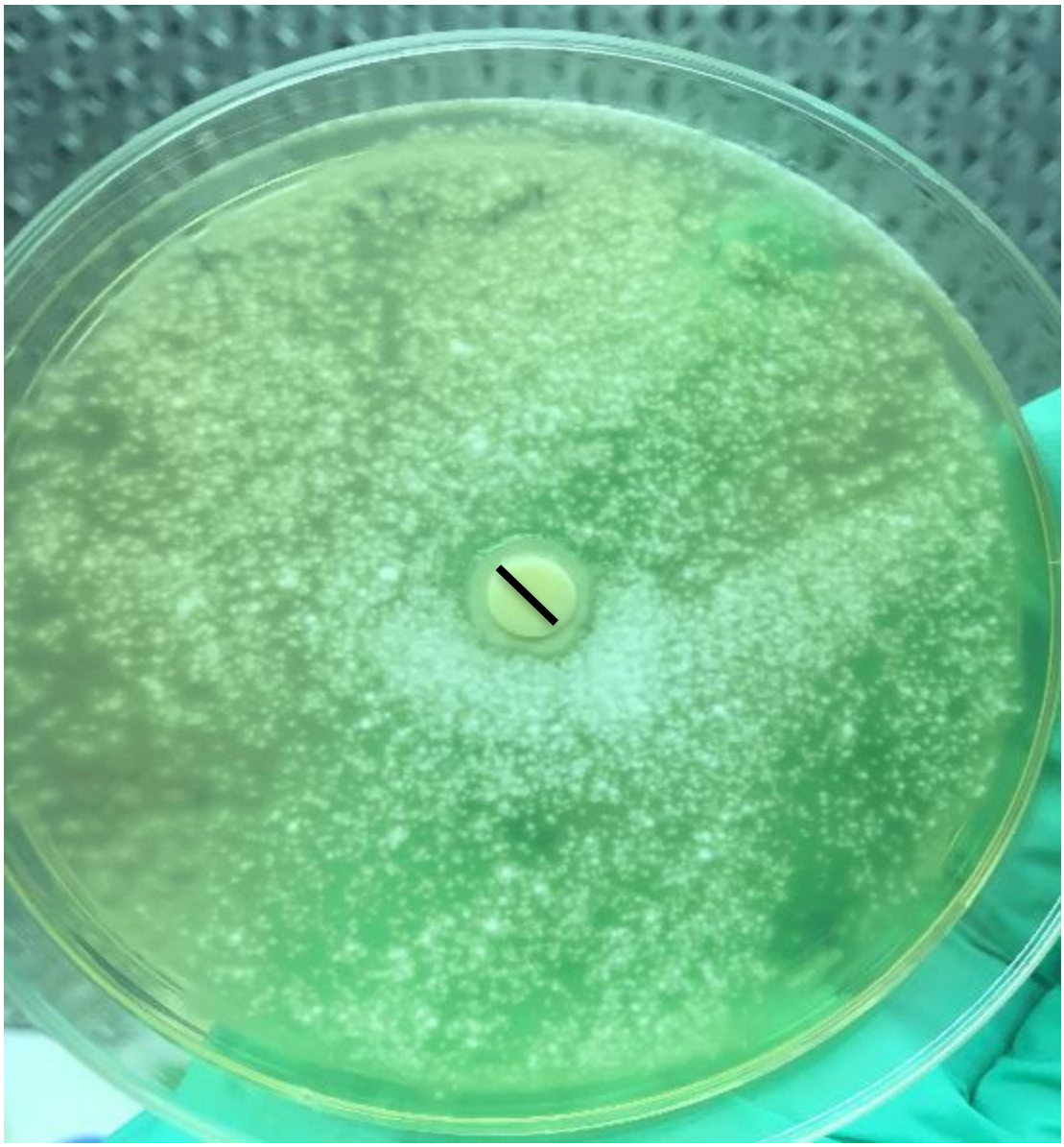

Figure 2-3. *Serratia marcescens* on a blank disc showing no zone of inhibition (solid black line) against *Beauveria bassiana* lawn, and also a halo of bacteria along the rim of the center disc.

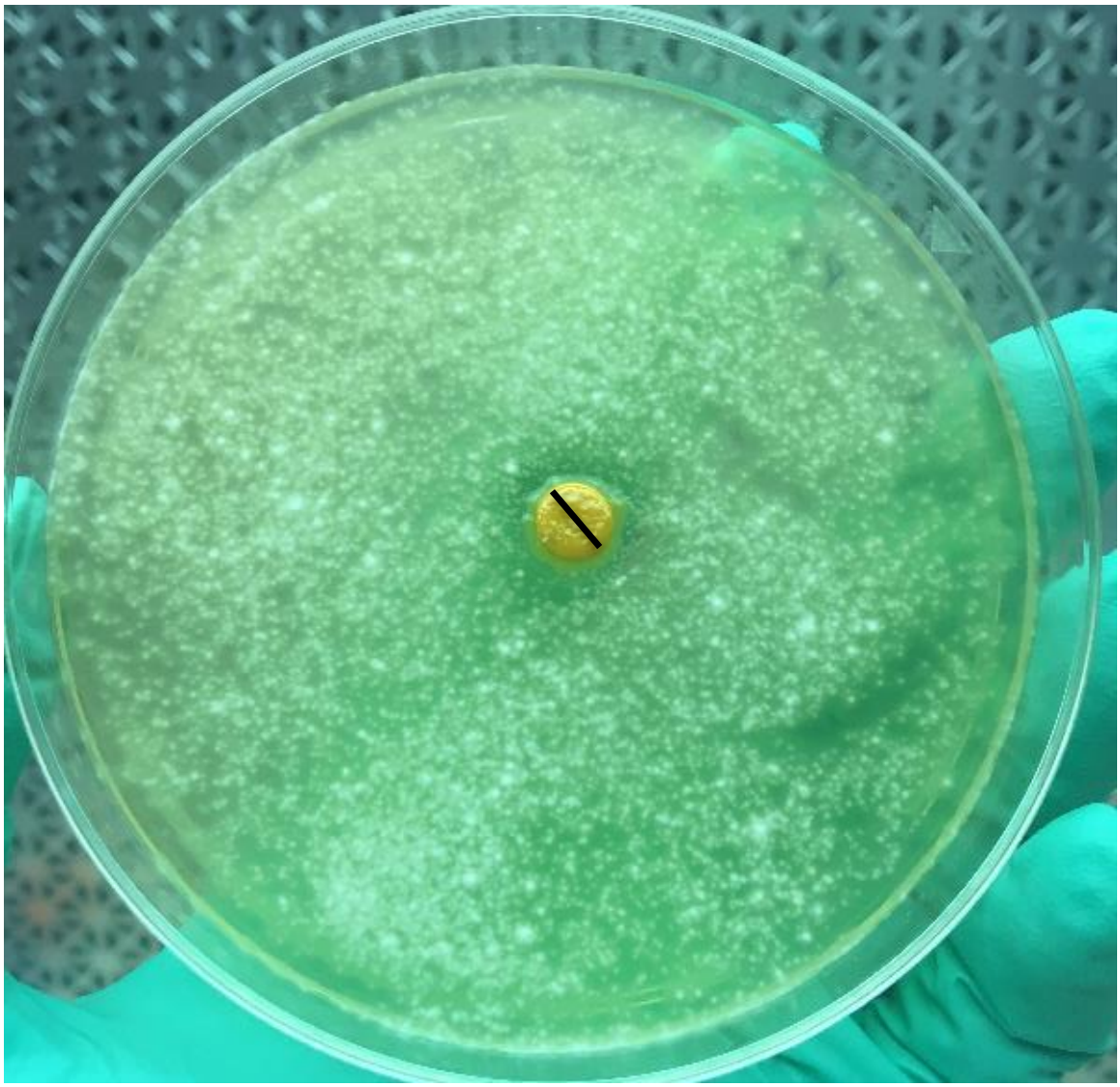

Figure 2-4. *Photorhabdus temperata* on the center disc showing a zone of inhibition (solid black line) against *Beauveria bassiana* lawn. Note the yellow coloration, as well as a visibly observable bacterial halo that grew past the disc.

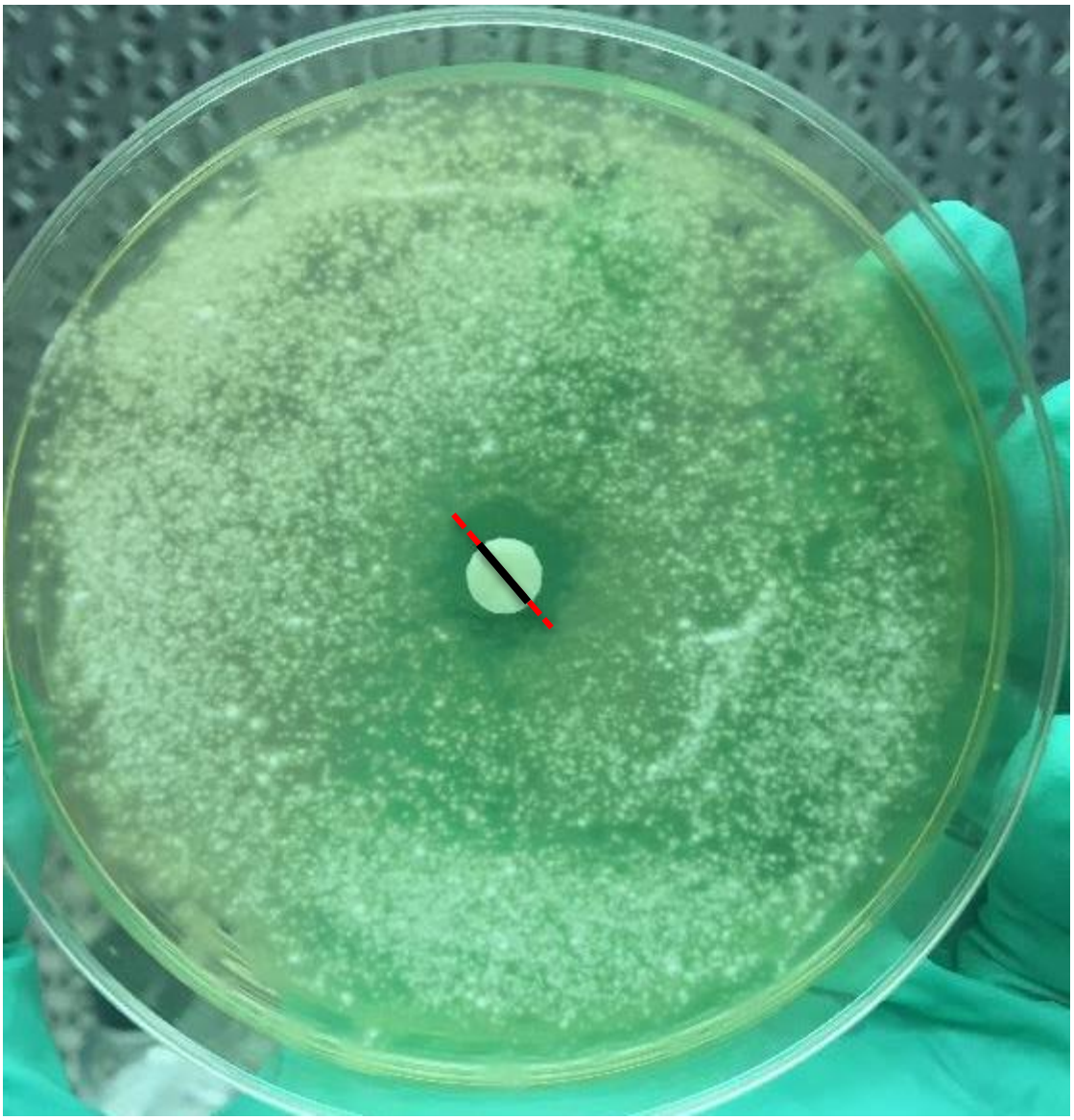

Figure 2-5. Amphotericin B in the center creating a clear zone of inhibition (red dotted line) against a *Beauveria bassiana* lawn. Black solid line indicates the diameter of the center disc (6 mm).

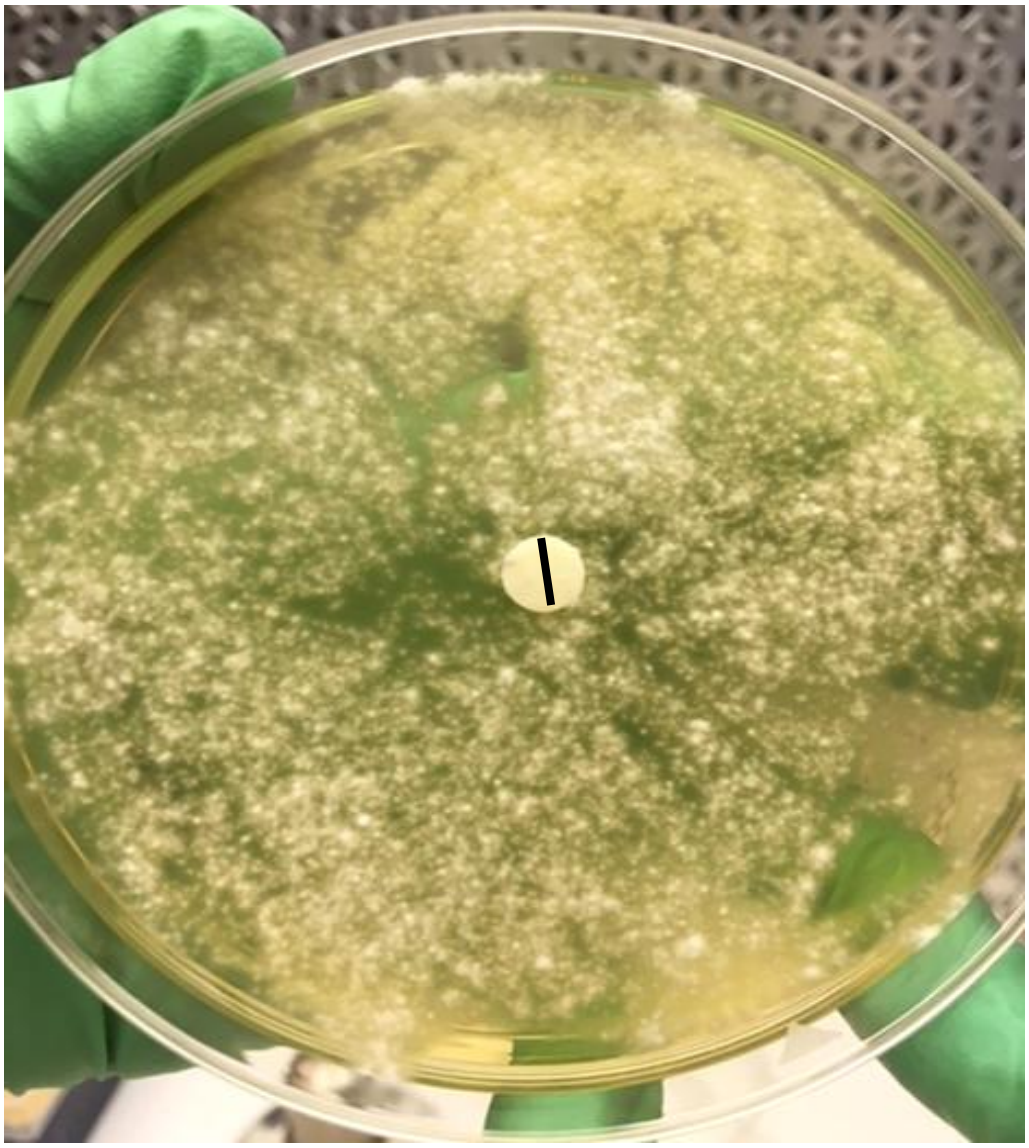

Figure 2-6. Blank disc control with no zone of inhibition against a *Beauveria bassiana* lawn. Black solid line indicates the diameter of the center disc (6mm).

CHAPTER 3
CHOICE OF AN APPROPRIATE CARRIER FOR HOUSE FLY SURVIVAL AND VIABILITY OF ALL PATHOGENS

Introduction

The overall objective of this research project was to test whether the efficacy of entomopathogenic fungi against house flies can be improved by using a combination product that pairs fungal conidia with bacterial pathogens, as the latter would otherwise have difficulty reaching the hemocoel of the fly. In order to develop such a combination product, it was important to find a suitable carrier that was safe for the flies**,** adhered to the fly cuticle, and was not harmful to either the fungal (i.e. *Beauveria bassiana*) or bacterial pathogens (i.e. *Serratia marcescens*, *Photorhabdus temperata*, and *Pseudomonas protegens*). This carrier would be useful both for the topical assays to be conducted in Chapter 4 and for future product formulation. An ideal combination formulation should have the following characteristics: high efficacy, ease of application, safety for organisms involved, and cost effectiveness.

The literature on formulation of *B. bassiana* is voluminous, as conidia can be formulated as dusts, baits, and water and oil suspensions (Wraight et al. 2001). The house fly thorax is rich in lipopolysaccharides, which are hydrophobic and repel water. Oils adhere well to insect cuticle and are attractive as formulation agents because of their long shelf life potential, but botanical oils vary considerably in the effects that they can have on spore viability (Moslim et al. 2004, Luz and Batagin 2005). In order to create a water-based formulation for dry spores, surfactants are needed to suspend the hydrophobic conidia (Jin et al. 2008). One way to achieve suspension is to employ nonionic surfactants that are often used as wetters and spreader-stickers for formulation of agricultural sprays applied to plant foliage. Nonionic surfactants do not contain a

charge, however, they are efficient at breaking the surface tension of water. These materials are amphiphilic, that is, they include both hydrophobic and hydrophilic components, and include well-known surfactants with agricultural, cosmetic, and industrial applications such as Triton X-100, the polysorbates such as Tween 20 and Tween 80, and nonoxynol-9.

Although numerous studies have examined the effect of surfactants on *B. bassiana* viability (Prasad 1993, Polar et al. 2005, Mishra et al. 2013) almost nothing has been published on their direct mortality effects when tested alone on house flies. For this research, a carrier was needed that could be easily and reliably applied, in that it would not be repelled by the cuticle or bead up on the cuticle after topical application, but also would not kill the fly directly. Although spreading is desirable, it is also important that the spread is limited. In preliminary studies with horticultural oil, I found that topical thoracic treatments with even small amounts were lethal because the oil spread too much and blocked the thoracic spiracles of the fly.

Even less is known about the compatibility of surfactants with bacteria. Bacterial cell walls are composed primarily of lipopolysaccharides that make up a bilayer to protect the interior of the cell (Costerton et al. 1974). This lipid bilayer is embedded with proteins that help modulate permeability (Costerton et al. 1974). The surfactants that we were considering contained both hydrophobic and hydrophilic properties. Although this is helpful for suspending *B. bassiana* conidia, the surfactant components could be detrimental to the bacterial cell wall because of the general principle that "like dissolves like". In contrast, the cell walls of *B. bassiana* consist mostly of chitin, a sturdier compound than lipopolysaccharides. Although there is some information on the effect of

nonionic surfactants for their cleansing and antibiotic properties and as carriers for pollutant bioremediation agents (Comas and Vives-Rego 1997, Tobe et al. 2015, Zhang et al. 2017), there is little known about their effects on the species under consideration for this project.

The objectives of this chapter were to evaluate several widely available agricultural surfactants for 1) spreadability on the house fly thorax; 2) acute mortality effects on the fly and 3) compatibility with *B. bassiana* and the bacteria *S. marcescens, P. temperata,* and *P. protegens.* This surfactant will be utilized in Chapter 4 in topical exposure bioassays. The results from this chapter also will provide information for manufacturing of fly pest control products that contain any of these agents.

Methods

House Fly Rearing

The Orlando Normal strain that has been reared at the Center for Medical, Agricultural and Veterinary Entomology, United States Department of Agriculture, Agricultural Research Service (Gainesville, FL) since 1958 was utilized for these studies. These flies were maintained at 28° C in wire mesh cages (37.5 x 37.5 x 37.5 cm), and fed a diet consisting of dried milk, dried egg, and sugar. Flies used in this bioassay were less than a week old. To keep the flies under anesthesia for the duration of the experiment, a CO_2 exposure chamber was fabricated. This consisted of a shallow dish fashioned from a 2 liter container (Instawares, Kennesaw, GA) cut in half (Fig. 3-1), utilizing the bottom only with a 1 cm diameter hole on the side into which the CO_2 hose fits (Fig. 3-2). Inside of the dish was a large Petri dish lid (14 cm) with small holes (Fig. 3-1). When the flies are placed on the perforated petri dish, the CO_2 is forced through the perforation and spreads evenly over the anesthetized flies.

Carriers

Kinetic® (Aquatrols, Paulsboro, NJ) is a non-ionic, synthetic surfactant used as a spreader sticker for pesticides and fungicides onto plants. CapSil® (Aquatrols, Paulsboro, NJ) is another non-ionic surfactant that is an organo-silicone. DyneAmic® (Helena, Collierville, TN) is a surfactant containing mostly fatty acids that is used to enhance the spread of insecticides on waxy plant surfaces. Induce® (Helena, Collierville, TN) is a nonionic blend containing high amounts of fatty acids; it aids in the adherence of insecticide onto plants and is known for its resistance to wash-off.

Various dilutions of each carrier were made in distilled water and mixed thoroughly. Three doses were tested for each carrier: minimum, medium, and maximum, based on the range of recommended dilution rates listed on the product labels. Each carrier was diluted in sterile distilled water following product label instructions to prepare 10 mL of solution. For CapSil® and Kinetic®, the minimum, medium and maximum concentrations were 0.01, 0.10 and 1.00%, respectively. For DyneAmic® the concentrations were 0.04, 0.40 and 4.00%, and for Induce® they were 0.03, 0.30 and 3.00%. Tween® 80 also was included as a standard and was tested at 0.01, 0.10 and 1.00%.

Application of Carriers to Flies

Utilizing a P-2 pipette (Gilson PIPETMAN™, Atlanta, GA), 1 µL was topically applied to the anterior thorax of five female flies individually with each dose of the carriers. All topical treatments were replicated four times (20 flies). Two measurements were recorded; the spreadability onto the house fly thorax for each carrier and fly mortality. Spreadability was determined subjectively by making direct observations of the dispersion of the applied droplet immediately after application. A scale of 1 to 3 was

used where a score of 1 indicated poor spreading (i.e. product bubbled or beaded off), a score of 2 indicated a medium spread, in that the product spread out but some bubbling and beading still occurred, and a score of 3 indicated a rapid and even dispersion over the thorax. Once flies were treated, they were placed in a 500 mL (Instawares, Kennesaw, Ga) plastic container with a 30 mL cup (Instawares, Kennesaw, GA) of diet (described above) and water. The water container had a lid with a 2.5 cm dental wick inserted. Fly mortality was observed at one hour after topical applications and then every 24 hours for three days total.

Bacterial and Fungal Cultivation

The bacteria and fungal strains were as described in Chapter 2: *B. bassiana* (strain L90), *S. marcescens* (strain Db11), *P. temperata* (strain NC19), and *P. protegens* (strain Pf5). Bacteria were cultured in Luria Bertani (LB) broth shaken at room temperature (RT) as described in Chapter 2. *Beauveria bassiana* were grown, harvested and counted as described in Chapter 2.

Viability Bioassay for Pathogens

Based on the results from the fly spreadability/mortality bioassay, three surfactant solutions were chosen for viability testing with bacteria and fungi, CapSil® 0.1%, CapSil® 0.5% and Kinetic® 0.5%. Bacterial strains were grown overnight (see Chapter 2 for methods), and the next day 1 mL of stationary phase culture containing $1x10^8$ cfu/mL was moved to a sterile microcentrifuge tube. The tube was centrifuged for 1 minute at 13,200 rpm to form a pellet, the supernatant was removed, and the pellet was re-suspended in the respective surfactant. Once each bacterial strain was suspended in the surfactants mentioned above, the high concentration ($1x10^8$ cfu/mL) was diluted in its respective surfactant to $1x10^2$, $1x10^3$, and $1x10^4$ cfu/mL, inoculated onto an LB plate in

10 µL spots, and held at 25 °C (time point 0). The dilutions were re-plated at 24 hours after they were prepared. As a control, bacterial strains were suspended in 1X phosphate buffered saline (PBS) and cultured using the same plating timeline as the cultures that were held in the surfactants. Initial assays with *B. bassiana* in PBS were problematic because conidia could not be kept in suspension to draw 10 µL aliquots reliably for spot plating. Therefore, for this species, 0.1% Tween® 80 was used instead to compare with the other surfactants. Each LB plate was observed for bacterial viability to determine survival in the surfactants. On each plate, the number of cfu were counted and recorded from spots that produced 3 to 30 cfu, and from this number the antibacterial actions of the carrier could be determined.

For culturing of *B. bassiana*, fresh conidia were harvested from dried Sabouraud's dextrose agar with yeast extract (SDY) plates, placed in a sterile container and stored at 4 °C. From this stock, 1 mg of dried *B. bassiana* conidia was weighed and suspended in each surfactant, and the highest concentration ($1x10^9$ conidia/mL) was diluted to $1x10^2$, $1x10^3$, and $1x10^4$. An aliquot of 10 µL of each suspension was plated onto SDY agar (time point 0 equals 1 hour), and then plated again at 24 hours (time point 1) post-suspension.

Statistical Analysis

Application of Carriers to Flies

Four replications were performed with five individual flies for each surfactant (20 flies per treatment). For the comparison of carrier spreadability scores, a Wilcoxon Mann Whitney test was done using the NPARONEWAY procedure of the Statistical Analysis System (SAS), version 9.4 (SAS Institute, Cary, NC). Fly mortality at one hour

and 24 hours was assessed using the general linear models procedure (Proc GLM) of SAS, and means were separated using the Means/Tukey statement of Proc GLM.

Viability Bioassay for Pathogens

The number of cfu for each pathogen and carrier combination was tested for normality. A linear mixed model was fitted with the effects of treatment, time and the interaction analyzed using repeated measures ANOVA using the Mixed Procedure as implemented in SAS (Proc Mixed), version 9.4 (SAS Institute, Cary, NC). Residual terms were modelled by considering an autoregressive order one error structure and the degrees of freedom were adjusted using the Kenward-Roger method. LSMEANS statements were used to obtain the adjusted means for the effects of treatment, time and the interaction, which were compared using Tukey's honest significant difference separation test method at $P<0.05$.

Results

Application of Carriers to Flies

Spreadability scores on fly thoraces varied significantly among the products and concentrations tested (Kruskal-Wallis Chi-Square = 45.9; df = 14; $P<0.01$). The highest scores (all flies scoring three out of three on the scale) were observed for Kinetic® at 1.0% and CapSil® at 0.1 and 1.0%, although these did not differ significantly from DyneAmic® at 4.0% (mean score 2.50), Induce® at 3.0% (2.50), or Kinetic® at 0.01 or 0.10% (2.25 and 2.00, respectively) (Fig. 3-3). Tween® 80 had the lowest score (1.75) of the five products tested at their highest rates of application, and did not differ significantly in spreadability over the range of concentrations examined (0.01-1.00%). A dose response was seen with the other four carriers, with all of them having significantly higher spreadability scores at the highest rate compared with the lowest.

Initial fly mortality after treatment was significantly higher for CapSil® at 1.0% (45%) than any other treatment except Kinetic® at 1.0% (35%) and DyneAmic® at 4.0% (15%) (Overall ANOVA $F = 3.60$, $df = 14, 45$, $P<0.01$) (Table 3-1). Initial mortality was $\leq$5% for all of the other treatments and was zero for many of the lower doses of each treatment. There was nearly no additional mortality when flies were examined 24 hours after treatment (Table 3-1). No additional mortality was observed on days two and three after treatment.

Viability Bioassay for Pathogens.

Test statistics for the viability assays are presented in Table 3-2, and viability means in the surfactants are shown in Figures 3-4 to 3-7. Viability for all four pathogens was high in all solutions and there were no significant negative viability effects between the initial time point and 24 hours later. Colony forming units of *Photorhabdus temperata* increased marginally during the 24 hour holding period (Table 3-2), and growth was significantly higher in Capsil® 0.5% than in Kinetic® 0.5% (Fig 3-4). Colony forming unit counts of *S. marcescens* (Fig 3-5) and *P. protegens* (Fig. 3-6) generally increased two- to four-fold after being held for 24 hours in the suspensions, and there was no significant surfactant effect on viability for either species. The only instance where substantial growth was not observed with these species was with *S. marcescens* held in Kinetic® 0.5%. In contrast, there were no significant time or surfactant effects for *B. bassiana*, with similar conidia counts at time point 1 and at 24 hours post-suspension for all of the surfactants tested (Fig. 3-7).

Discussion

Entomopathogenic fungi have long been studied for their potential as biological control agents for arthropod pests (Butt et al. 2001, Shah and Pell 2003, Lacey et al.

2015). Although at least 700 entomopathogenic species have been identified (Sandhu et al 2012) and they are ubiquitous in essentially all insect ecosystems (Lacey et al. 2015), several species have received the majority of the attention for development as microbial insecticides. *Beauveria bassiana* is one of the most widely studied species and accounts for over one-third of the 171 commercial myco-biocontrol products available globally (Sandhu et al 2012). There are several reasons why *B. bassiana* is attractive for use as a biocontrol agent. It has a wide host range, is easily and economically produced, and the conidia have a long shelf life and can be formulated in a variety of ways such as food baits, dusts and sprays (Feng et al. 1994). Because of this flexibility, it has been used against a wide array of target pests in their respective habitats (Lacey et al. 2015). Natural *B. bassiana* infections of house flies have been observed (Steinkraus et al. 1990, Skovgärd and Steenberg 2002), and flies are readily infected after exposure to treated surfaces or contaminated food (Geden et al. 1995, Weeks et al. 2016). Furthermore, Kaufman et al. (2005) observed satisfactory fly suppression after applying a commercial *B. bassiana* spray (balEnce™; (Terregena, Inc., Raleigh, NC, U.S.A.)) in poultry facilities in New York.

For this research project, the addition of a bacterial agent to *B. bassiana* was the goal with the ultimate aim of accelerating pathogen-induced mortality. I hypothesized that *B. bassiana* could act as a vehicle to deliver a bacterial agent into the house fly hemocoel that would cause rapid death from sepsis. Testing this hypothesis required identifying a carrier that would not be inimical to either fungi or bacteria. Despite sensitivity to environmental conditions during germination, *Beauveria bassiana* conidia can tolerate a wide range of conditions, and there is a great deal of information available

on methods to formulate them for application (Wraight et al. 2001). In contrast, little is known about formulation of bacterial insect pathogens. Other than the spore-forming *Bacillus thuringiensis* and its relatives, the only bacterial pathogen that has been developed as a live microbial insecticide is *Serratia entomophila*, which was developed for grass grub control (Jackson 2007). Non-spore-forming bacteria generally have a narrower range of tolerance for their environment than *B. bassiana* conidia, and it was unknown whether the bacterial species chosen for study in this project would survive in any carrier. This was a particular concern for *Photorhabdus temperata*, which lives exclusively in host environments provided by its symbiotic relationship with entomopathogenic nematodes of the genus *Heterorhabditis* (Waterfield et al. 2009). When combining two different pathogens, the minimum requirement was determining a carrier that would ensure viability of both organisms and not be harmful to flies when applied topically.

The use of oils would have been the simplest method for suspension of the pathogens because they can be used as is, i.e. no need for an adjuvant. Although oils are useful and promote spreadability (Polar et al. 2005), they vary a great deal in their effects on germination of *B. bassiana* conidia (Luz and Batagin 2005). My experiments were a proof of concept that required a carrier that was safe for all organisms, including the fly. Although I recognize that additional mortality caused by the carrier on the pest organism might not be considered a negative aspect of a fly control product, low control mortality was required for comparison with pathogen treatments. While the data are not included herein, preliminary tests of horticultural oil indicated that it could not be used safely on flies in the topical bioassay. As little as 1 µL applied to the house fly thorax rapidly

caused unacceptably high levels mortality. Observations of dead flies indicated that the oils had spread evenly over the anterior thorax and ran down the sides, covering the thoracic spiracles and thus preventing proper gas exchange.

Non-ionic surfactants include spreader stickers, wetting agents, and detergents. They are composed of hydrophobic and hydrophilic components that aid in breaking the surface tension of water (van Os et al. 1993). Because of these properties, they have a variety of uses and are currently in many industrial products. However, their chemical nature varies depending on the intended use of the products (van Os et al. 1993). We concentrated on horticultural wetting agents and spreader stickers because of their known safety profiles and availability (reviewed in Krough et al. 2003). These products allow the preparation of aqueous solutions of otherwise-insoluble active ingredients and aid in adherence to the hydrophobic surfaces of plant foliage. The latter was an attractive trait for the application on the house fly thorax, which is also highly hydrophobic. Moreover, a number of nonionic surfactants are already known to be compatible with *B. bassiana* conidia (Prasad 1993, Polar et al. 2005, Mishra et al. 2013).

In the spreadability bioassay, there were numerous promising candidate surfactants, with all five types scoring well at higher concentrations for their ability to provide even dispersion over the dorsal thoracic cuticle of the house fly (Fig 3-3). Several of these, however, resulted in >10% mortality immediately after treatment (Table 3-1). It is uncertain whether this mortality was due to toxicity of the solutions or whether, as with the oils, the surfactants interfered with gas exchange through the thoracic spiracles. Spiracle coverage was harder to observe with these materials because, unlike the oils, they did not leave a visible residue on the fly once the water evaporated.

The spreadability scores for Tween® 80 were surprisingly low, considering that this surfactant is commonly used in laboratory bioassays with *B. bassiana* (Mishra et al. 2013, Immediato et al. 2015, Andreadis et al. 2016). Perhaps the hydrophobicity of the house fly cuticle did not interact well with the long chains of polyethylene glycol of Tween®, a hydrophilic compound. I chose to move forward with CapSil® and Kinetic® because of their high spreadability scores. Furthermore, adjustments were made to the concentrations of CapSil® and Kinetic® to prevent unacceptable fly mortality without compromising high spreadability before conducting the viability assays.

All of the carriers tested supported viability of all four pathogens (Figs 3-4 to 3-7). No reduction in cfu counts were observed after 24 hours suspension in all surfactants. Indeed, there was an increase in bacterial (but not *B. bassiana*) cfu after 24 hours. This was probably due to cell division of the bacteria, which were harvested from stationary-phase cultures and presumably had sufficient reserves to divide. Although the bacteria also could have been utilizing the long chain components of the surfactants as a carbon source for further growth and division, growth in the surfactants was not significantly higher than in the PBS control. The surfactants differed little in their effects on viability, except for *P. protegens* and CapSil® where significantly higher bacterial growth was observed after 24 hours post-suspension than in at least one other carrier, e.g. Kinetic®. Based on these results, CapSil® was chosen as the surfactant for the topical applications of pathogens described in the next chapter.

In summary, this study demonstrated that a common non-ionic surfactant carrier (CapSil®) can be used for preparing viable suspensions of four very different microorganisms, including one that does not exist in nature outside of its hosts (*P.*

temperata). This allows for formulation of combined-species products that are safe for use with both pathogens and flies. In the next chapter, these combinations with CapSil® are tested for efficacy against living flies.

Table 3-1. Effect of different surfactants (i.e. CapSil®, DyneAmic®, Induce®, Kinetic® and Tween® 80) on house fly, *Musca domestica*, mortality up to three days post topical application.

		Mean (SE) % mortality at time after treatment			
Treatment		1 hour	1 day	2 days	3 days
CapSil	0.01%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	0.10%	5 (2.5) b	5(2.5) b	5(2.5) b	5(2.5) b
	1.00%	45 (9.7) a	50 (9.7) a	50 (9.7) a	50 (9.7) a
DyneAmic	0.04%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	0.40%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	4.00%	15 (4.9) ab	25 (4.9) ab	25 (4.9) ab	25 (4.9) ab
Induce	0.03%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	0.30%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	3.00%	5 (2.5) b	5 (2.5) b	5 (2.5) b	5 (2.5) b
Kinetic	0.01%	5 (2.5) b	5 (2.5) b	5 (2.5) b	5 (2.5) b
	0.10%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	1.00%	35 (8.8) a	35 (8.8) a	35 (8.8) a	35 (8.8) a
Tween	0.01%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	0.10%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b
	1.00%	0 (0.0) b	0 (0.0) b	0 (0.0) b	0 (0.0) b

Means within columns followed by the same letter are not significantly different ($P>0.05$, two-way ANOVA with repeated measures)

Table 3-2. ANOVA results for effects of different surfactants (i.e. Tween® 80, CapSil®, and Kinetic®) on viability of *Beauveria bassiana, Photorhabdus temperata, Pseudomonas protegens,* and *Serratia marcescens* 1 and 24 hours after being placed in the surfactants.

Model term	ANOVA F-test value for species			
(df)	*B. bassiana*	*P. temperata*	*P. protegens*	*S. marcescens*
Treatment (3,6)	1.24ns	6.20*	0.10ns	2.63ns
Time (1,8)	0.36ns	6.96*	18.02**	15.15**
Treatment x Time (3,8)	1.01ns	1.80ns	0.56ns	3.67ns

** = P<0.01; * = P<0.05, ns = P>0.05 (two-way ANOVA with repeated measures)

Figure 3-1. Top view of the apparatus used to anesthetize house flies. Small holes in a 14 cm Petri dish lid that nestles into a halved 2 liter container.

Figure 3-2. Side view of container used with a 1 cm diameter hole to insert CO_2 hose. This creates a blanket of CO_2 over house flies to prevent premature waking.

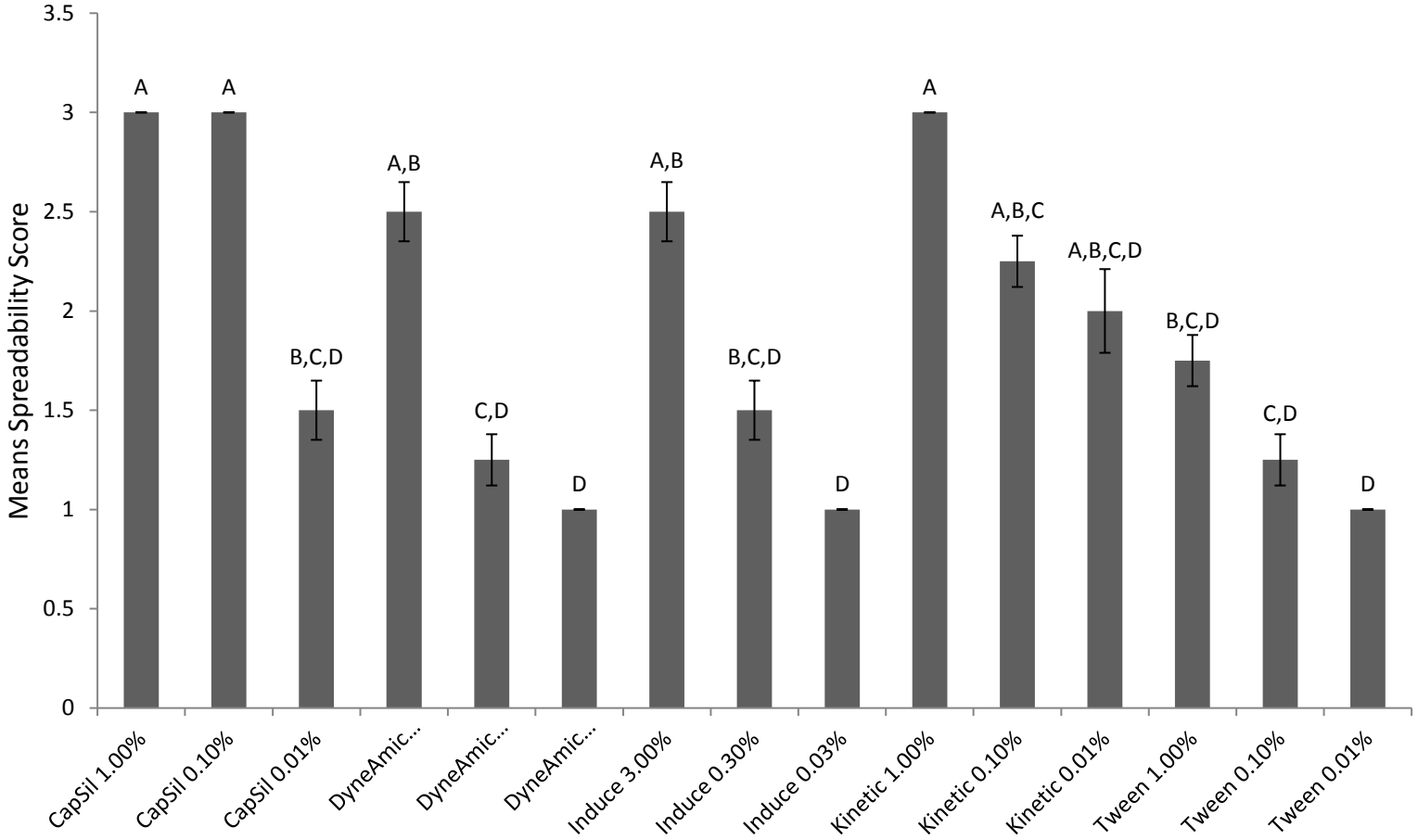

Figure 3-3. Spreadability scores for surfactants after application to female house fly, *Musca domestica*, thoraces. Possible scores ranged from one (i.e. surfactant beaded up and rolled off the fly) to three (i.e. rapid and even dispersion of the droplet). Bars represent mean ± standard errors; letters above bars indicate significance, means with the same letter are not significantly different (Tukey's HSD test at $P<0.05$)

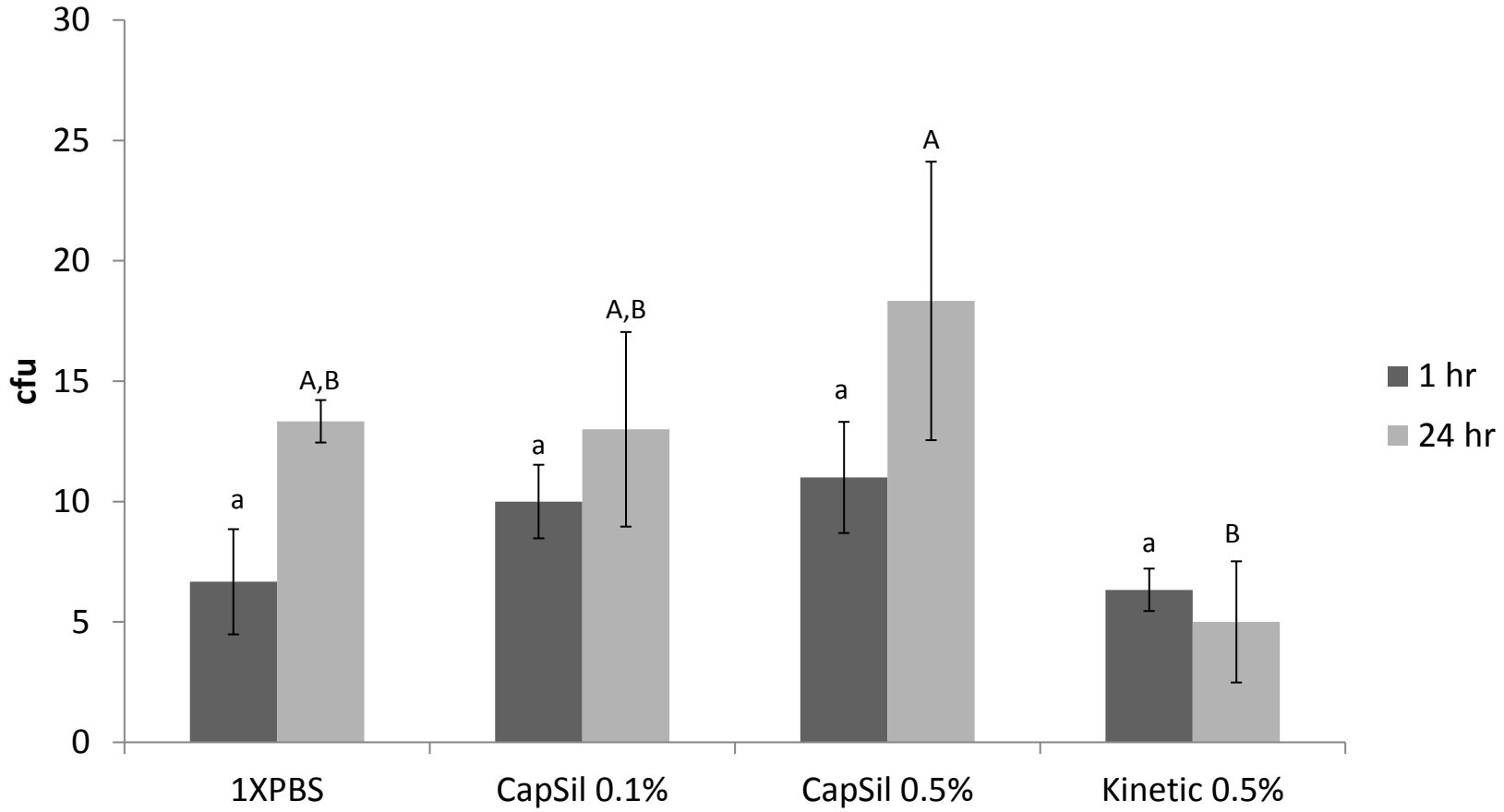

Figure 3-4. Colony forming units (cfu) of *Photorhabdus temparata* on Luria Bertani (LB) agar plates after being held in surfactant (CapSil® or Kinetic®) solutions briefly (1 hr) or for 24 hrs. Controls were held in 1X phosphate buffered saline (PBS) Bars represent the mean number of cfu ± standard error; means with the same letter at the same time point (1 and 24 hrs) are not significantly different (Tukey's HSD test at $P < 0.05$). Lower case letters denote significant differences between 1 hr treatments, while upper case letters describe significant differences among 24 hr treatments.

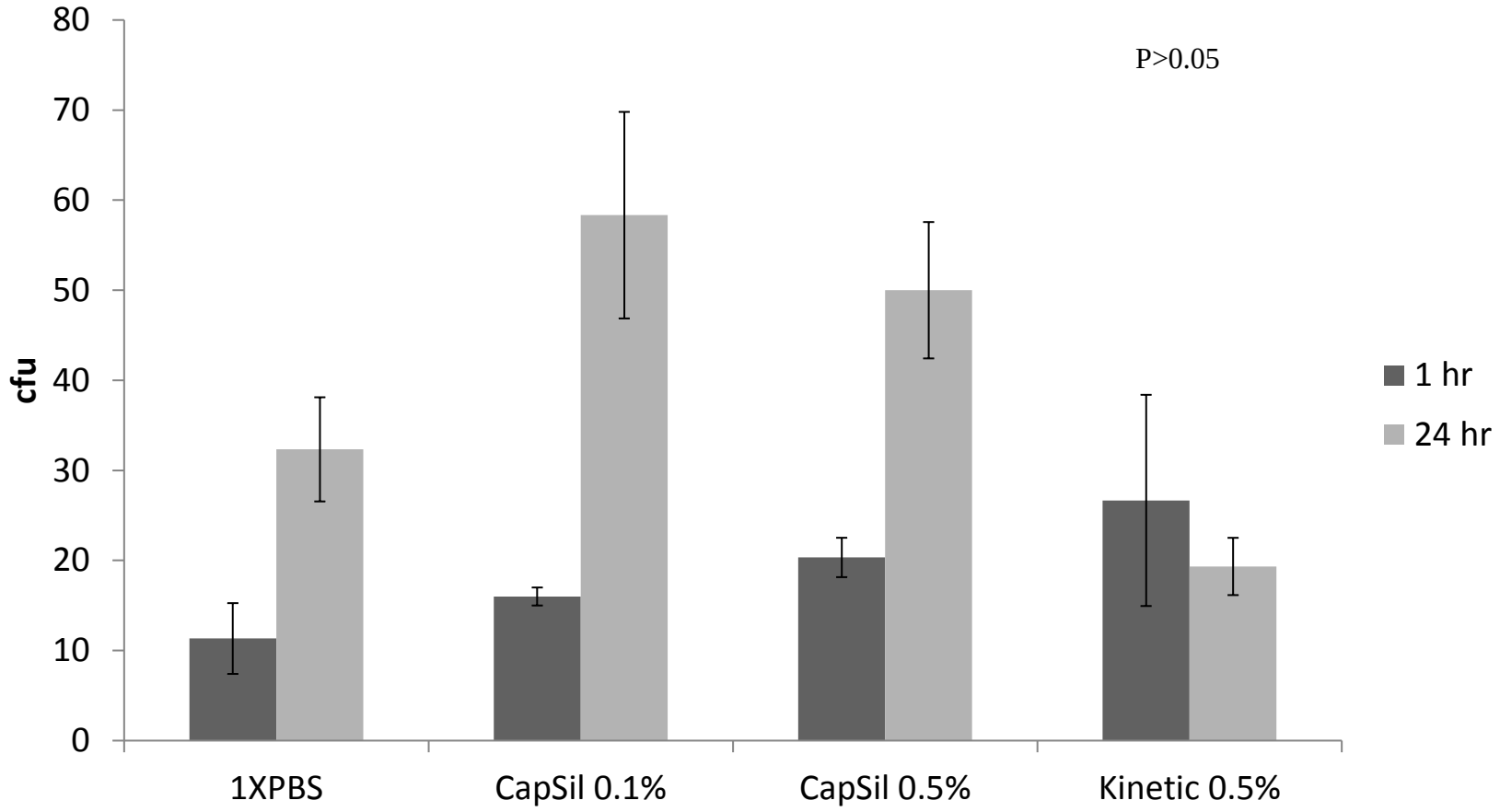

Figure 3-5. Colony forming units (cfu) of *Serratia marcescens* on Luria Bertani (LB) agar plates after being held in surfactant solutions briefly (1 hr) or for 24 hrs. Bars represent the mean number of cfu ± standard errors; means with the same letter at the same time point (1 and 24 hrs) are not significantly different (Tukey's HSD test at $P < 0.05$)

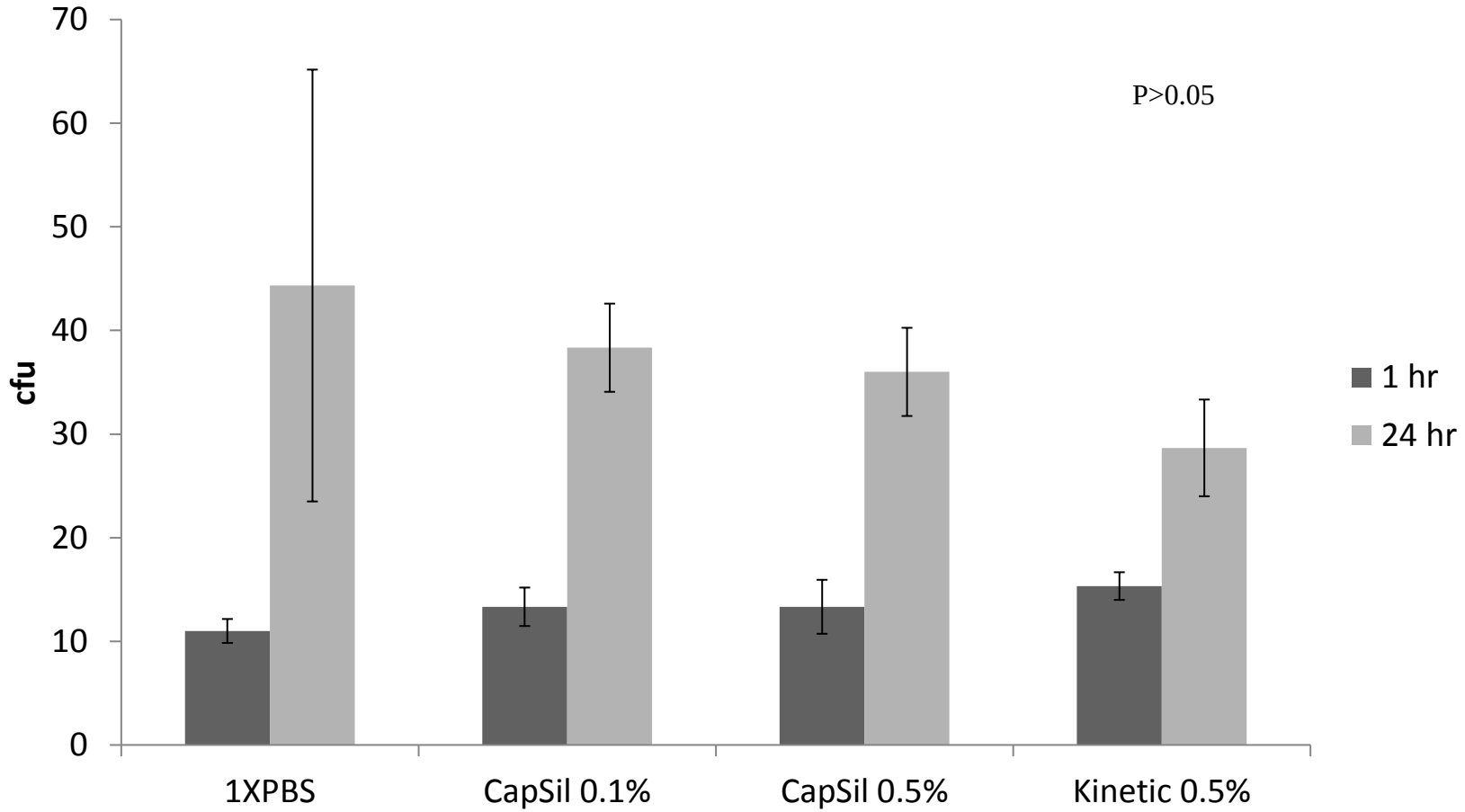

Figure 3-6. Colony forming units (cfu) of *Pseudomonas protegens* on Luria Bertani (LB) agar plates after being held in surfactant solutions briefly (1 hr) or for 24 hrs. Bars represent the mean number of cfu ± standard errors; means with the same letter at the same time point (1 and 24 hrs) are not significantly different (Tukey's HSD test at $P < 0.05$).

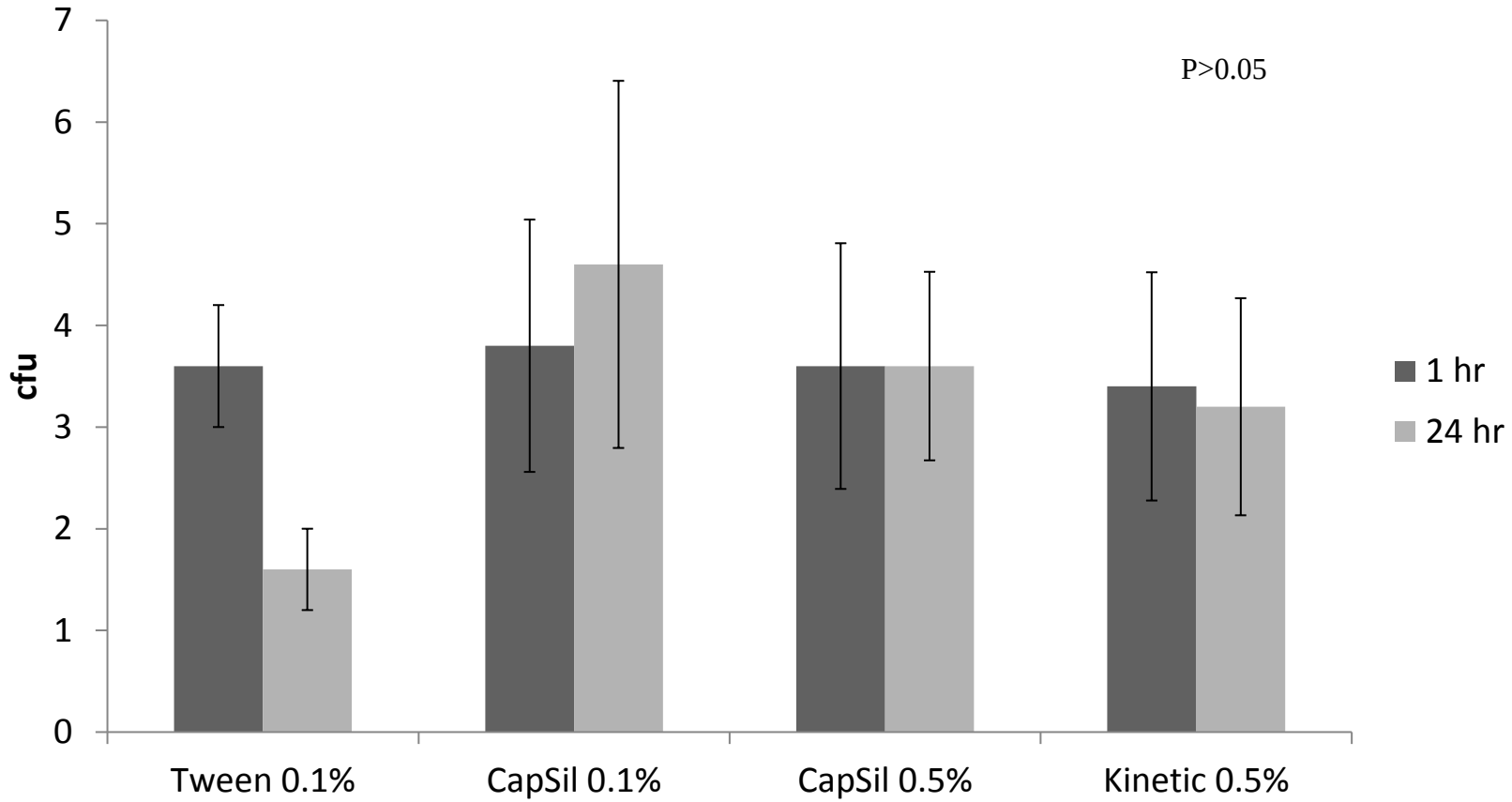

Figure 3-7. Colony forming units (cfu) of *Beauveria bassiana* on Sabouraud's dextrose agar with yeast extract (SDY) plates after being held in surfactant (CapSil® or Kinetic®) solutions briefly (1 hr) or for 24 hrs. Controls were held in Tween 80. Bars represent the mean number of cfu ± standard errors; means with the same letter at the same time point (1 and 24 hrs) are not significantly different (Tukey's HSD test at $P < 0.05$)

CHAPTER 4
EFFECT OF COMBINING THE BACTERIAL PATHOGENS *Photorhabdus temeperata, Serratia marcescens,* AND *Pseudomonas protegens* WITH *Beauveria bassiana* ON HOUSE FLY MORTALITY

Introduction

The common house fly, *Musca domestica* L., is a major nuisance pest and carrier of human and animal pathogens throughout the world (West 1951, Scott et al. 2009). The entomopathogenic fungus *Beauveria bassiana* has long been suggested as a potential biological control agent for this pest (Dresner 1950). Watson et al. (1995) exposed flies to *B. bassiana*-treated plywood, and the highest dose $1x10^8$ conidia/cm^2 caused $\geq$ 90% mortality. Similar results were seen when flies were confined with *B. bassiana*-treated plywood by Geden et al. (1995). Lecuona et al. (2004) tested 19 different strains of *B. bassiana* on house flies and observed 90% mortality after 15 days post fly exposure to conidia. Although these and other studies have provided ample evidence that house flies are susceptible to *B. bassiana,* the chief disadvantage of this pathogen is the long time required to kill the host after exposure to conidia (St. Leger et al. 1996). High mortality in house flies is often not reached until six to seven days after exposure (Geden et al. 1995), and even longer latency times have been observed in some studies (Lecuona et al. 2004). This slow kill rate may not be adequate for practical field use because of the rapid development rate and high fecundity of house flies (Lysyk and Axtell 1986, Malik et al. 2007).

The long interval between exposure to *B. bassiana* and host death has prompted several investigations into combinations of this species with other entomopathogens for potential synergistic effects. Bacteria, other fungi and nematodes have been tested in combination with *B. bassiana* in efforts to increase virulence or shorten the time to host

death (Wraight and Ramos 2005, Mwamburi et al. 2009, Senthrilraja et al. 2010, Prabhukarthikeyan et al. 2014). Research into bacterial partners for *B. bassiana* has concentrated on *Bacillus thuringiensis*, which must be ingested to cause host mortality. Wraight and Ramos (2005) tested individual and combined treatments of *B. bassiana* and *B. thuringiensis* var. *tenebrionis* on larval field populations of Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), and discovered that mortality in the combinations far exceeded mortality caused by either pathogen tested alone. Additionally, Mwamburi et al. (2009) observed larvicidal and adulticidal effects against house flies in poultry houses when chicken feed was treated with *B. bassiana* and *Bacillus thuringiensis* var. *israelensis*. In a study with another fungal pathogen, *Metarhizium robertsii*, a synergistic relationship was observed when the fungus was paired with *B. thuringiensis* var *morrisoni* against Colorado potato beetle larvae (Yaroslavtseva et al. 2017). These instances may reflect reduced host immune responses to the fungus caused by infection with *B. thuringiensis* (Yaroslavtseva et al. 2017).

Combinations of two entomopathogenic fungi have been tested as well. Treating lettuce disks with *B. bassiana* combined with another fungal entomopathogen, *Metarhizium flavorviride*, resulted in higher grasshopper mortality than when the individual fungi were tested alone (Inglis et al. 1997). In other cases, combinations of two fungal pathogens resulted in mortality that was at most additive and sometimes only equal to mortality caused by the more virulent pathogen when tested alone (Devi et al. 2006, Santos et al. 2006).

Combinations of *B. bassiana* or *M. anisopliae* with entomopathogenic nematodes (*Heterorhabditis* and/or *Steinernema* spp.) have been found to work synergistically

against white grubs, *Hoplia philanthus* Fuessly (Coleoptera: Scarabaeidae) (Ansari et al. 2006) and black vine weevil (Ansari et al. 2010), but not against cattle fever ticks (Monteiro et al. 2013) or masked white chafer grubs (Wu et al. 2014). The efficacy of combinations involving entomopathogenic nematodes is interesting in that the nematodes themselves act as host and vector for bacterial symbionts of the genera *Xenorhabdus* and *Photorhabdus* (Atwa 2014). The role of the nematode is to invade the hemocoel of the host and release the bacteria, which generally results in host death 2 to 3 days later (Kaya and Gaugler 1993). Combinations of such nematodes with fungi, therefore, involve the net effect of three pathogenic organisms rather than two.

Moreover, the mode of action of entomopathogenic nematodes provide an example of one organism that is highly pathogenic if it can reach the hemocoel (*Photorhabdus* or *Xenorhabdus* spp.) being delivered by another that is capable of gaining entry (the nematode itself). Can *B. bassiana*, with its ability to penetrate the host cuticle, be used to deliver fast-killing bacterial pathogens in an analogous manner? This was the primary question of my MS research, using house flies as a model. To my knowledge, the only precedent in the literature was a study by Ledvev et al. (2008), who examined combinations of a *Pseudomonas* spp. with *B. bassiana* and *M. anisopliae* against migratory locust nymphs. Pathogen combinations caused substantially higher mortality and greatly shortened the time to death compared with individual pathogens tested alone. The locusts who died from mixed infections showed symptoms of bacteriosis, supporting the idea that the bacteria, delivered into the host hemocoel by *B. bassiana*, were responsible for the faster kill times (Lednev et al. 2008).

Successful use of combination products in a topical form requires adequate penetration of the house fly cuticle by *B. bassiana,* and this involves several steps. Conidia must adhere to the fly cuticle, which depends on the charge, hydrophobicity and other surface properties of both the host and pathogen. The next step is to overcome several obstacles to gain entry into the host. First, the conidia must survive the house fly's extensive grooming activities (Jacques et al. 2017). Second, the environment and surface chemistry of the epicuticle must be supportive of conidial germination (Pedrini et al. 2013). Third, the developing fungus must penetrate the heavily sclerotized exoskeleton. Once in the hemocoel, fungal survival depends on avoiding the immune system (Fleming et al. 2014, Nayduch and Burrus 2017, Sackton et al. 2017) and the ability of the fly to use behavior to raise its body temperature, a phenomenon known as behavioral fever (Anderson et al. 2013).

In the previous chapters, I determined the compatibility of bacterial pathogens with *B. bassiana* (Chapter 2) and identified a suitable carrier for both house fly survival and pathogen viability (Chapter 3). The selection of the three bacteria (*Pseudomonas protegens, Photorhabdus temperata,* and *Serratia marcescens*) for this research was based on expected virulence if they gained access to the host hemocoel. However, no information was available about the virulence of these pathogens to house flies by either injection or when applied topically. The objectives of this study were to determine: 1) virulence of *B. bassiana* and the three bacterial pathogens when injected into flies; 2) virulence of *B. bassiana* and the bacterial pathogens alone or in combinations when applied topically at the same time; and 3) virulence of *B. bassiana* and the bacterial

pathogens alone or in combinations when applied topically in sequence, with two days between treatments with the individual pathogens.

Methods

House Fly Rearing and Handling

House flies were reared and anesthetized as described in Chapter 3. Flies (two- four days old) were removed from rearing cages using a vacuum and placed at -20 °C for 10 minutes to lightly sedate the flies for injecting and topical treatments. Each replicate consisted of 20 female flies, after treatment this group of flies was placed into a 500 mL plastic container containing 10 mg of diet (1:1 dry milk, sugar granules, and dry egg) and a 30 mL plastic container with water and a lid with a dental wick (to prevent flies from drowning), held at 29 °C.

Bacterial and Fungal Cultivation

Before moving forward with bioassays, it was necessary to enumerate the bacteria in the media as bacteria per mL of Luria Bertani (LB broth, Fisher BioReagents, Pittsburgh, PA) broth. This was determined for each bacterial species by making 10-fold dilutions of a culture that reached an optical density (OD) of 0.5 measured using a spectrophotometer (Biochrom LKB Ultrospec II; Cambridge, UK) at an absorbance of 600 nm and plating dilutions onto LB agar. From this, bacterial counts could be estimated for future bioassays. Once bacterial enumeration was completed, bacteria were grown until an OD of 0.5 was reached, and ratio calculations were used to determine the concentration (bacteria/mL) for each species.

For bioassays, bacterial strains were cultured on LB agar plates and incubated at 28 °C, overnight and then placed at 4 °C. From these plates, the respective bacterial colony forming unit (cfu) was picked with a sterile loop and placed into a 10 mL conical tube

containing 3 mL of LB broth to start an "overnight culture". The overnight culture was then placed in a controlled environment shaker (New Brunswick Scientific, Edison, NJ) at 250 rpm and 28 °C, overnight. The bacteria would grow overnight and reach a level where the replication would cease, known as the "stationary phase". The following morning, the overnight culture was transferred into a 50 mL glass flask in a 1:20 dilution with LB broth and grown to an OD of 0.5 measured using a spectrophotometer (Biochrom LKB Ultrospec II; Cambridge, UK) at an absorbance of 600 nm. Once OD 0.5 was reached, the ratio (OD 0.5/ OD measurement of culture x ratio of bacterial enumeration = $1x10^8$ cfu/mL) calculation from the bacterial enumeration step was used to prepare known concentrations of each bacterium for topical applications.

Beauveria bassiana was cultured on Sabouraud's dextrose agar with yeast extract (SDY; 2% glucose, 1% peptone, 0.5% yeast extract; pH 7.0) at room temperature (23 °C) for seven days to obtain heavily sporulating cultures. After one week, the plates were dried in a sterile hood for an additional week. After drying, conidia were scraped from each plate with a sterile small metal spatula and stored at -4 °C in a sterile glass vial. For conidial counts, 10 mg of dried harvested conidia were suspended into 0.1% Tween® 20 (Sigma-Aldrich, Saint Louis, MO) and distilled water. Then 10 µL aliquots of this suspension were taken and the conidia were counted using an Automatic Cell Counter (Cellometer® Vision HSL; Nexcelom Bioscience LLC, Lawrence, MA) to determine the conidial concentration.

Injections

A spectrophotometer (Biochrom LKB Ultrospec II; Cambridge, UK) was used to measure the optical density of each bacteria absorbance at 600 nm. Bacterial cells were cultured until OD 600 = 0.5, and appropriate dilutions were made to acquire the

following concentrations: $1x10^4$, $1x10^5$, $1x10^6$, and $1x10^7$ cfu/mL. Each dilution was centrifuged for one minute at 13,200 rpm to pellet and resuspended in 1X phosphate buffered saline (PBS). Dried *B. bassiana* conidia (10 mg) were weighed out and placed in a sterile 1.5 mL tube. Conidia were resuspended in 1 mL of 0.1% Tween® 20 and conidial counts were completed with an automated cell counter (Cellometer® Vision HSL; Nexcelom Bioscience LLC, Lawrence, MA). The spectrophotometer was used to measure the optical density of each bacterial strain absorbance at 600 nm, and this was used to determine the number of cfu/mL as in Chapter 2. Dilutions were made for bacterial (in 1X PBS) and fungal (in 0.1% Tween® 20) isolates to inject 1 µL of the following doses: $1x10^1$, $1x10^2$, $1x10^3$, and $1x10^4$ cfu/µL or conidia/µL.

After removing sedated flies from the freezer, they were placed on a small laboratory chill table (BioQuip® Products, Inc. Rancho Dominguez, CA) for continuous sedation. Each of the 20 female flies per replicate were injected with a microinjector (Nanomite™, Harvard Apparatus, Holliston, MA) with a 1 cc syringe and a 28-gauge needle. The microinjector was set to release 1 µL into each fly. The injection site was the thoracic mesopleuron adjacent to the wing base.

For each trial, there were three replications of each treatment (60 flies per treatment, n = 3) and fly mortality was monitored daily for four days. *Beauveria bassiana* was observed for an additional 24 hours compared to other pathogens due to its long time to death when applied topically. In each trial there were also two negative controls. The first was 20 female flies (per replicate) injected with 1 µL 1X PBS, and the second was 20 female flies (per replicate) injected with 1 µL *E. coli* strain DH5α in the following dosages: $1x10^1$, $1x10^2$, $1x10^3$, and $1x10^4$ cfu/µL. This strain of *E. coli* was

known to be avirulent for house flies (Geden, personal communication) and it was included as a control for injection with bacteria.

Dose-Response Topical Applications Individual Pathogens

Bacteria and fungi were tested by individual topical application on house fly thorax. Dilutions were made of each pathogen in 0.5% CapSil® (Aquatrols, Paulsboro, NJ) mixed with 1X PBS, the chosen carrier in Chapter 3, to prepare suspensions containing $1x10^3$ to $1x10^6$ cfu/µL. One µL of each suspension was pipetted onto the anterior thorax of 20 female house flies, and replicated three times (60 flies per treatment, n=3). CapSil 0.5% with no micoorganisms was applied to an equal number of flies as a control.

Topical Application of Pathogen Combinations

Results of dose response tests of individual pathogens indicated that $1x10^6$ conidia/µL of *B. bassiana* caused high but less than 100% mortality, and so this dose was selected to be used in combination trials. The same concentration ($1x10^6$ cfu/µL) of bacteria was used in the combinations. For each trial, 0.5% CapSil was prepared that contained no microorganisms (control), *B. bassiana* alone ($1x10^6$ conidia/µL), the bacteria alone ($1x10^6$ cfu/µL), and the two pathogens combined so that 1 µL of the combination contained the same number of cells of each as were used in the single-pathogen suspensions. Groups of 20 female flies were treated with 1 µL of the suspensions and monitored for mortality daily for seven days. The experiment was replicated three times (60 flies per treatment, n=3).

Sequential Topical Applications

This bioassay was set up the using the same doses that were used in the combination trials. In these tests, flies were first treated with one pathogen and then with

the second one two days after the first application. That is, flies were either treated with *B. bassiana* first and the bacteria two days later or with the bacteria first and the *B. bassiana* two days later. Three controls were conducted at the time of the initial treatment including *B. bassiana* alone, bacteria alone, and CapSil 0.5% alone. The experiment was run for seven days as with the previous experiment, but it should be noted that the design meant that the pathogens that were applied second in the sequence had two days less to manifest their effects than when they were applied first. Twenty female flies were sedated and topically treated on the anterior thorax with 1 μL of the suspensions, and mortality was monitored. Trials were replicated three times for each bacteria and *B. bassiana* combination (60 flies per treatment, n=3).

Statistical Analysis

For all topical application bioassays (individual, combinations, and sequential), comparisons of fly mortality between time points were analyzed using a linear mixed model fitted with the effects of treatment, time and the interaction using repeated measures ANOVA through the Mixed Procedure as implemented in SAS (Proc Mixed), version 9.4 (SAS Institute, Cary, NC). Residual terms were modelled by considering an autoregressive order 1 error structure and the degrees of freedom were adjusted using the Kenward-Roger method. Adjusted treatment means were compared using Tukey's honest significant difference tests at $\alpha=0.05$. For combinations and sequential treatments, Tukey's tests were only done at three time points three, five, and seven days after treatment.

Results

Fly Mortality from Injections

All pathogens at the higher doses caused high mortality but at different time points. *Photorhabdus temperata* caused around 40% mortality at the lowest dose ($1x10^1$ cfu) 48 hours post injection, whereas the highest dose ($1x10^4$ cfu) caused roughly 98% mortality after only 24 hours (Table 4-1, Fig. 4-1). *Serratia marcescens* caused half (20%) the mortality at the lowest dose compared to *P. temperata*, and about 90% mortality at the highest dose at 24 hours (Table 4-2, Fig. 4-2). In contrast, *P. protegens* at the lowest dose caused <10% mortality initially (24 hours), but mortality increased to 78% after 48 hours. Mortality at the highest dose was 100% at 24 hours post injection (Table 4-3, Fig. 4-3). Mortality from *B. bassiana* at the lowest dose was <5% after 24 hours and only reached ca. 15% after 96 hours (Fig. 4-4). At the highest dose of *B. bassiana*, a sudden increase in fly mortality was observed (82%) at 72 hours post injection when compared to 48 hours (<15%). Mortality at 96 hours was similar to 72-hour mortality at the high dose (Table 4-4, Fig. 4-4).

Topical Applications of Individual Pathogens

A dose-response relationship for mortality was observed for all four pathogens when applied topically, although the relationship was stronger with some pathogens than with others (Figures 4.5-4.8). Mortality due to *P. temperata* was low at all doses throughout the test and only reached 17 and 22% mortality on day 7 at the high doses of 10^5 and 10^6 cfu, respectively (Figure 4-5). Results with *P. temperata* were variable, and mortality was similar at the two high doses (10^5 and 10^6 cfu).

Similarly, *Serratia marcescens* at the higher doses ($1x10^5$ and $1x10^6$ cfu) killed more flies (13%) on day two compared to lower doses (ca. 7%) (Fig. 4-6). Fly mortality

increased to 18 and 22% at 10^5 and 10^6 cfu, respectively, on day five and increased little on subsequent days (Fig. 4-6).

Pseudomonas protegens had the highest topical virulence of the three bacterial pathogens (Fig. 4-7). Mortality at $1x10^6$ cfu was 49% two days after application and reached 60% by day seven. Mortality at the two lower doses ($1x10^3$ and $1x10^4$ cfu) never reached 35% throughout the test (Fig. 4-7).

All doses of *B. bassiana* killed <20% of the flies until day five, when mortality at $1x10^5$ and $1x10^6$ conidia increased to 40 and 68%, respectively. Mortality increased until day seven, reaching 70% at 10^5 conidia and 90% at 10^6 conidia (Fig. 4-8).

Topical Applications of Combined Pathogens

Results of topical applications with single versus combined pathogens are presented in Figures 4-9 through 4-14. Data for each *B. bassiana*-bacteria pairing are presented in two figures. The first figure shows daily mortality with standard errors for the duration of the seven-day trial (Fig. 4-9, 4-11 and 4-13). The second figure shows a snapshot comparing treatment means at the three selected time points of days three, five, and seven after treatment (Fig. 4-10, 4-12, and 4-14).

Topical applications of *B. bassiana* and *P. temperata* did not result in substantial mortality, either alone or in combination, until day five, and there was no indication that the two-pathogen combinations caused higher mortality than *B. bassiana* alone throughout the experiment (Fig. 4-9). Significant differences were seen with treatment over time ($F_{21, 64} = 6.84$, $P<0.001$). There was no significant treatment effect on day three ($P>0.05$) (Fig 4-10). On day five, mortality in the *B. bassiana* alone and combination treatments (40-53%) did not differ significantly from each other but both treatments caused significantly higher mortality than the control ($P<0.05$). On day seven, mortality

in the *B. bassiana* alone and combination treatments (65-85%) did not differ significantly from each other but both treatments caused significantly higher mortality than the control or *P. temperata* alone ($P<0.001$).

Results with *S. marcescens* combinations (Figs. 4-11 and 4-12) were somewhat similar to those with *P. temperata*, with low mortality until day five and no evidence of higher mortality in the two-pathogen combination compared to the treatment with *B. bassiana* alone. A significant interaction was observed between treatment and time ($F_{21, 64} = 4.31$, $P<0.001$). There was no significant treatment effect on day three ($P>0.05$) (Fig. 4-12). On day five, mortality in the *B. bassiana* alone and combination treatments (38-54%) did not differ significantly from each other and the only treatment that was significantly different from the control was *B. bassiana* alone ($P<0.05$). On day seven mortality in the *B. bassiana* alone and combination treatments (65-85%) did not differ significantly from each other but both treatments caused significantly higher mortality than the control ($P<0.001$).

In contrast, *P. protegens* combined with *B. bassiana* caused 50% mortality after 48 hours, and continued in an upward trend throughout the seven-day observation time (Fig. 4-13). There was a significant difference with treatment over time ($F_{21, 64} = 10.52$, $P<0.0001$). On day three, the two treatments with *P. protegens* caused significantly higher mortality (53-60%) than the control or *B. bassiana* alone ($P<0.01$) (Fig 4-14). Mortality in the three-pathogen treatments was similar but significantly higher than the control on days five ($P<0.001$) and seven ($P<0.001$).

Sequential Applications of Pathogens

Results of topical applications of pathogens in a sequential manner are presented in Figures 4-15 through 4-20. As in the previous section, data for each *B. bassiana*-

bacteria pairing are presented in two figures, one showing the daily mortality over seven days (Figures 4-15, 4-17, and 4-19) and the other a snapshot of the results on days three, five, and seven for means comparison (Figures 4-16, 4-18, and 4-20).

Mortality due to *P. temperata* or *B. bassiana* applied alone were similar to the previous tests, with maximum mortality on day seven of 21% and 85%, respectively (Fig. 4-15). Treating flies with *B. bassiana* followed two days later with *P. temperata* resulted in an apparent increase in mortality on day four compared with *B. bassiana* alone (37% and 20%, respectively), but not on days five through seven. Treatment with *P. temperata* followed by *B. bassiana* resulted in mortality on day seven (five days after *B. bassiana* application) that was similar to mortality due to *B. bassiana* alone on day five (ca. 60% in both cases). A significant difference was observed with treatment over time ($F_{28, 80}$ = 8.94, P<0.0001). When data were examined at set time points there were no significant treatment effects on day three (Figure 4-16) (P>0.05). On day five, mortality in the two treatments that included initial *B. bassiana* applications were similar to each other and significantly higher than the other three treatments (P<0.01). On day seven mortality in the three treatments that included *B. bassiana* were similar to each other and significantly higher than the controls and *P. temperata* alone (P<0.01).

Similar results were observed in sequential treatments of *S. marcescens and B. bassiana,* with no evidence of accelerated mortality in species combinations compared with *B. bassiana* alone (Figures 4-17 and 4-18). There was a significant difference with treatment over time ($F_{28, 80}$ = 2.7, P<0.0001). No significant treatment effects were observed on days 3 (P>0.05) or 5 (P>0.05) (Figure 4-18). On day seven, mortality in the

three treatments that included *B. bassiana* were similar to each other and significantly higher than the controls and *S. marcescens* alone ($P<0.01$).

Mortality in the treatments that included initial application of *P. protegens* were noticeably higher (35%) than the others (<17%) at two days post-treatment, with considerable convergence on day four onwards (Figure 4-19). Similarly, like all other treatments, there was a significant difference with treatment over time ($F_{28, 80} = 2.19$, $P<0.0001$). When data were examined at set time points there were no significant treatment effects on day three (Figure 4-20) ($P>0.05$). On day five, mortality was highest when *P. protegens* was followed by *B. bassiana*, but this was only significantly different from the control ($P<0.001$). Mortality on day seven was highest in the three treatments that included *B. bassiana* ($P<0.05$).

Discussion

Entomopathogens occur in every ecosystem on all continents and are a highly diverse group that includes fungi, bacteria, viruses, microsporidia, entomopathogenic nematodes, and protists (Vega and Kaya 2012). Many of them have been studied and developed for their potential as biological control agents against insect pests (Ravensberg 2011, Sundh and Goettel 2013). The concept of combining two distinctly different emtomopathogens to improve efficacy against target pests has been considered and tested (Inglis et al. 1997, Koppenhöfer and Kaya 1997, Ansari et al. 2006, Kwak et al. 2015, Wakil et al. 2017, Yaroslavtseva et al. 2017), but such combinations can be a complicated due to some important factors.

Perhaps the most important consideration is whether the organisms will be antagonistic or competitive with each other to an extent that one or the other cannot sustain life. For example, associations in nature between fungi and bacteria are complex

and they can influence each other's survival and pathogenicity via resource competition, antifungal compounds, antibiotics, and toxins (Frey-Klett et al. 2011). Compatibility between *B. bassiana* and the three bacteria species used here was addressed in Chapter 2, where I observed that *P. protegens* and *P. temperata* inhibited *B. bassiana* growth on Sabouraud's dextrose agar with yeast extract (SDY) plates, whereas *S. marcescens* did not inhibit *B. bassiana*. Another factor in combining two entomopathogens is that they may require different nutrients, and finding media that suits both without compromising their pathogenicity can be a challenge. For instance, carbon sources serve fungal and bacterial pathogens differently (Zhou et al. 2016) and changing the carbon source can change genetic expression of necessary virulence factors (Görke and Stülke 2008, Ene et al. 2014). Another factor is the identification of surfactant solutions that can be used with both organisms without affecting viability. Application of pesticides and biopesticides typically involve spray application with a water carrier. Because many pesticides do not mix well with water, an adjuvant is usually added to ease mixing and application. There are many types of adjuvants that can be added to a water carrier to create formulations. A type of adjuvant known as a surfactant/wetting agent/spreader physically alters the surface tension of droplets improving pesticide application. These surfactants differ in size of lipid and charge, and are mostly used for application in agricultural pest management. Because of the hydrophilic and hydrophobic components of water carriers with surfactants, they are a useful resource for applying microorganisms directly onto insects (addressed in Chapter 3). Lastly, when combining different pathogens, the mechanism and timing of their individual modes of infection may affect whether simultaneous or sequential treatments would result in higher or faster host mortality.

The bacteria chosen for this study were selected because all were expected to have high virulence when injected but little virulence when applied topically. As predicted, all caused nearly 100% mortality within 24 hours of injection at higher concentrations. Results with topical applications were more variable. Mortality was low when *P. temperata* and *S. marcescens* were applied topically and remained <26% even after treatment with $1x10^6$ cfu. In contrast, topical application of *P. protegens* resulted in surprisingly high (ca. 50%) house fly mortality after only 48 hours (Fig. 4-7).

Because *P. protegens* are motile (Song et al. 2016), topical mortality may have been partially due to bacteria entering the fly through natural openings such as the spiracles, anus, or mouthparts. Another factor could be the toxins associated with this species, especially FitD (for *P. fluorescens* insecticidal toxin) (Péchy-Tarr et al. 2008), which might have been responsible for high fly mortality. Rangel et al. (2016) observed that *P. protegens* strain pf-5 exhibited significant oral toxicity against *D. melanogaster* and attributed the mortality to FitD. In preliminary experiments, that are not presented herein, I observed very high initial fly mortality after topical treatment with *P. protegens*. In many cases the treated flies never recovered from the anesthetic after application (controls recovered). I suspected that exotoxins present in the *P. protegens* growth media were responsible and began rinsing the bacterial pellets during processing with 1X PBS before adding the surfactant (CapSil) used for the suspensions. This greatly reduced immediate fly mortality to acceptable levels, but toxins may still have played a role in the topical mortality seen with this species. At this point I do not know whether *P. protegens* or its toxins entered the fly at/near the point of application or whether they were transferred to the mouthparts, and possibly the gut, by grooming. To my knowledge this

is the first report of *P. protegens* affecting house flies. Further research with this species and associated toxins could lead to its development as a biocontrol agent for the house fly.

Combinations of *P. protegens* with *B. bassiana* suggested a complementary effect on house fly mortality. These combinations resulted in much higher mortality than *B. bassiana* alone through four days after topical application, with *B. bassiana* accounting for additional mortality on subsequent days (Fig. 4-12, and Fig. 4-13). In contrast, no such complimentary effect was observed in combinations of *B. bassiana* with *P. temperata* or *S. marcescens*. Fly mortality after exposure to combinations with the latter two species never differed significantly from treatment with *B. bassiana* alone.

The overall hypothesis of my project was that germinating conidia of *B. bassiana* on the fly cuticle would create wounds that would allow other pathogens to reach the fly hemocoel, and that these secondary pathogens would result in faster host death than when *B. bassiana* was used alone. As noted above, the experiments with simultaneous pathogen treatment did not support that hypothesis. One possible reason for this lack of synergy was timing. Conidia of *B. bassiana* generally take 14 to 24 hours to germinate (Hywel-Jones and Gillespie 1990, Luz and Farques 1997, Iskandarov et al 2006), and it was uncertain whether the bacteria would survive long enough to exploit the cuticular insult caused by fungal penetration. Moreover, normal grooming behavior can result in substantial removal of bacteria from the cuticle (Zukovskaya et al 2013). In subsequent tests with sequential treatments I gave the *B. bassiana* a 48 hour "head start" to breach the cuticle before applying the bacteria. Once again, I saw no evidence for higher or faster mortality than when *B. bassiana* was used alone. It is possible that 48 hours was

too long, and that any breach in the cuticle caused by *B. bassiana* penetration had already been repaired by the fly before application of the second pathogen. House flies can recover from mechanical wounds very rapidly, as evidenced by their low mortality after being injected with a 28-gauge syringe in the first experiment of this chapter (control mortality <3%). Rapid wound healing is critical for flies under field conditions such as poultry houses, where they are constantly exposed to injury and potentially harmful bacteria (Nayduch and Burrus 2017). Such septic conditions may be more favorable to bacterial invasion after fungal treatment than those used in our bioassay, which involved discrete bacterial challenges at fixed times. It would be interesting to observe the effects of adding the bacteria at shorter intervals of two to four hours after *B. bassiana* conidia treatment to determine whether there is a narrow window after fungal application when the fly is vulnerable to invasion by a secondary pathogen.

Another factor that may have influenced my results was the application method of the pathogens. I chose an assay that involved placement of a small volume of inoculum (1 µL) on the dorsal thorax of the fly, which allowed precise control of the timing, dose, and location of the treatment. The epicuticle of the fly thorax is particularly well-sclerotized and presumably more resistant to fungal penetration than other body regions such as intersegmental membranes, the ventral side of the abdomen, or the soft portions of the labellum (Ortiz-Urquiza and Keyhani. 2013). Grooming could involve another potential route of infection via contamination of the mouthparts by tarsi and possible entry into the alimentary tract. Whole-body treatments such as sprays or dips might be more permissive of secondary infection by the bacteria because of the opportunities for more vulnerable body regions to be exposed to the pathogens, but this method prevents

the application of precise doses, results in unnatural exposure and causes spurious mortality in controls. Another experimental approach that would be useful would be to treat flies with *B. bassiana* and then place the treated flies in septic field habitats such as poultry, dairy, or pig housing where they would be exposed to natural bacteria in the environment.

In summary, I found that *P. temperata*, *S. marcescens* and *P. protegens* were all highly virulent when injected into the fly. Unfortunately, there was little evidence to support the hypothesis that *B. bassiana* could be used as a "Trojan horse" to deliver bacteria into the host hemocoel of the fly in a manner analogous to the way in which entomopathogenic nematodes naturally deliver *Photorhabdus* spp. and *Xenorhabdus* spp. into the host (Atwa 2014). Further studies using other pathogens and alterations in the timing and method of application may yet uncover vulnerabilities to species combinations that were not observed in this study. Perhaps the most exciting discovery of this research was the high virulence of *P. protegens* and its toxins for house flies, both alone and in concert with *B. bassiana*. Future work should elaborate on this observation and include assays for oral toxicity in adult flies, potential use as a larvicide, and impact on beneficial arthropods in livestock and poultry systems. Development of *P. protegens* as a biological control agent for house flies could result in a welcome and needed additional tool for management of this difficult pest.

Table 4-1. Mortality of female house flies for three days after receiving 1 µL injections of 1X phosphate buffered saline (PBS) alone, with $1x10^4$ cfu of an avirulent strain of *Escherichia coli*, and four doses of *Photorhabdus temperata.*

Dose (cfu)	Mean (SE) % mortality at day post-injection*		
	1 day	2 days	3 days
$1x10^1$	0.0 (0.0) a	38.3 (6.7) a	46.7 (9.0) a
$1x10^2$	1.7 (0.6) a	88.3 (2.8) b	90.0 (2.2) b
$1x10^3$	15.0 (4.0) a	85.0 (4.0) b,c	100.0 (0.0) b
$1x10^4$	97.0 (1.2) b	97.0 (1.2) c	97.0 (1.2) b
Control (PBS)	0.0 (0.0) a	0.0 (0.0) a	1.7 (1.6) a
Control (10^4 cfu *E. coli*)	3.0 (2.3) a	5.0 (3.5) a	5.0 (3.5) a

* Means within columns followed by the same letter are not significantly different (P>0.05, Tukeys HSD)

Table 4-2. Mortality of female house flies for three days after receiving 1 µL injections of 1X phosphate buffered saline (PBS) alone, with $1x10^4$ cfu of an avirulent strain of *Escherichia coli*, and four doses of *Serratia marcescens*.

Dose (cfu)	Mean (SE) % mortality at day post-injection*		
	1	2	3
$1x10^1$	1.7 (1.6) a	13.9 (4.0) a	13.9 (0.0) a
$1x10^2$	17.2 (5.4) a	62.2 (2.8) b	74.4 (5.7) b
$1x10^3$	52.2 (1.5) b	95.6 (1.2) b	100.0 (4.4) b
$1x10^4$	90.0 (0.0) b	93.3 (0.0) b	93.3 (0.0) b
Control (PBS)	0.0 (0.0) a	0.0 (0.0) a	1.7 (1.6) a
Control (10^4 cfu *E. coli*)	3.0 (2.3) a	5.0 (3.5) a	5.0 (3.5) a

* Means within columns followed by the same letter are not significantly different (P>0.05, Tukeys HSD)

Table 4-3. Mortality of female house flies for three days after receiving 1 µL injections of 1X phosphate buffered saline (PBS) alone, with $1x10^4$ cfu of an avirulent strain of *Escherichia coli*, and four doses of *Pseudomonas protegens*.

Dose (cfu)	Mean (SE) % mortality at day post-injection*		
	1 day	2 days	3 days
$1x10^1$	8.3 (6.0) a	77.8 (6.1) b	95.0 (2.8) b
$1x10^2$	7.8 (4.0) a	90.0 (10.0) b	93.3 (6.6) b
$1x10^3$	94.4 (2.9) b	100.0 (0.0) b	100.0 (0.0) b
$1x10^4$	100.0 (0.0) b	100.0 (0.0) b	100.0 (0.0) b
Control (PBS)	0.0 (0.0) a	0.0 (0.0) a	1.7 (1.6) a
Control (10^4 cfu *E. coli*)	3.0 (2.3) a	5.0 (3.5) a	5.0 (3.5) a

* Means within columns followed by the same letter are not significantly different (P>0.05, Tukeys HSD)

Table 4-4. Mortality of female house flies for four days after receiving 1 µL injections of Tween 0.1% alone, with four doses of *Beauveria bassiana*, or with $1x10^4$ cfu of an avirulent strain of *Escherichia coli* in 1X phosphate buffered saline (PBS)

Dose (cfu)	Mean (SE) % mortality at day post-injection*			
	1	2	3	4
$1x10^1$	2.5 (2.5) a	10.0 (5.0) a	10.0 (5.0) a	15.0 (5.0) a
$1x10^2$	2.5 (2.5) a	2.5 (2.5) a	2.5 (2.5) a	17.5 (12.5) a
$1x10^3$	0.0 (0.0) a	10.0 (0.0) a	22.5 (2.5) a	45.0 (10.0) a
$1x10^4$	5.0 (0.0) a	12.5 (2.5) a	82.5 (12.5) b	90.0 (5.0) b
Control (Tween 0.1%)	2.5 (0.0) a	5.0 (2.5) a	5.0 (2.5) a	5.0 (2.5) a
Control (10^4 cfu *E. coli*)	3.5 (2.3) a	5.0 (3.5) a	5.0 (3.5) a	5.0 (3.5) a

* Means within columns followed by the same letter are not significantly different (P>0.05, Tukeys HSD)

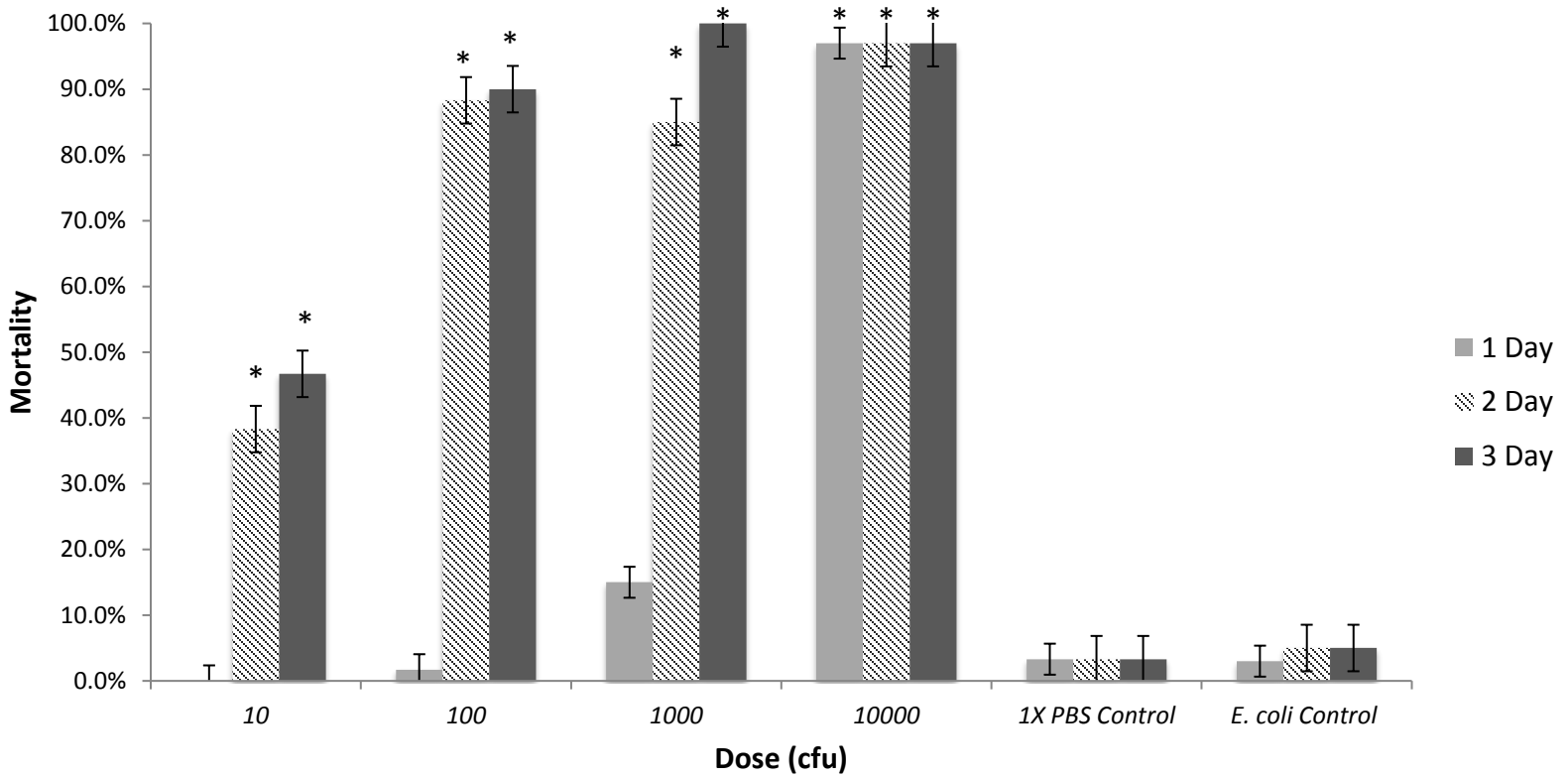

Figure 4-1. Mortality of adult house flies, *Musca domestica*, for three days following injection into the thoracic mesopleuron adjacent to the wing base with 1 µL of *Photorhabdus temperata* at different doses. Controls were 1X PBS (phosphate buffered saline) and *Escherichia coli*. Bars represent the mean percent cumulative mortality ± standard errors. Asterix * denotes $P<0.05$ compared to 1x PBS control within a time point.

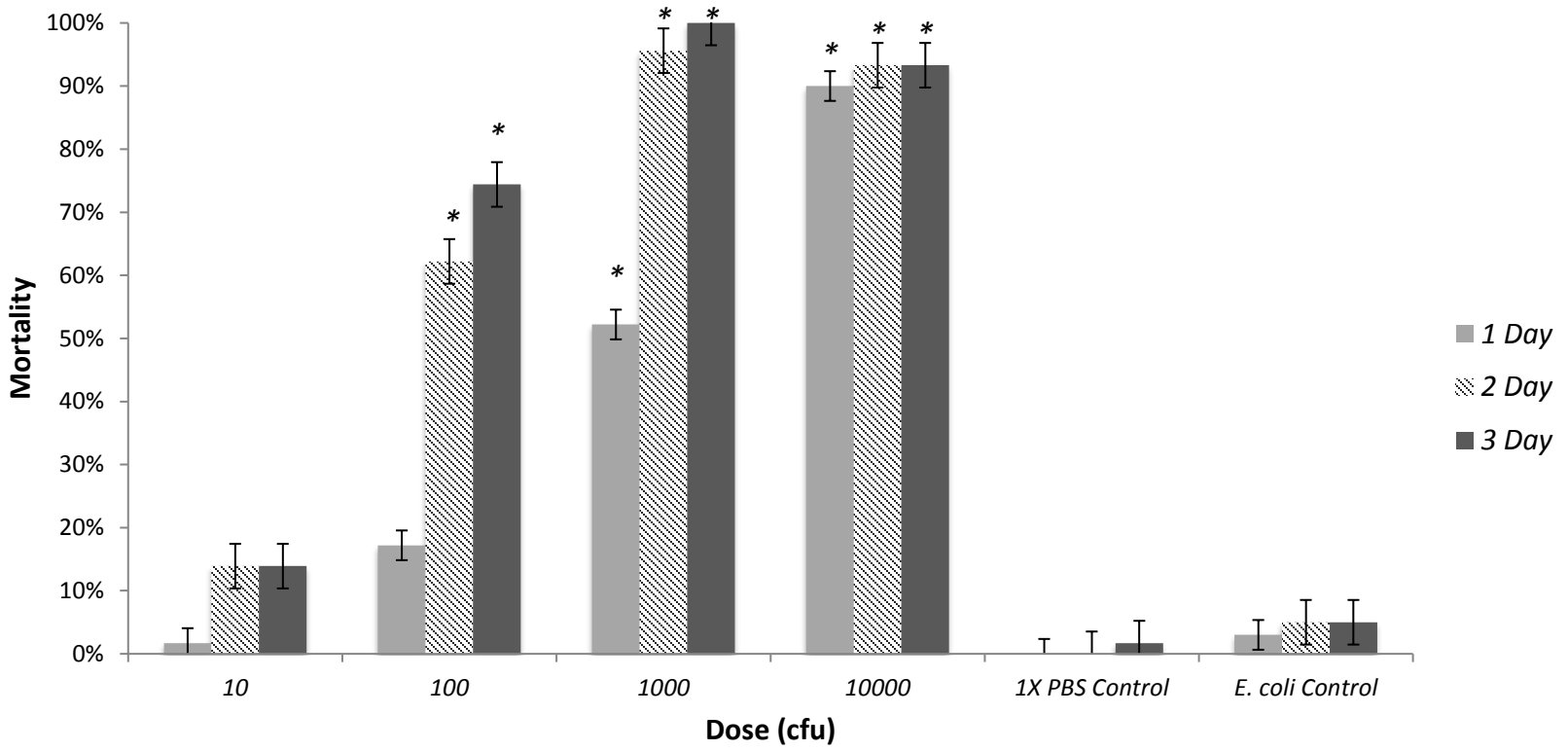

Figure 4-2. Mortality of adult house flies, *Musca domestica*, for three days following injection with 1 µL of *Serratia marcescens* at different doses. Controls were 1X PBS (phosphate buffered saline) and *Escherichia coli*. Bars represent the mean percent cumulative mortality ± standard errors. Asterix * denotes $P<0.05$ compared to 1x PBS control within a time point.

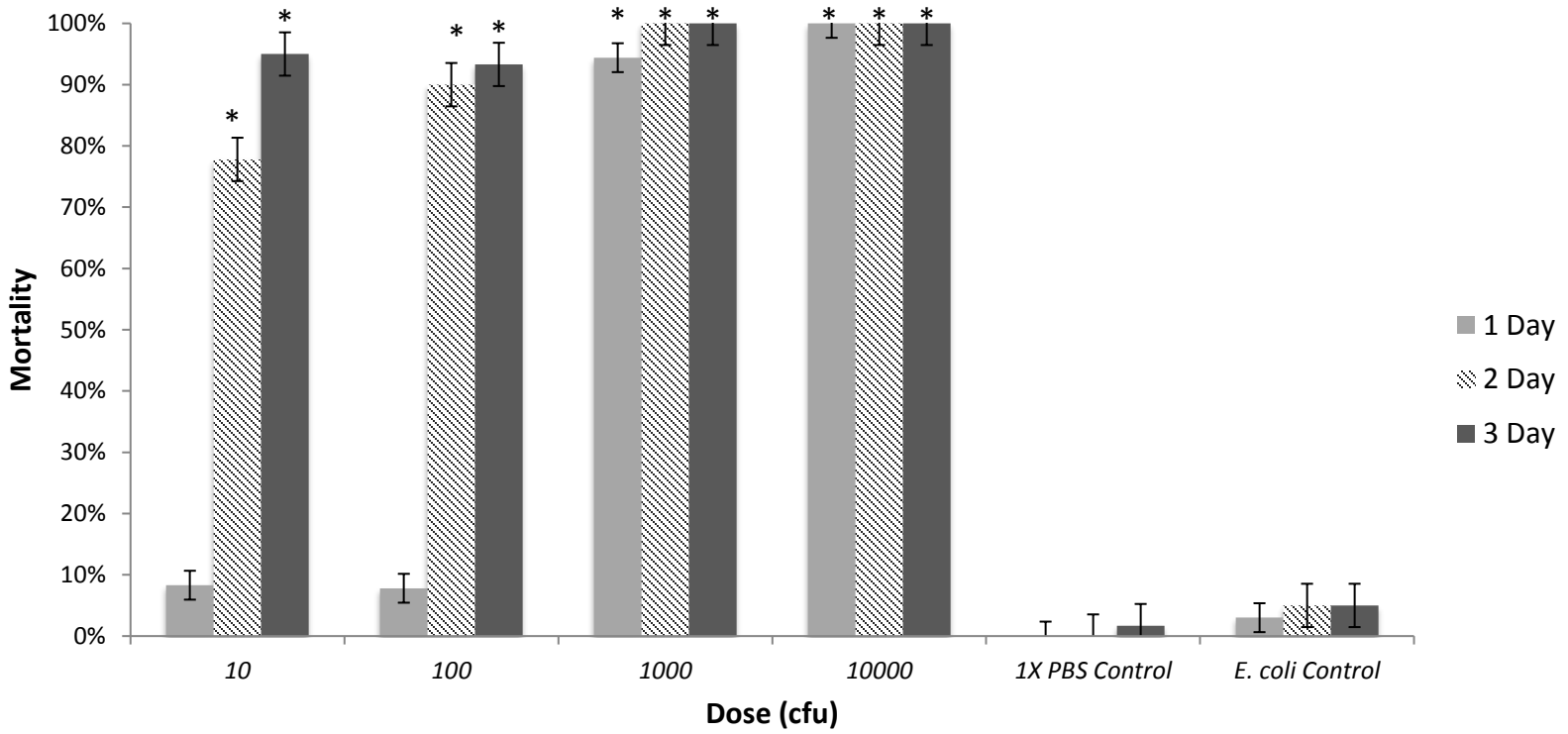

Figure 4-3. Mortality of adult house flies, *Musca domestica*, for three days following injection with 1 µL of *Pseudomonas protegens* at different doses. Controls were 1X PBS (phosphate buffered saline) and *Escherichia coli*. Bars represent the mean percent cumulative mortality ± standard errors. Asterix * denotes $P<0.05$ compared to 1x PBS control within a time point.

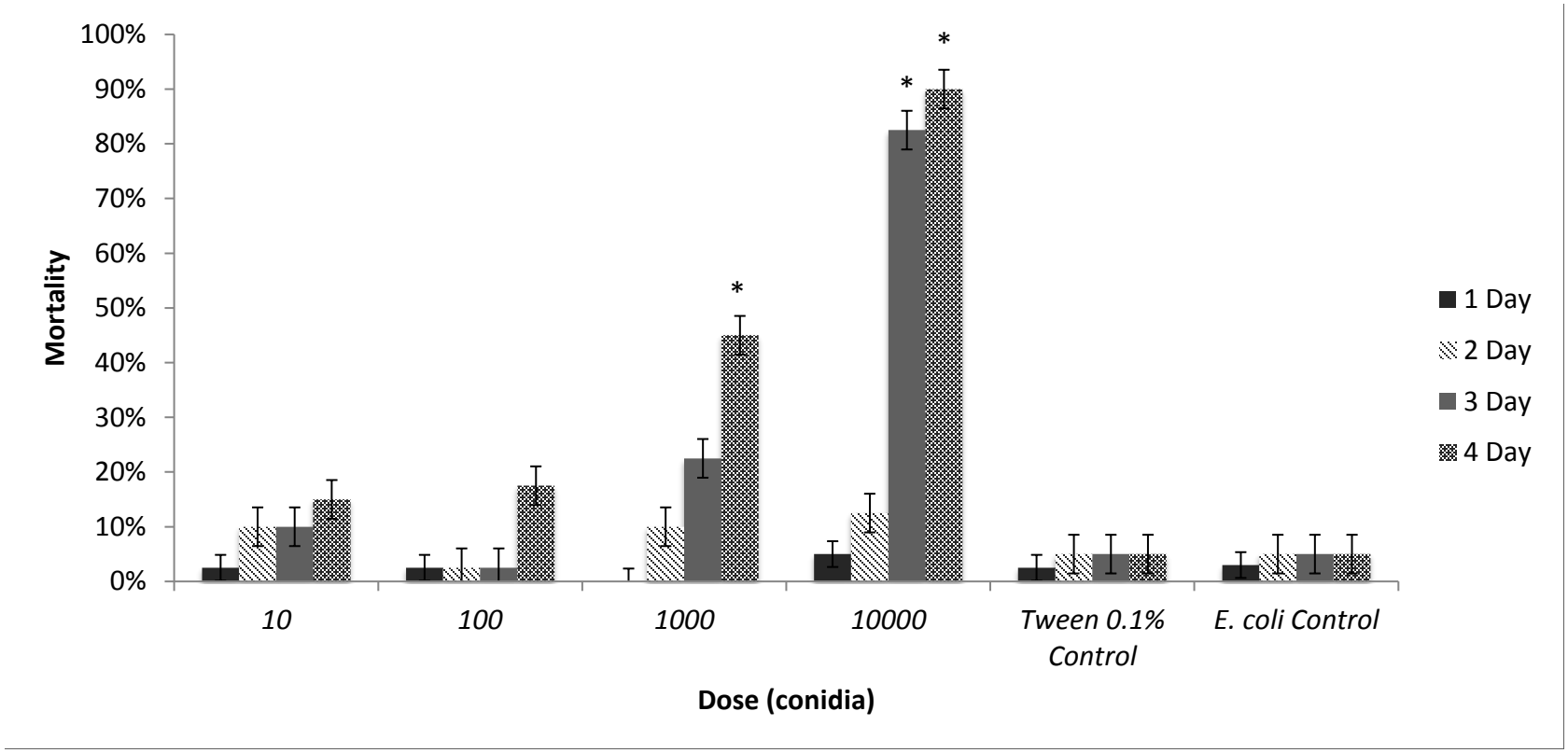

Figure 4-4. Mortality of adult house flies for four days following injection with 1 µL of *Beauveria bassiana* at different doses. Controls were 0.1% Tween 20 and *Escherichia coli.* Bars represent the mean percent cumulative mortality ± standard errors. Asterix * denotes P<0.05 compared to 1x PBS control within a time point.

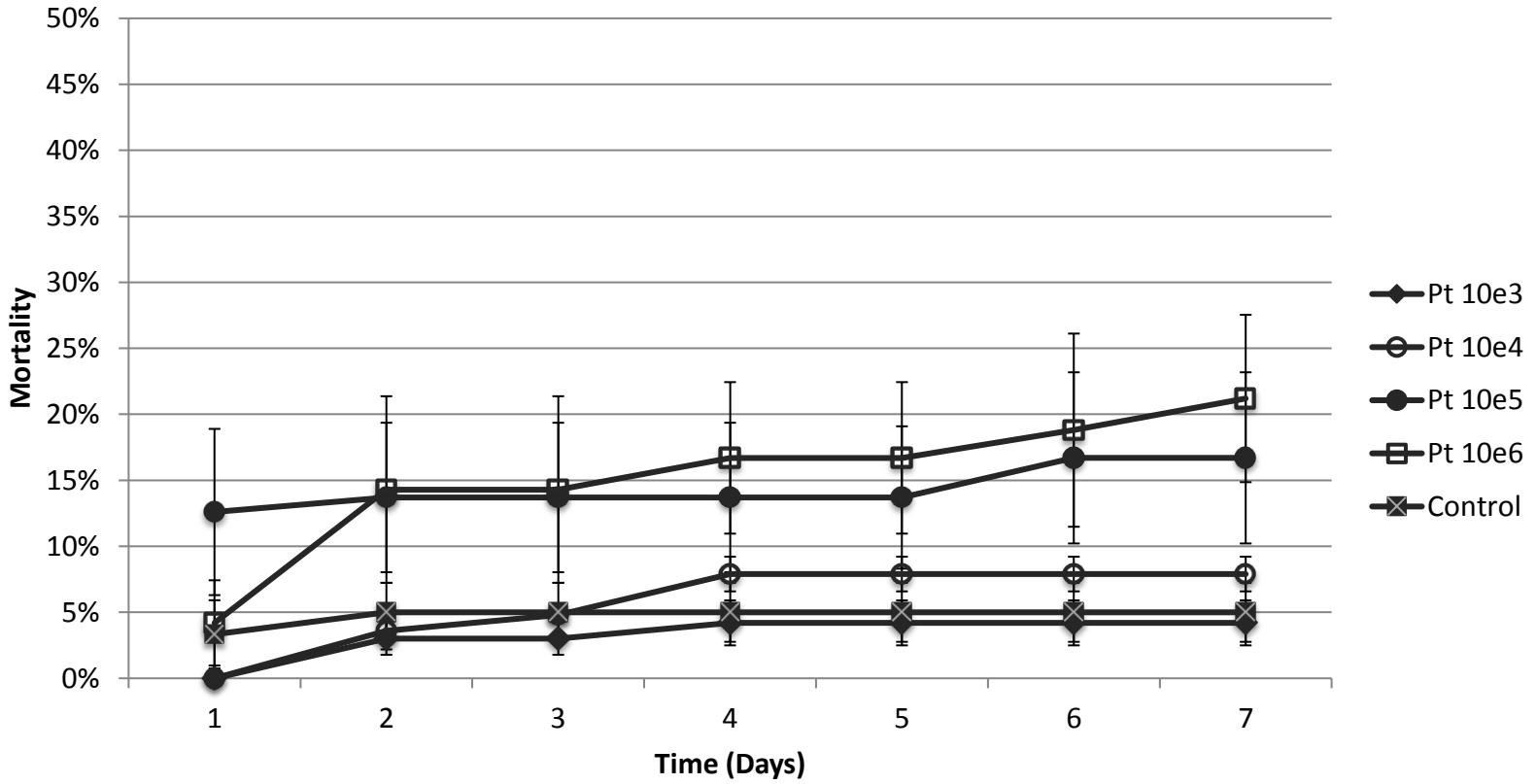

Figure 4-5. Mortality of adult house flies, *Musca domestica*, for seven days after topical application of 1 µL of 0.5% CapSil containing $1x10^3$ through $1x10^6$ *Photorhabdus temperata* colony forming unit (Pt). Control = CapSil 0.5% alone. Points on the line represent mean percentage mortality ± standard errors.

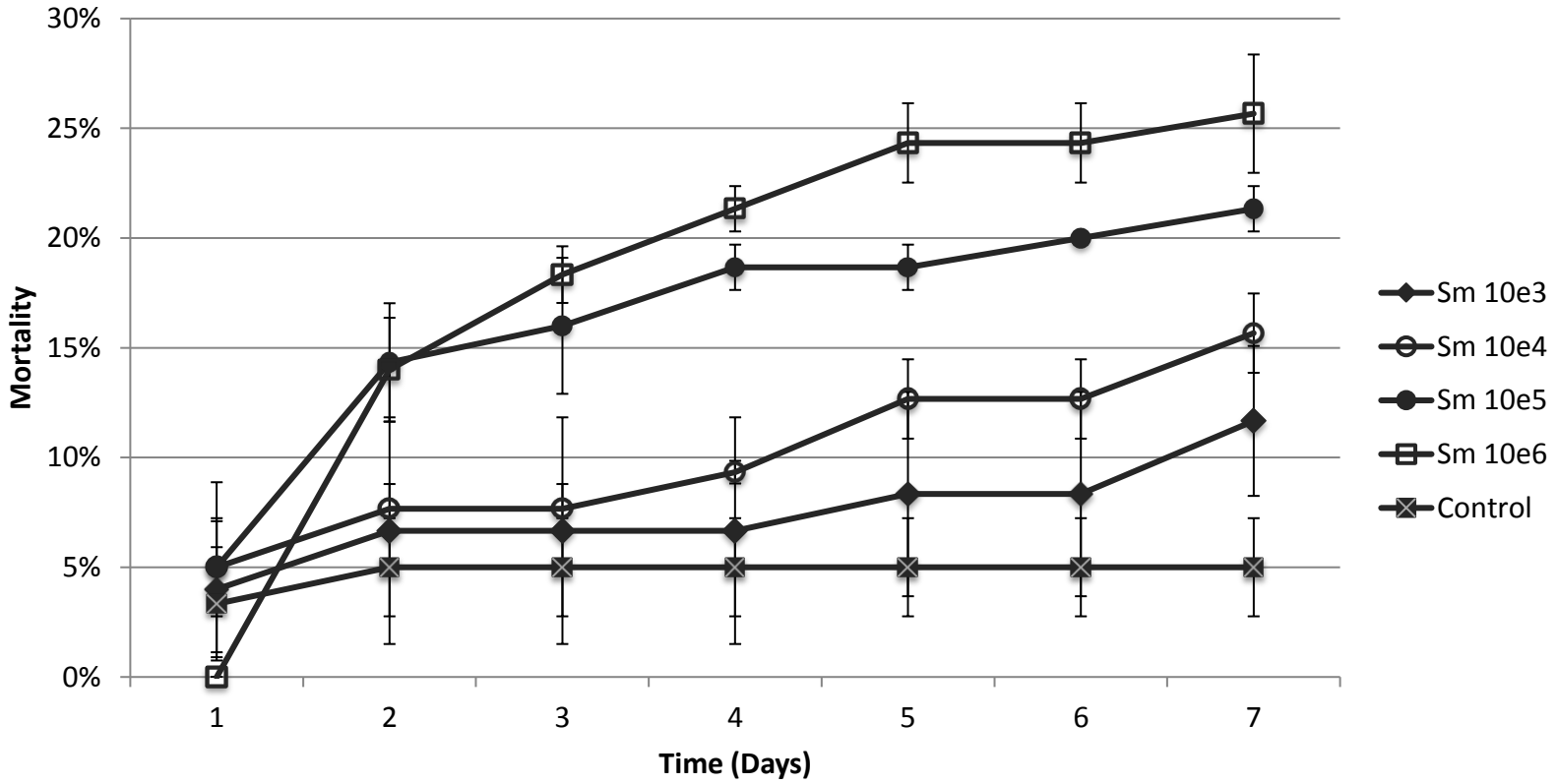

Figure 4-6. Mortality of adult house flies, *Musca domestica*, for seven days after topical application of 1 µL of 0.5% CapSil containing $1x10^3$ through $1x10^6$ *Serratia marcescens* colony forming unit (Sm). Control = CapSil 0.5% alone. Points on the line represent mean percentage mortality ± standard errors

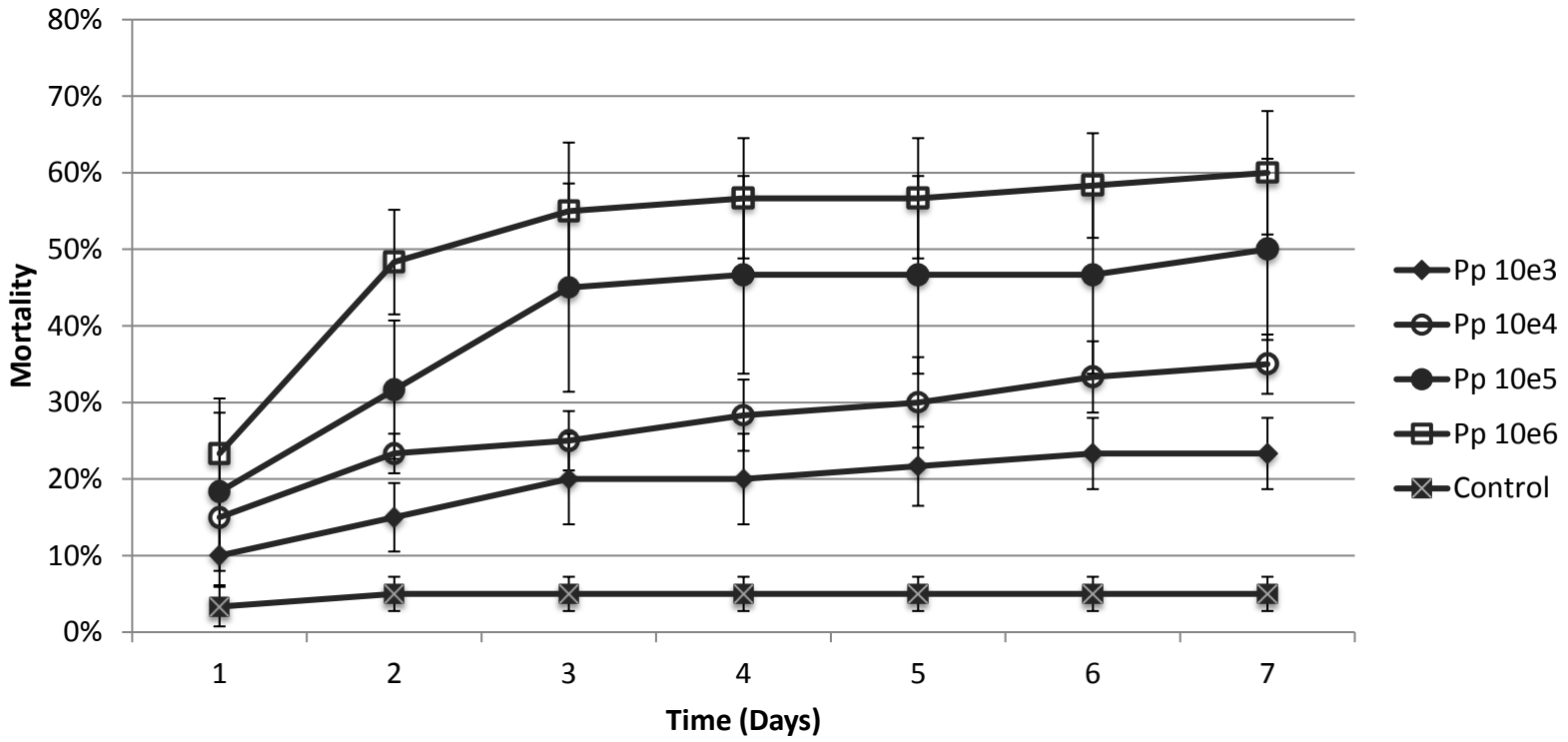

Figure 4-7. Mortality of adult house flies, *Musca domestica*, for seven days after topical application of 1 µL of 0.5% CapSil containing $1x10^3$ through $1x10^6$ *Pseudomonas protegens* colony forming unit (Pp). Control = CapSil 0.5% alone. Points on the line represent mean percentage mortality ± standard errors.

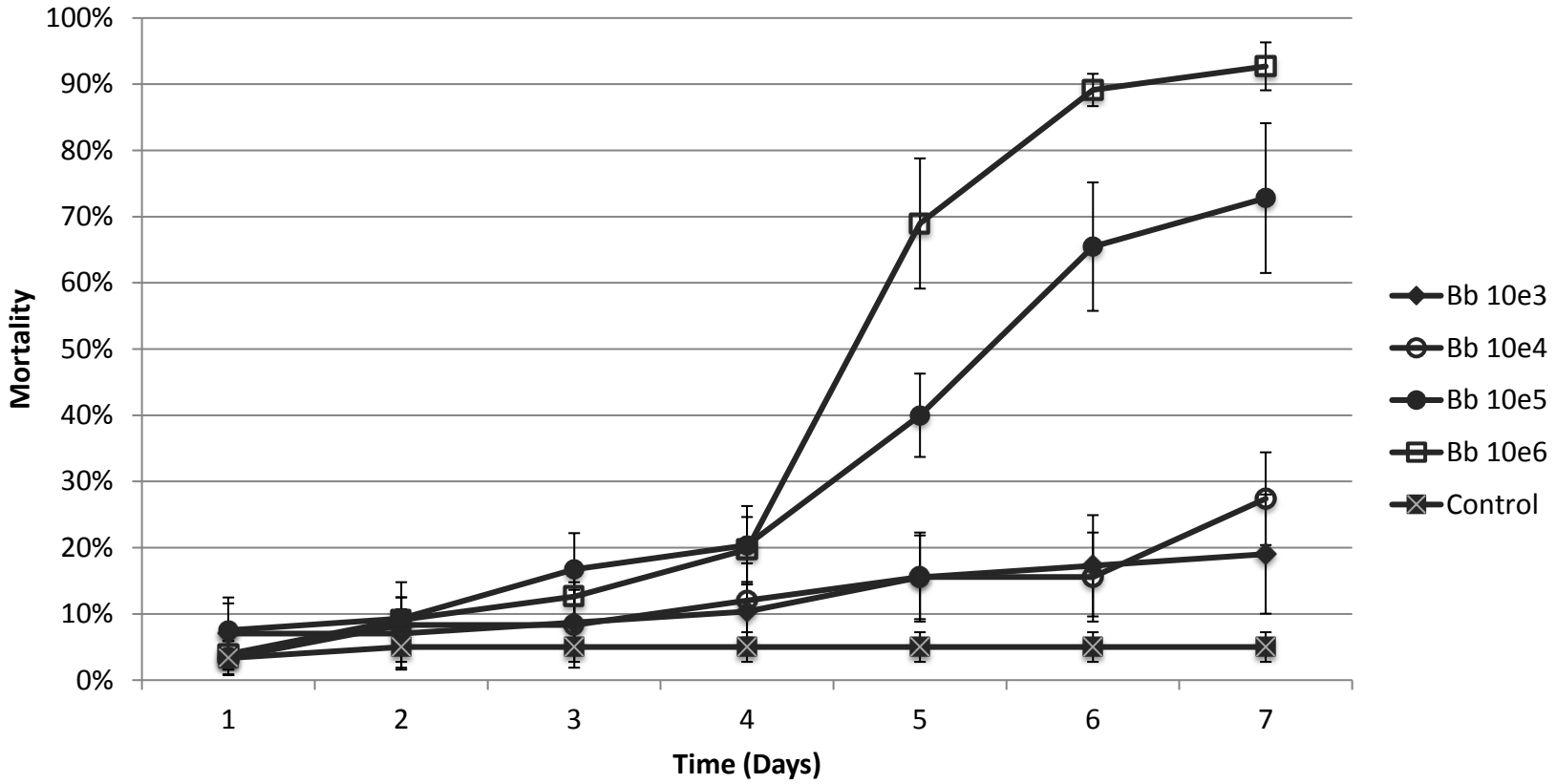

Figure 4-8. Mortality of adult house flies, *Musca domestica*, for seven days after topical application of 1 µL of 0.5% CapSil containing $1x10^3$ through $1x10^6$ *Beauveria bassiana* conidia (Bb). Control = CapSil 0.5% alone. Points on the line represent mean percentage mortality ± standard errors.

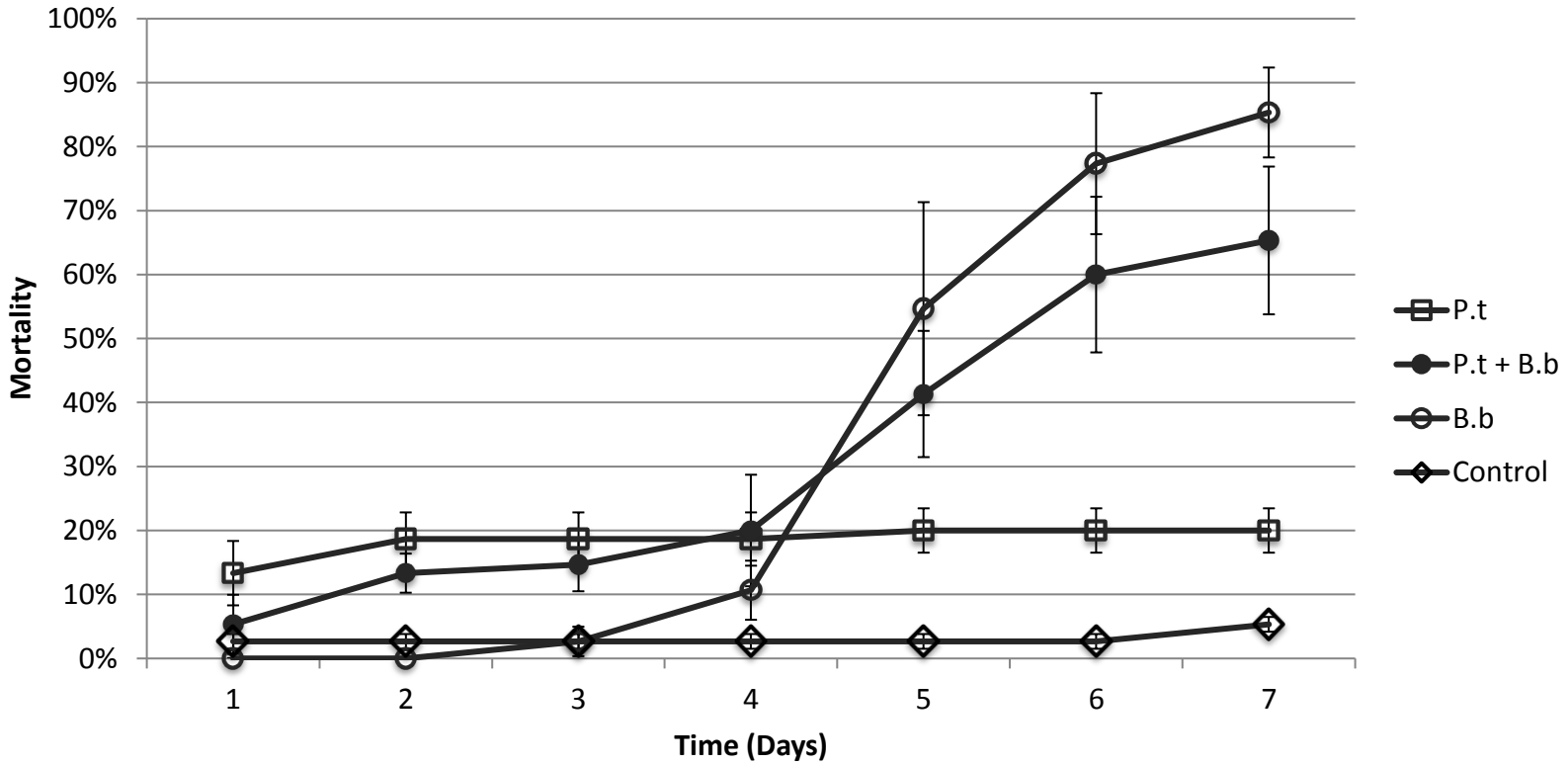

Figure 4-9. Mortality (%) of adult house flies, *Musca domestica*, for seven days after 1 µL topical application of 0.5% CapSil containing *Photorhabdus temperata* ($1x10^6$ colony forming unit) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in combination. Points on the line represent mean percentage mortality ± standard errors. P.t = *Photorhabus temperata* alone, B.b = *Beauveria bassiana* alone, P.t + B.b = two-pathogen combination, and Control = CapSil 0.5% alone.

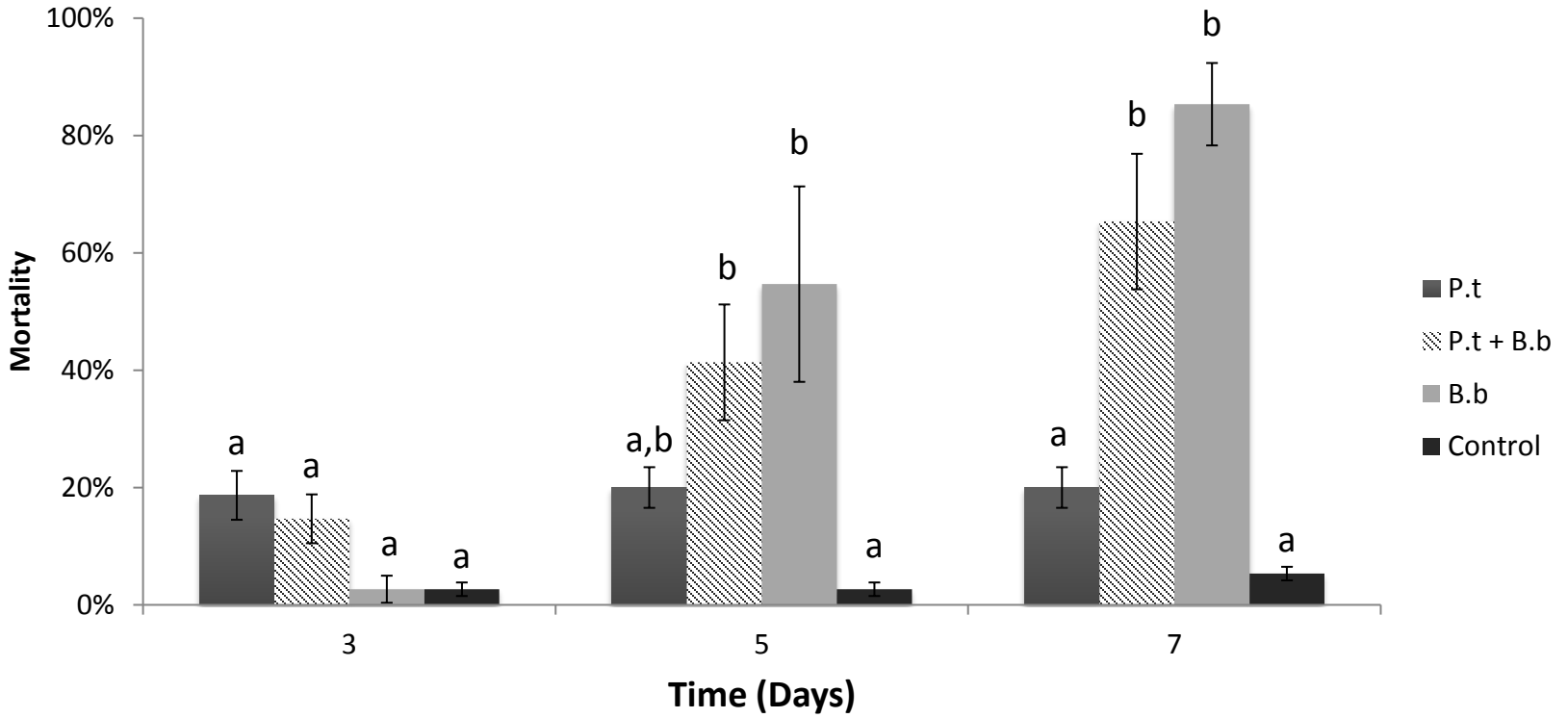

Figure 4-10. Mortality (%) of adult house flies, *Musca domestica*, three, five, and seven days after 1 µL topical application of 0.5% CapSil containing *Photorhabdus temperata* ($1x10^6$ colony forming) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in combination. Bars represent mean percentage mortality ± standard errors, and different letters within a time point denote significant differences (Tukeys HSD $P<0.05$) among treatments. P.t = *Photorhabus temperata* alone, B.b = *Beauveria bassiana* alone, P.t + B.b = two-pathogen combination, and Control = CapSil 0.5% alone.

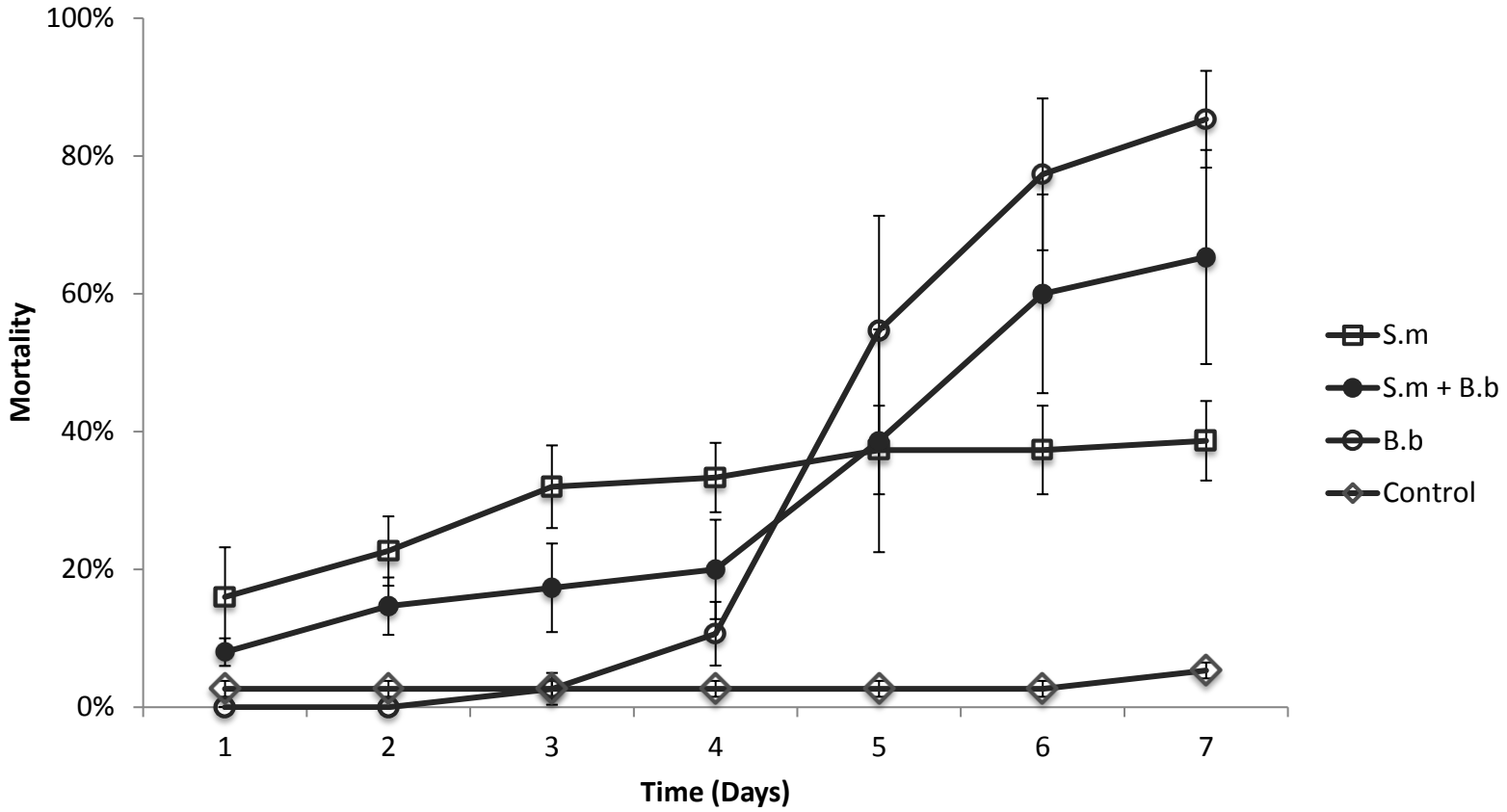

Figure 4-11. Mortality (%) percent mortality of adult house flies for seven days after 1 µL topical application of 0.5% CapSil containing *Serratia marcescens* ($1x10^6$ colony forming unit) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in combination. S.m = *Serratia marcescens* alone, B.b = *Beauveria bassiana* alone, S.m + B.b = two-pathogen combination, and Control = CapSil 0.5% alone. Bars represent mean percentage mortality ± standard errors.

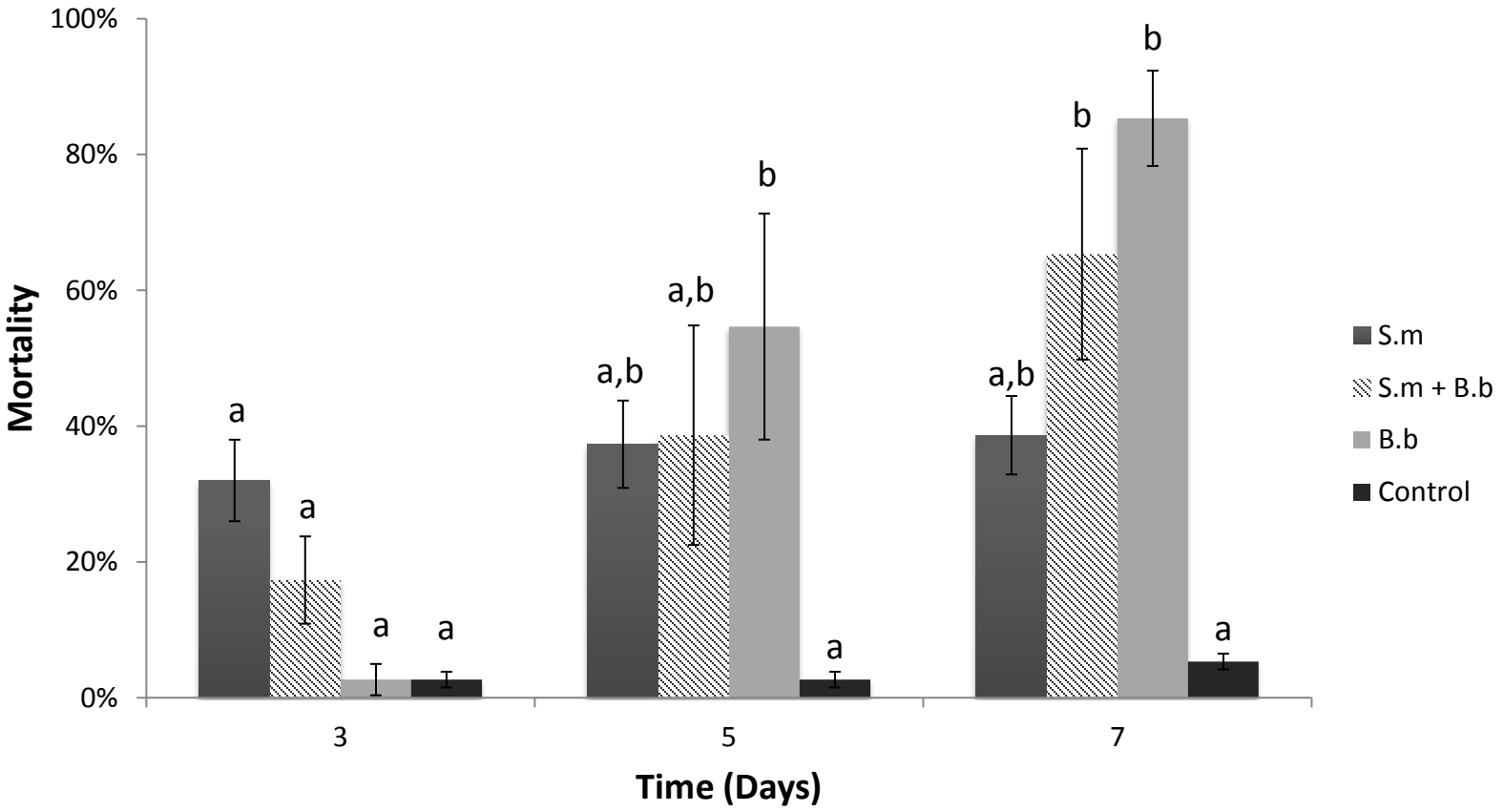

Figure 4-12. Mortality (%) of adult house flies three, five, and seven days after 1 µL topical application of 0.5% CapSil containing *Serratia marcescens* ($1x10^6$ colony forming units) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in combination. Bars represent mean percent mortality ± standard errors, and different letters within a time point denote significant differences (Tukeys HSD $P<0.05$) among treatments. S.m = *Serratia marcescens* alone, B.b = *Beauveria bassiana* alone, S.m + B.b = two-pathogen combination, and Control = CapSil 0.5% alone.

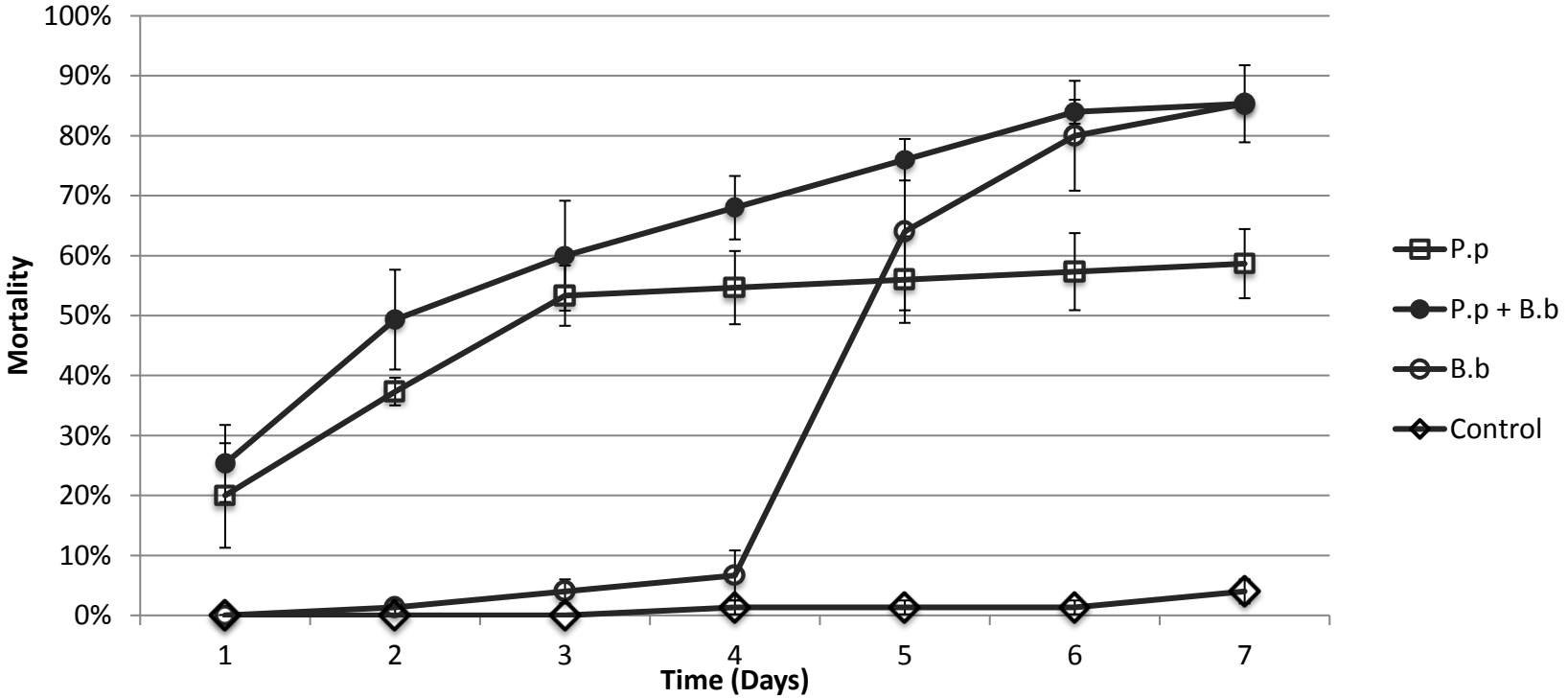

Figure 4-13. Mortality (%) of adult house flies for seven days after 1 µL topical application of 0.5% CapSil containing *Pseudomonas protegens* ($1x10^6$ colony forming units) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in combination. P.p = *Pseudomonas protegens* alone, B.b = *Beauveria bassiana* alone, P.p + B.b = two-pathogen combination, and Control = CapSil 0.5% alone. Bars represent mean percentage mortality ± standard errors.

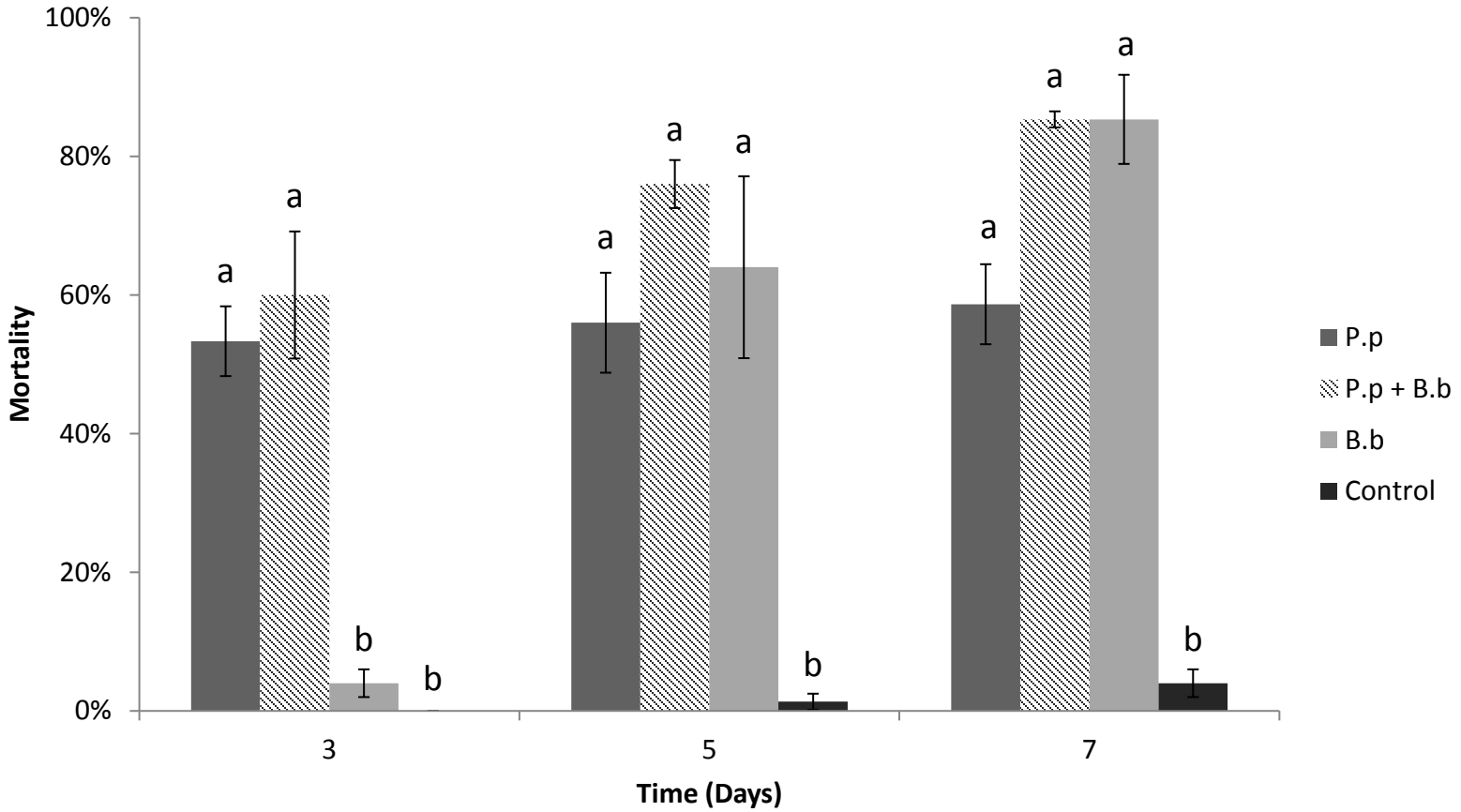

Figure 4-14. Mortality (%) of adult house flies, *Musca domestica*, three, five, and seven days after 1 µL topical application of 0.5% CapSil containing *Pseudomonas protegens* ($1x10^6$ colony forming units) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in combination. Bars represent mean percentage mortality ± standard errors, and different letters within a time point denote significant differences (Tukeys HSD $P<0.05$) among treatments. P.p = *Pseudomonas protegens* alone, B.b = *Beauveria bassiana* alone, P.p + B.b = two-pathogen combination, and Control = CapSil 0.5% alone.

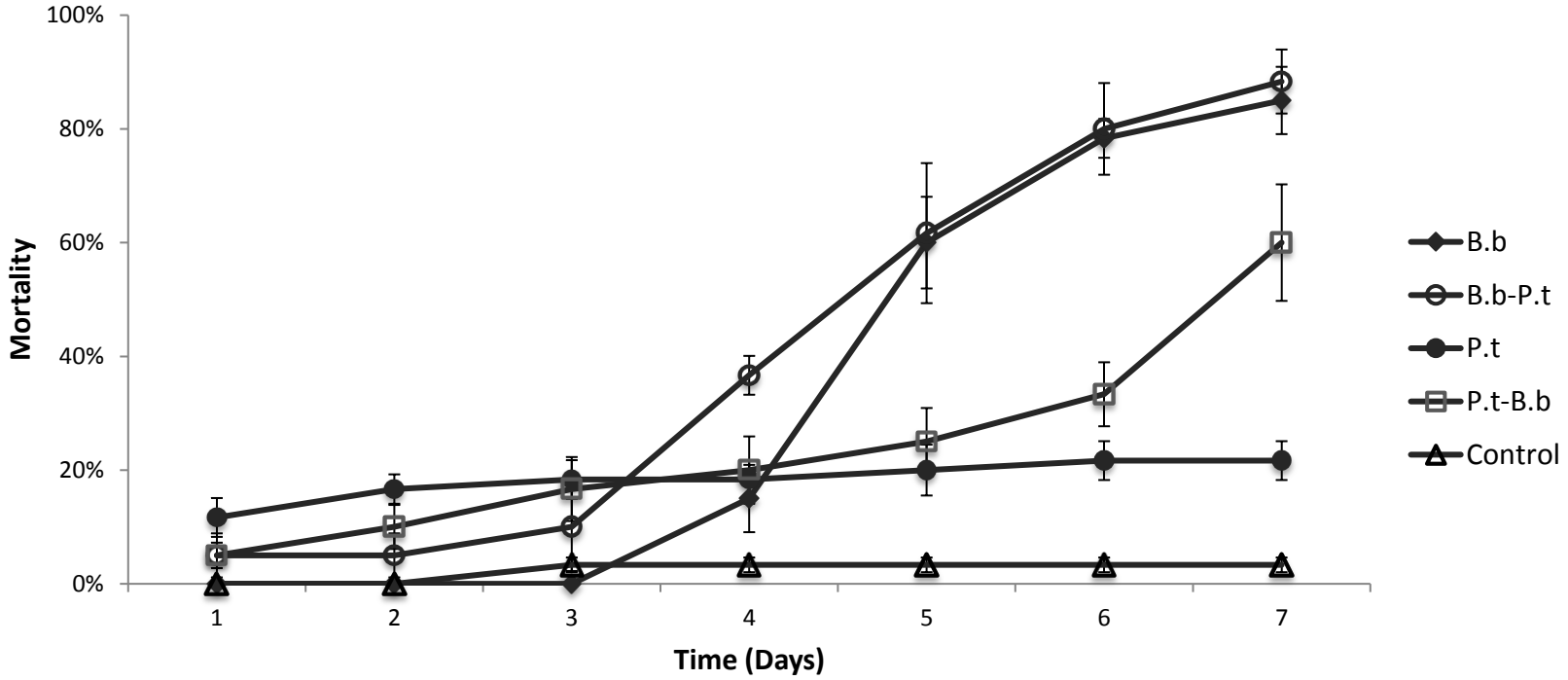

Figure 4-15. Mean (SE) percent mortality of house flies when treated topically with *Photorhabdus temperata* and *Beauveria bassiana* alone or sequentially with 48 hrs between applications of the first and second pathogen. Flies were treated individually with 1 µL of 0.5% CapSil containing no microorganisms (controls) or $1x10^6$ colony forming units (*P. temperata*) or conidia (*B. bassiana*). Bars represent mean percentage mortality ± standard errors. P.t = *P. temperata* alone, B.b = *B. bassiana* alone, B.b-P.t = *B. bassiana* first with *P. temperata* two days later, P.t-B.b = *P. temperata* first with *B. bassiana* two days later, and Control = CapSil 0.5% alone.

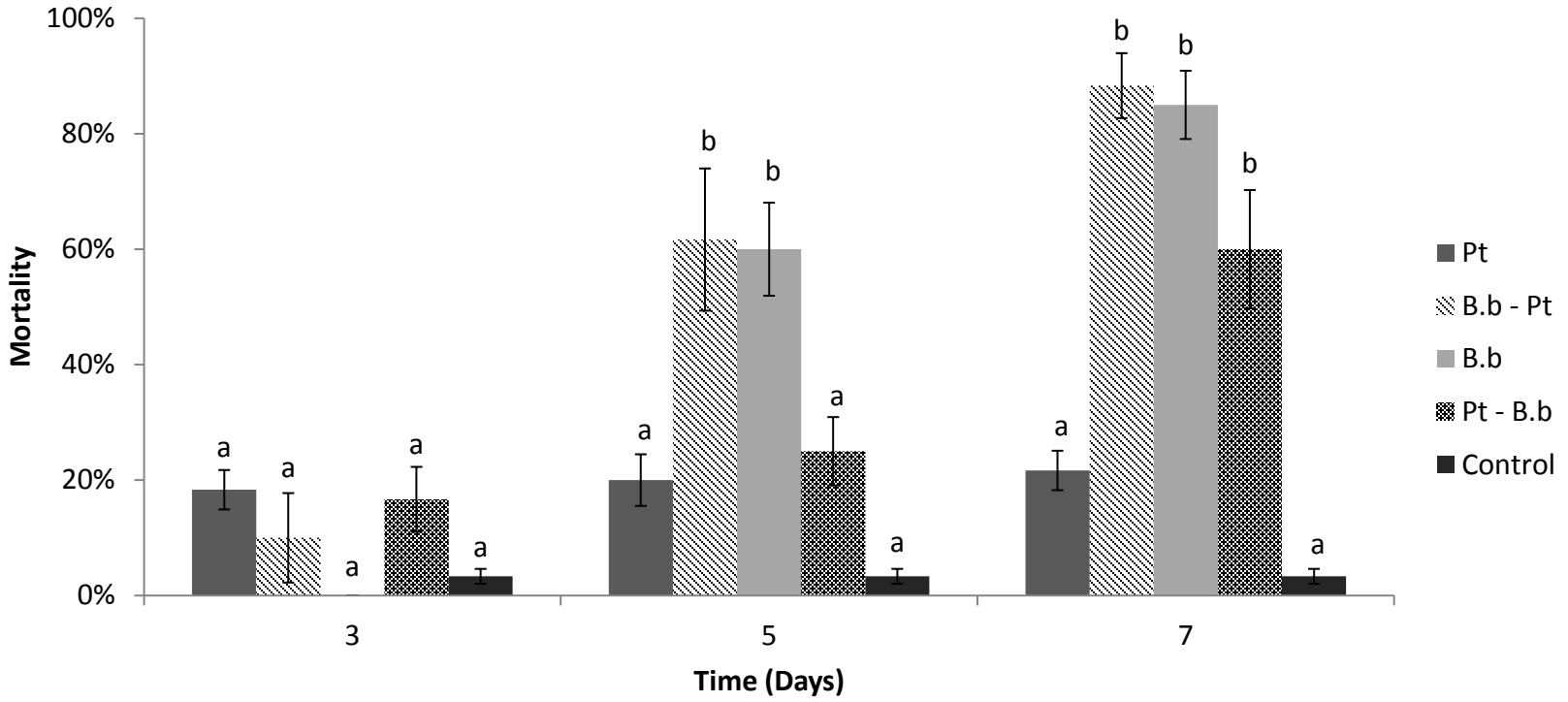

Figure 4-16. Sequential applications for *Photorhabdus temperata* at three time points: three, five, and seven days. Topical application of 1 µL of 0.5% CapSil containing *Photorhabdus temperata* ($1x10^6$ colony forming units) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in or in sequence with 48 hours between treatments. Bars represent mean percentage mortality ± standard errors, and different letters within a time point denote significant differences (Tukeys HSD $P<0.05$) among treatments. P.t = *P. temperata* alone, B.b = *B. bassiana* alone, B.b - Pt = *B. bassiana* first with *P. temperata* two days later, P.t - B.b = *P. temperata* first with *B. bassiana* two days later, and Control = CapSil 0.5% alone.

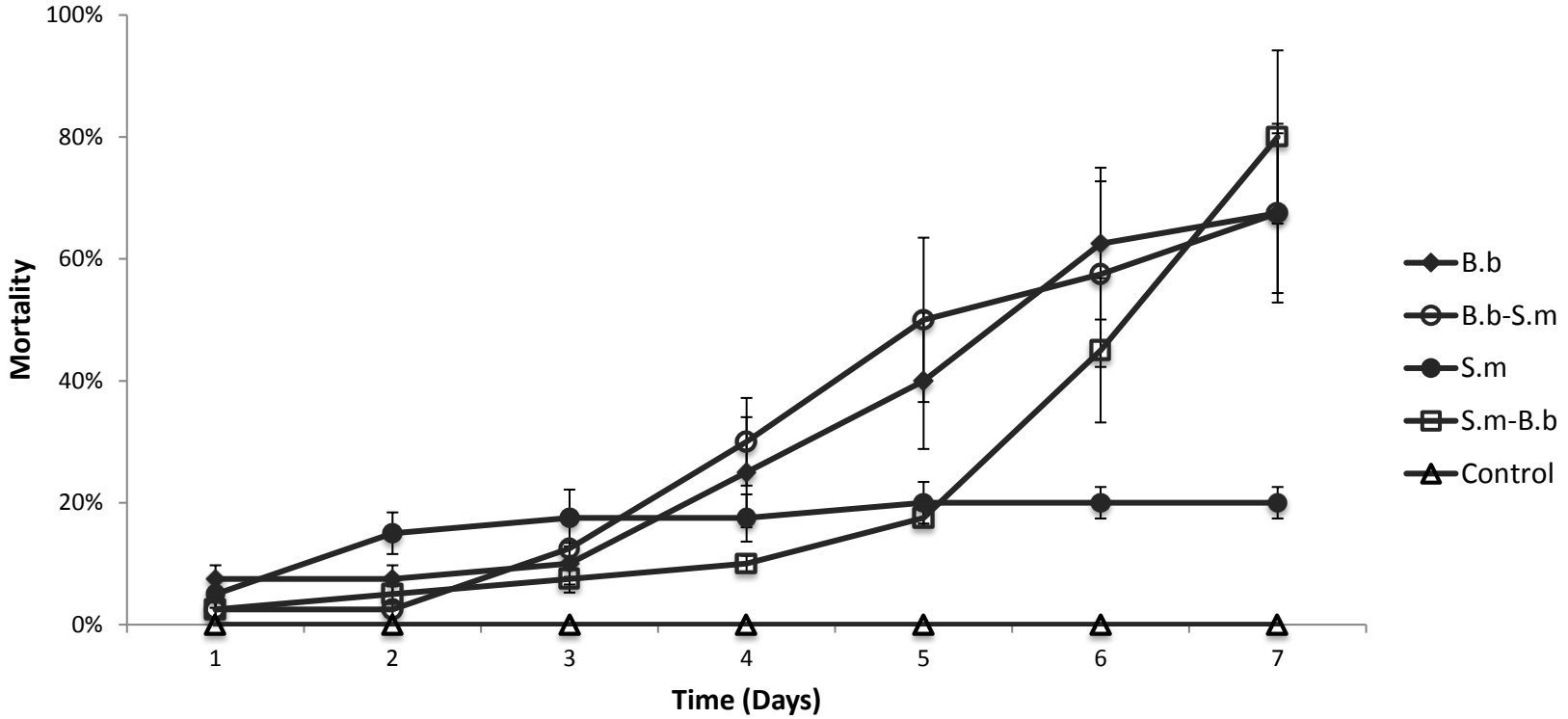

Figure 4-17. Mean (SE) percent mortality of house flies when treated topically with *Serratia marcescens* and *Beauveria bassiana* alone or sequentially with 48 hrs between applications of the first and second pathogen. Flies were treated individually with 1 µL of 0.5% CapSil containing no microorganisms (controls) or $1x10^6$ colony forming units (*S. marcescens*) or conidia (*B. bassiana*). Bars represent mean percentage mortality ± standard errors. S.m = *S. marcescens* alone, B.b = *B. bassiana* alone, B.b-S.m = *B. bassiana* first with *S. marcescens* two days later, S.m-B.b = *S. marcescens* with *B. bassiana* two days later, and Control = CapSil 0.5% alone.

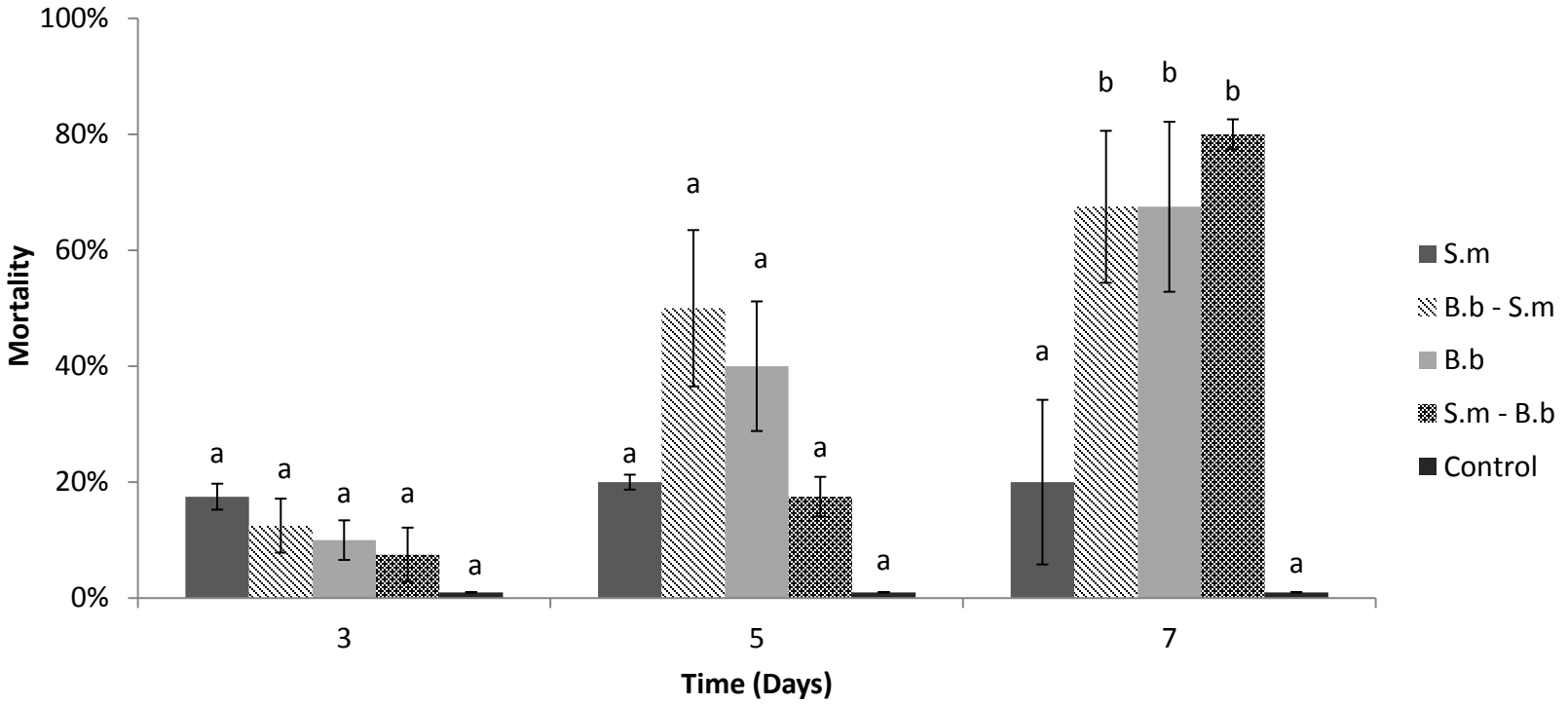

Figure 4-18. Sequential applications for *Serratia marcescens* at three time points: three, five, and seven days. Topical application of 1 µL of 0.5% CapSil containing *Serratia marcescens* ($1x10^6$ colony forming units) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in or in sequence with 48 hours between treatments. Bars represent mean percentage mortality ± standard errors, and different letters within a time point denote significant differences (Tukeys HSD $P<0.05$) among treatments. S.m = *S. marcescens* alone, B.b = *B. bassiana* alone, B.b - S.m = *B. bassiana* first with *S. marcescens* two days later, S.m - B.b = *S. marcescens* with *B. bassiana* two days later, and Control = CapSil 0.5% alone.

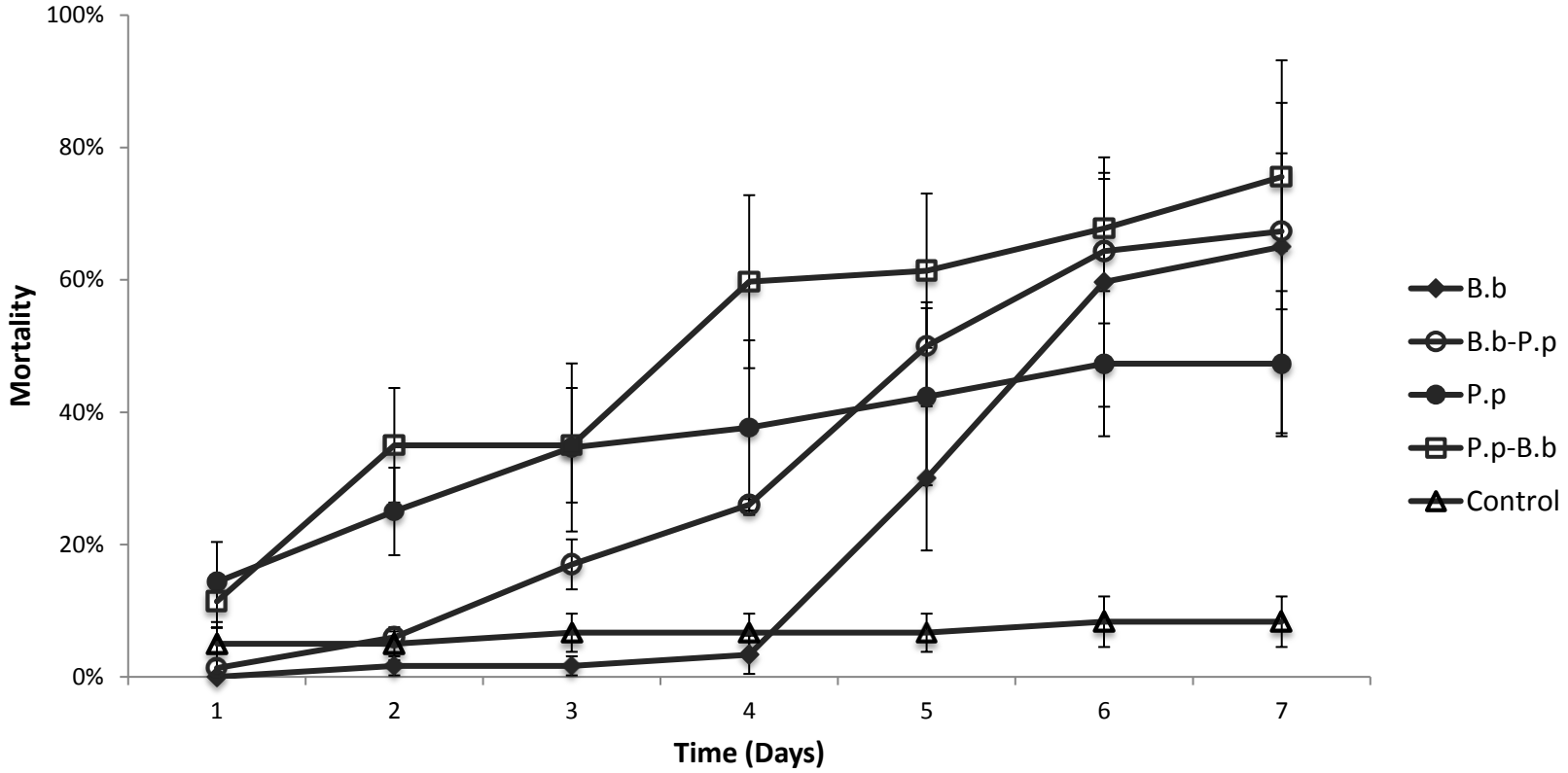

Figure 4-19. Mean (SE) percent mortality of house flies when treated topically with *Pseudomonas protegens* and *Beauveria bassiana* alone or sequentially with 48 hrs between applications of the first and second pathogen. Flies were treated individually with 1 µL of 0.5% CapSil containing no microorganisms (controls) or $1x10^6$ colony forming units (*P. protegens*) or conidia (*B. bassiana*). Bars represent mean percentage mortality ± standard errors. P.p = *P. protegens* alone, B.b = *B. bassiana* alone, B.b-P.p = *B. bassiana* first with *P. protegens* two days later, P.p-B.b = *P. protegens* with *B. bassiana* two days later, and Control = CapSil 0.5% alone.

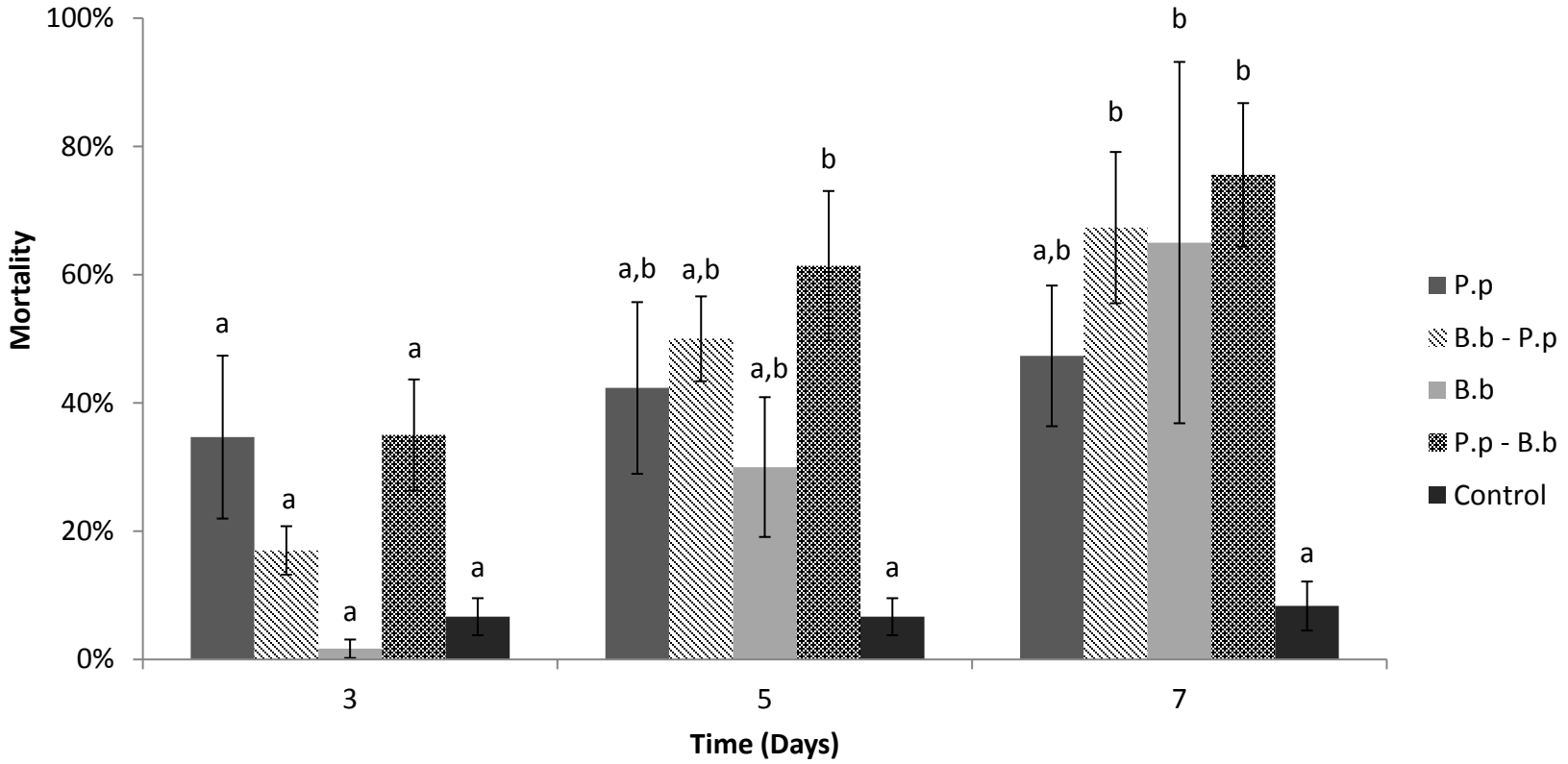

Figure 4-20. Sequential applications for *Pseudomonas protegens* at three time points: three, five, and seven days. Topical application of 1 µL of 0.5% CapSil containing *Pseudomonas protegens* ($1x10^6$ colony forming units) and *Beauveria bassiana* ($1x10^6$ conidia) alone or in or in sequence with 48 hours between treatments. P.p = *P. protegens* alone, B.b = *B. bassiana* alone, B.b - P.p = *B. bassiana* first with *P. protegens* two days later, P.p - B.b = *P. protegens* with *B. bassiana* two days later, and Control = CapSil 0.5% alone. Bars represent the mean percentage mortality ± standard errors and lower case letters denote significant differences (Tukeys HSD $P<0.05$) among treatments within a time point

CHAPTER 5
DISCUSSION OF FINDINGS AND FUTURE RESEARCH DIRECTIONS FOR COMBINING BACTERIAL PATHOGENS WITH *Beauveria bassiana* FOR HOUSE FLY MANAGEMENT.

The house fly, *Musca domestica* L. is a major synanthropic pest that is of public health and agricultural concern (West 1951, Scott et al. 2009). Fly larvae typically develop in the manure of livestock and poultry facilities, and the resulting adults can invade surrounding areas, potentially carrying animal and human pathogens to nearby farms and neighborhoods. To make matters worse, house flies are increasingly resistant to most of the conventional insecticides commercially avaialable, making them a difficult pest to control. Biological control methods offer an alternative to insecticides, and house flies have many natural enemies to choose from that can be explored and enhanced. With few exceptions, such as *Bacillus thuringiensis* (Tabashnik et al. 1990), resistance to biological control agents develops slowly and does not lead to outright failure, as is often the case with insecticides. All biological control agents have some advantages and liabilities, and a better understanding of their limitations is critical to their successful use.

Entomopathogenic fungi, especially *Beauveria bassiana*, are among the most promising biological control agents for house flies. House fly susceptibility to *B. bassiana* has been demonstrated in numerous studies. This pathogen can be easily mass-produced, has a good storage stability, and can be applied in a variety of ways that include sprays, dusts and baits. Once conidia have made contact with the host cuticle, they germinate, penetrate the exoskeleton, and proliferate within the hemocoel. The main drawback to *B. bassiana* is the long time that it takes to kill the host. This latency time varies among hosts but is typically 5 to 7 days for house flies. Long latency times are more acceptable with pests that are long-lived or slow to develop such as ticks or litter

beetles. House flies, however, have a relatively short development time and adult lifespan, and lay most of their eggs within seven to ten days after emergence. Managing this pest requires control agents that can act quickly to kill female flies before they can lay most of their eggs, and *B. bassiana* is generally too slow-acting to accomplish this. Other pathogens, especially bacteria, have the ability to cause fast mortality once they reach the hemocoel but have challenges gaining entry through the exoskeleton or overcoming the fly's defense mechanisms if they are ingested. In other words, *Beauveria bassiana* has the ability to penetrate the cuticle but kills the fly slowly whereas bacteria can kill the fly rapidly but cannot breach the outer defenses of the host. The ultimate goal of my MS research was to test whether opportunistic bacterial pathogens could trail behind through entry wounds made by *B. bassiana* and kill the fly quickly.

The concept of synergizing *B. bassiana* with a second biocontrol agent has been explored with other pathogens. Other fungi and entomopathogenic nematodes have been combined with *B. bassiana* in efforts to increase virulence or shorten the time to host death (Wraight and Ramos 2005, Mwamburi et al. 2009, Senthrilraja et al. 2010, Prabhukarthikeyan et al. 2014). Work with bacterial pathogens as synergists has largely been limited to *Bacillus thuringiensis* (Wraight and Ramos 2005, Mwamburi et al. 2009). Spores of *B. thuringiensis* must be ingested to kill the host and their mode of action involves release of toxins within the midgut of the insect (Bravo et al. 2007). Synergy with *B. bassiana* in these cases is thought to be related to the combined effects of pathogens with two different host entry mechanisms affecting the insect's immune system. In contrast, my research concentrated on three pathogens that, like *B. bassiana*, proliferate in the host hemocoel: *Photorhabdus temperata, Serratia marcescens*, and

Pesudomonas protegens. The goal was to combine these pathogens with *B. bassiana* and apply them to the cuticle in the hope that fungal penetration would act as a "Trojan horse" to deliver the bacteria into the hemocoel. This work required several steps and will be summarized in the next paragraphs.

In Chapter 2, I examined potential negative interactions between *B. bassiana* and the bacteria. Each of the bacteria (*P. temperata*, *S. marcescens*, and *P. protegens)* were placed on Sabouraud's dextrose agar with yeast (SDY) agar plates with growing *B. bassiana* cultures to observe potential inhibition of fungal growth. *Photorhabdus temperata* and *P. protegens* inhibited *B. bassiana* growth whereas *Serratia marcescens* did not. These observations were consistent with the known activity of the first two species. *Photorhabdus temperata* is a symbiont of the entomopathogenic nematode *Heterorhabditis bacteriophora* (Poinar, 1975) and one of its roles is to prevent the host cadaver from being overwhelmed by saprophytic fungi and bacteria before the nematode and associated bacteria have completed their life cycle (Boemare 2002). *Pseudomonas protegens* is known as a plant-protecting species because of its ability to produce antimicrobial metabolites (Ramette et al. 2011). In spite of this observed inhibition I moved forward with all three bacterial species because it was uncertain how the pathogens would interact on the cuticle of a live host insect, which is a very different environment than artificial media in a Petri dish.

My prior knowledge of microbes lead to the concern of carefully strategizing a technique of topically applying pathogen combinations without impeding the ability of pathogens to adhere to the house fly thorax. In Chapter 3, I discuss the differences between *B. bassiana* (eukaryote) and the three bacterial strains (prokaryotes)

in terms of nutritional needs and mechanisms of infecting insect pests. An appropriate surfactant was needed for aqueous suspensions of pathogens that would not be harmful to either of the pathogens or the fly. Five nonionic surfactants (CapSil®, DyneAmic®, Induce®, Kinetic®, and Tween® 80) were evaluated for house fly and pathogen survival. Pathogen viability remained high after 24 hours of being held in surfactant solutions, and cell counts of the bacteria species actually increased during that time. Additionally, each surfactant was graded on a scale of one to three (one = poor, two= medium, and three= ideal spreadability) on its spreadability capabilities onto the house fly thorax. Both CapSil and Kinetic had performed the highest in spreadability scores but caused higher initial mortality in house flies compared to the rest of surfactants. CapSil at 0.5% ended up being chosen for subsequent topical applications of combined pathogens because this was the dose that caused the least amount of initial fly mortality with no hindrance of spreadability once the pathogens were added.

In Chapter 4, I first examined the virulence of all four pathogens if they were to gain entry into the house fly hemocoel. This was by injecting flies with 1 µL dose response of each of the pathogens, and all of the bacterial species caused high and rapid mortality, with *P. protegens* killing the fastest at <24 hours. Having established that the pathogens were virulent when injected, the next phase was to determine whether they would penetrate through the exoskeleton after being applied topically to the thorax. Each pathogen was then applied topically in 1 µL drops containing $1x10^3$ through $1x10^6$ cfu or conidia, and mortality was observed for seven days after treatment. Topical mortality was generally low with *P. temperata* and *S. marcescens* but was surprisingly high with *P. protegens*. When bacteria were combined with *B. bassiana* and applied simultaneously,

only *P. protegens* showed a complementary effect, with higher initial mortality in the combination than with *B. bassiana* alone. Taking time into consideration, sequential topical applications were performed and higher mortality was observed in treatments that were first treated with *P. protegens* and then with *B. bassiana* 48 hours later.

Overall, the results did not support the hypothesis that bacterial pathogens could be driven in the fly hemocoel via the cuticular insult caused by *B. bassiana* penetration. As discussed in Chapter 4, this could be related to method of testing used here, where pathogens were applied as singular events, either simultaneously or 48 hours apart. Because *B. bassiana* conidia do not germinate until 14-24 hours after adhering to the host cuticle it is possible that the bacteria were no longer viable by the time the damage to the cuticle was occurring when the two pathogens were applied at the same time. Timing also could have been problematic with tests involving 48 hour intervals between *B. bassiana* and bacterial treatments because the cuticular wound caused by the fungus may have already been repaired by the time the bacteria were applied. Under septic field conditions where flies are constantly exposed to bacteria, there would be greater opportunities for secondary infection after fungal exposure. Future research could evaluate this by narrowing the window between sequential pathogen treatments or housing *B. bassiana*-treated flies under field conditions where they would be exposed to challenge from natural opportunistic bacteria.

Although the original hypothesis was not confirmed in the final experiments, this research resulted in a number of useful findings. The compatibility of the pathogens was established when they were grown together in media that was suitable to both bacterial and fungal pathogens (Chapter 2). A number of potential carriers were evaluated for

their effects on the fly and the pathogens, which resulted in the selection of CapSil at 0.5% for further testing (Chapter 3), and this could prove helpful for future research with these pathogens. Although we found little evidence for synergistic effects, results in Chapter 4 suggest that combinations *of P. protegens* and *B. bassiana* have a complimentary effect, with an initial jump in mortality caused by *P. protegens* and a second jump several days later caused by *B. bassiana*. Future research could build on these findings by evaluating other doses and treatment schedules that could improve the effectiveness of such combinations. Finally, the discovery that *P. protegens* was virulent to the flies when used alone raises intriguing possibilities for developing this species as a bacterial biocontrol agent. Future research on this species and its associated toxins are needed to determine its potential uses, especially as a larvicide and in bait form for adult flies.

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BIOGRAPHICAL SKETCH

Dana Johnson was born and raised in a small, rural town called Eustis, Florida. She spent the majority of her childhood outside, exploring the nearby woods and playing in the orange groves adjacent to her house. In 2003, she finished high school and spent many years as a cosmetologist until her mid-twenties. At 25, she enrolled at Daytona State College in Daytona Beach, Florida to begin her bachelor's in biology where she discovered her passion for science. In 2011, she transferred to the University of Florida in Gainesville, Fl. to complete her undergrad. During her transition, Dana fell very ill and was diagnosed with multiple tick-borne diseases. During her rigorous 2 year treatment plan, her interest in vector borne diseases and public health peaked and she started participating in research at the microbiology department at UF. Around the same time, she began working part time at the USDA under Dr. Christopher Geden, performing entomological research. Dana received her bachelor's from the University of Florida in 2014.

Dana worked up a full time position at the USDA as a research technician where she performed experiments with biological control methods for house fly management. Her passion for vector borne-diseases and public health led her to enroll in graduate school under Dr. Christopher Geden to further her knowledge in medical entomology in 2015. She finished her Master of Science in December 2017 from the University of Florida and continues to work at the USDA in hopes to expand her research career.