Design Brief

A fungal-derived enzymatic anti-tick spray: Targeting the *Ixodes scapularis* population*

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Ticks are a type of arachnid with species found on six continents worldwide. There are over 800 species but only a few can transmit disease to humans. One of those species is Ixodes scapularis, the black-legged tick native to the eastern half of the United States. This species is responsible for transmitting several severe illnesses, including Lyme disease. The Centers for Disease Control estimates that over 400,000 people are diagnosed and treated for Lyme disease each year in the United States. The ticks attach themselves to a host to consume and store blood using a tube-like mouth; this allows ticks to spread infectious diseases from host to host. In the past decade, tick populations and the prevalence of tick-borne diseases have spiked worldwide, largely due to increasing global temperatures and precipitation resulting from climate change. Here, we outline the development of a bio-friendly super-spray against I. scapularis that is e safe for human use. The spray contains fungal enzymes derived from Beauveria bassiana, which effectively break down cuticles of I. scapularis decreasing the viability of the ticks. In addition, by combining the enzymes with a natural repellant, the incidence of tick bites and tick-borne disease cases can be significantly reduced. Overall, the spray can be used on clothing or other areas susceptible to ticks to control the I. scapularis population, thereby controlling the spread of tick-borne diseases.

Keywords: *Ixodes scapularis, Beauveria bassiana,* enzymatic anti-tick spray, Lyme disease, transformed *E. coli* DH5α

Ticks are a species of arachnids responsible for transmitting over fifteen different bacterial and viral diseases in the United States. Over the past decade, tick populations in North America have been increasing dramatically (*How Many*, n.d.). They spread a notorious collection of diseases that commonly pass through parasitism, a form of symbiotic relationship in which one species benefit at the expense of another to improve the likeliness of survival (Overstreet & Lotz, 2016, pp. 28-29). In this relationship, ticks act as the symbiote or the resource taker, obtaining blood from the host organism as food to survive. Through this process, harm is inflicted on the host (Overstreet & Lotz, 2016, p. 29).

Ticks are biological vectors for diseases; when feeding, a tick may pick up disease-causing pathogens in the form of bacteria, viruses, or even parasites from the blood of the host organism, making the tick a reservoir for said pathogens Over the course of two to

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three years, ticks go through g four life stages: eggs, larvae, nymphs, and adults (Figure 1) (*How Ticks*, n.d.). In all of these stages, with an exception of the egg phase, ticks feed on the blood of a host by attaching themselves to them using a feeding tube-like mouth, which is inserted under the skin of the host. This is when infection-causing pathogens can get picked up by the ticks When feeding, these pathogens may make their way into the blood of the next host directly or transferred into the bloodstream of the next host when a tick expels saliva into the host after the attachment process (*Transmission*, n.d.). Ticks typically feed on a host for multiple days to become fully engorged and most of them have a different host in each life phase (*How Ticks*, n.d.). This frequent contact with multiple host organisms increases the chances of picking up and spreading diseases-causing pathogens. After an adult female tick feeds, it will mate once with a male and utilize nutrients obtained from different hosts to produce eggs. A few species can reproduce and lay eggs asexually. One tick can lay between a few hundred to a few thousand tick eggs at a time, allowing for a rapid increase in population size (*Reproduction*, n.d.).

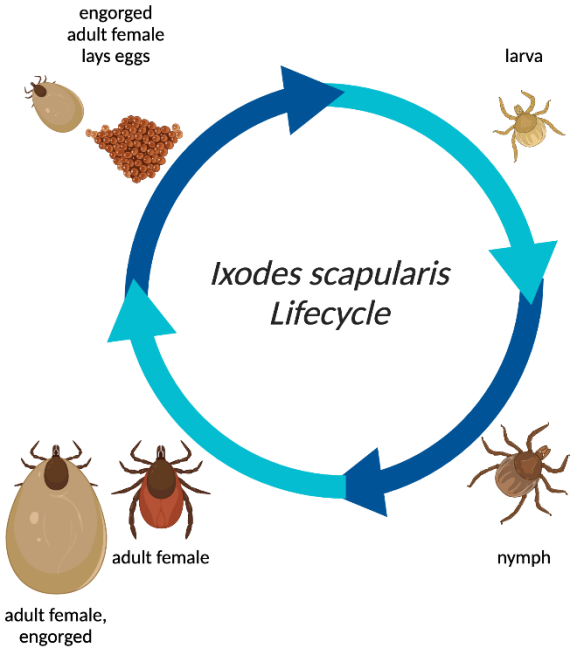

Figure 1. Life cycle of I. scapularis. This diagram shows the life cycle of black-legged ticks. Ticks feed on a host between each life stage. It takes about two years for a tick to reach reproductive maturity and lay eggs.

Diseases transmitted by ticks can be severe and even lead to death in both humans and animals. Before feeding, ticks–especially larvae and nymphs–are incredibly tiny and likely go unnoticed by a host for multiple days, increasing a tick's likelihood of spreading illness (*How Ticks*, n.d.). Their small size makes it difficult for a person to find and completely remove ticks. Also, their heads or feeding tubes may become stuck under the skin of hosts, making them difficult to eradicate. While tick-borne illness cases are reported throughout the United States, they are more common in rural or heavily wooded areas.

There are over 800 species of ticks globally, but few transmit bacteria, viruses, and other pathogens that cause disease. One of the most common ticks in the United States that transmit Lyme disease is the *I. scapularis*, also known as the black-legged tick. It can be found in the eastern half of the United States, and like most ticks, it thrives during warm seasons (from April to September) (Figure 2) (*Regions Where*, n.d.) (*Preventing Tick*, n.d.).

In North America, cases of tick-borne illnesses have more than doubled in the past two decades, and the continuing changes to the climate will likely increase numbers in the coming years (Winny, 2023). In 2019 alone, the CDC recorded over 50,000 cases of tick-borne diseases across the United States (Centers for Disease Control and Prevention, n.d.). Researchers predict that rising global temperatures, and increased humidity caused by climate change will promote the expansion of the tick population Longer and earlier warm seasons will extend the active tick season, providing more time and opportunity for ticks to transfer between hosts and transmit pathogens (Levi et al., 2015, p. 1). In tandem with the increased tick population Lyme disease was also observed to have risen in the United States, with the number of cases nearly doubling since 1991, from 3.74 reported cases per 100,000 people to 7.21 reported cases per 100,000 people in 2018 (*Climate Change*, n.d.).

The symptoms of Lyme disease include fevers, headaches, fatigue, and a highly characteristic "bull's-eye" rash called erythema migrans (EM) (*Lyme Disease*,

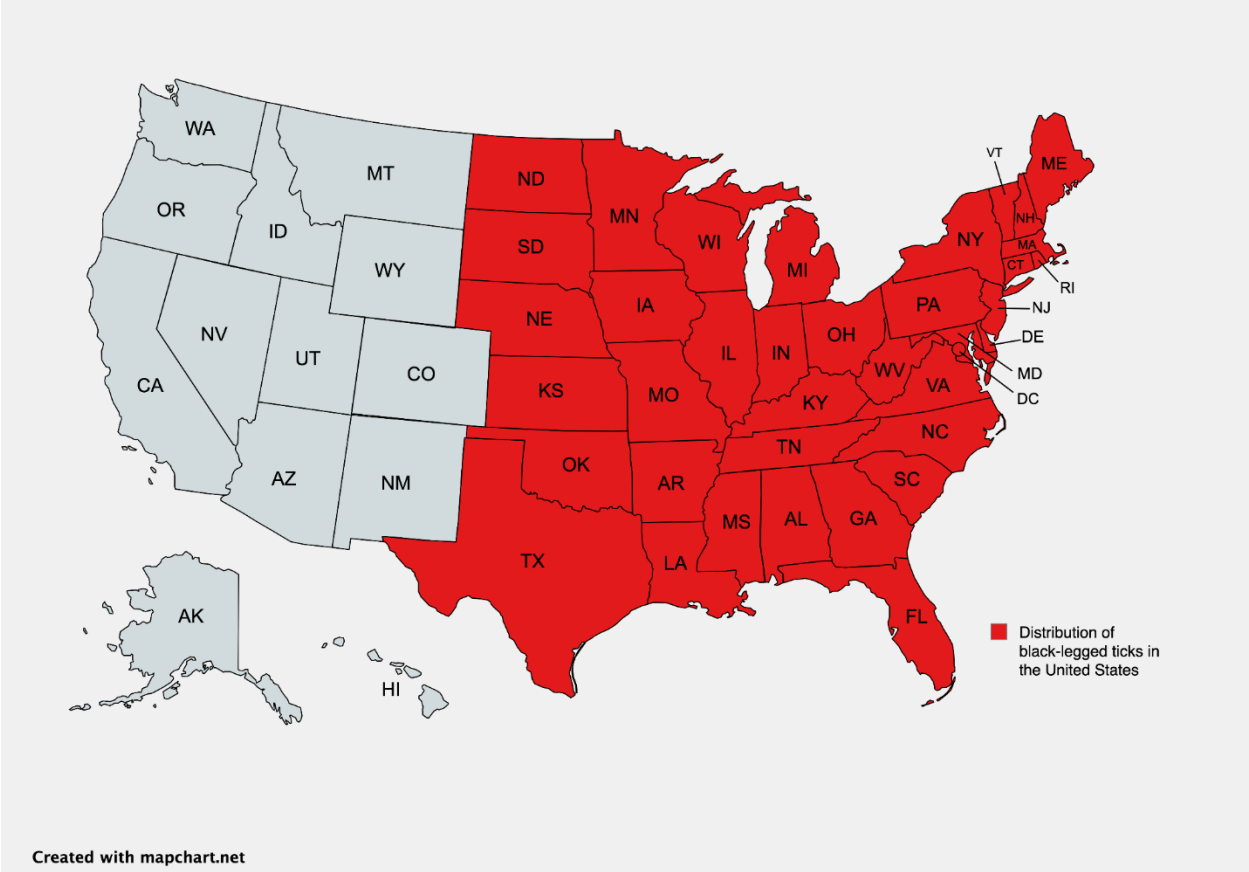

Figure 2. Distribution of I. scapularis in the United States. The black-legged tick is typically found in the Eastern half of the United States, although the abundance varies depending on the location.

n.d.). Other diseases commonly transferred through ticks are anaplasmosis, ehrlichiosis, babesiosis, tularemia, and Rocky Mountain Spotted Fever (*Tickborne Diseases*, n.d.). However, Lyme disease remains as one of the most common tick-borne diseases in the northern U.S. (Centers for Disease Control and Prevention, 2022, pp. 7-8).

Lyme disease is typically diagnosed based on physical symptoms such as the bull's-eye rash. Without treatment, which includes antibiotics, infected people may develop chronic symptoms or conditions that affect the organs of the cardiovascular, muscular, and nervous systems. Although rare, Lyme disease can be fatal (*Lyme Disease*, n.d.). Specific symptoms relating to Lyme disease are categorized into two groups: early signs and symptoms (3 to 30 days after tick bite) and later signs and symptoms (days to months after tick bite). Early signs include fever, chills, headache, fatigue, muscle and joint aches, swollen lymph nodes, and variations of expanding skin rashes, including EM (Figure 3) (*Signs and Symptoms*, n.d.). Later signs include severe headaches and neck stiffness, additional EM rashes on other areas of the body, facial palsy (loss of muscle tone or droop on one or both sides of the face), arthritis with severe joint pain and swelling in the knees, and other large joints, intermittent pain in tendons, muscles, joints, and bones, heart palpitations or an irregular heartbeat (Lyme carditis), dizziness or shortness of breath. inflammation of the brain and spinal cord, and nerve pain (*Signs and Symptoms*, n.d.).

Although the increasing tick populations pose a public health threat, the complete and instantaneous eradication of ticks would have negative consequences on their ecosystems. Most woodland animals living in tick-abundant habitats depend on ticks as a food source. These species specifically include a variety of birds, reptiles, and amphibians.

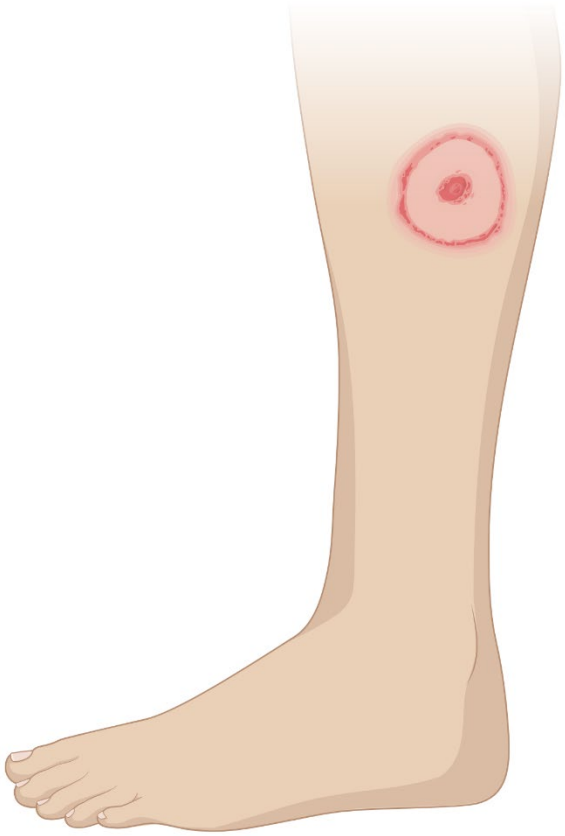

Figure 3. A bull's-eye rash appearing after a tick bite.

Some animal species eat ticks, preventing ticks from feeding on them. For example, opossums and squirrels eat 83–96% of the ticks that attempt to attack hosts and feed (Keesing et al., 2009, p. 3911). Therefore, there is a need to reduce the tick population in a well-controlled manner to not disrupt the ecosystem.

Many insects and arachnids, including ticks, possess a protective outer skin-like layer called a cuticle, which protects them from dehydration, mechanical injury, pathogens, toxins, and even predators (Muthukrishnan et al., 2020, p. 3546). The cuticle expands during feeding to aid the engorgement of ticks from blood storage (Flynn & Kaufman, 2015, p. 2806). Since the cuticle serves as a protective shield against the external environment, many studies have suggested that breaking the cuticle could lead to sustainable control of the tick population. Previous studies have shown that some fungal enzymes and toxins are effective in cuticle degradation (Arya & Cohen, 2022, p. 7).

Cuticles are made of chitin, a fibrous material consisting of polysaccharide, lipids, and proteins. Cuticles are composed of an epicuticle (the outermost layer) and a procuticle (also known as the endocuticle) (North Dakota State University, n.d., p. 1) and the endocuticle only contains chitin. Therefore, enzymes such as proteases and lipases are necessary to break cuticles down and each type of enzyme plays a respective role in the disintegration of cuticle layers. *B. bassiana* fungi are entomopathogenic (Pedrini, 2022, p. 1). Entomopathogenic fungi are parasitic microorganisms that can infect and kill a range of insects; some are known to cause disease in numerous tick species, including *I. scapularis* (Pedrini, 2022, p. 1). Therefore, *I. scapularis* is a naturally occurring biopesticide against *I. scapularis*. Enzymes produced by *B. bassiana* including proteases, chitinases, and lipases come into contact with the cuticle layers of ticks act to digest the proteins, chitin, and lipids respectively, effectively dissolving he cuticle (Figure 4) (Wang et al., 2021, pp. 2-3). Spore produced by *B. bassiana* could also penetrate the insect body and release toxins under the exoskeleton and epidermis of ticks, resulting in the liquidation of internal organs (Anderson, 2020).

The process by which *B. bassiana* invades ticks is demonstrated below in Figure 4. The conidia, or microscopic spore particles, are produced by the *B. bassiana* as a form of asexual reproduction (Malloch, n.d.). They attach themselves and penetrate the outermost epicuticle to reach the endocuticle of the insect, releasing chitinase, protease, and lipase enzymes that hydrolyze the cuticle (Wang et al., 2021, p. 3). Once the cuticle degrades, hyphal bodies, which are yeast-like cells, secrete toxins, including oosporein, beavericin, beaverolides, and tenellin (Ortiz-Urquiza, 2021, p. 3). The hyphal bodies eventually pass through the epidermis and intrude the hemolymph—the fluid equivalent of blood in insects and arachnids. These toxins either suppress the insect's immune system or directly destroy hemolymph cells, impacting hemolymph circulation (Wang et al., 2021, p. 3).

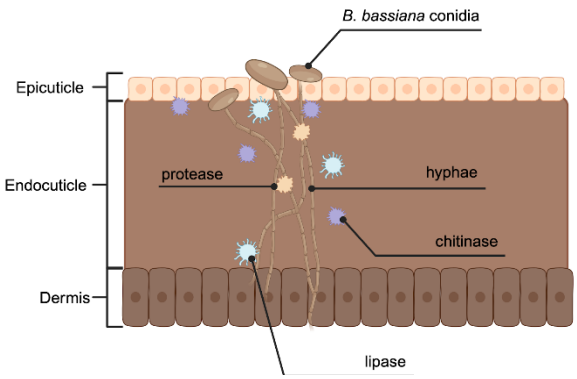

Figure 4. How B. bassiana invades and breaks cuticles of ticks down. The process begins with conidia, spores produced by the B. bassiana fungus and used in asexual reproduction, which physically penetrates through cuticle layers of ticks and release enzymes and fungal toxins.

The predominant toxin produced by *B. bassiana* is beauvericin, followed by beauverolides and oosporein. *Beauvericina* mycotoxin that is also produced by the Fusarium species of fungi, is a biopesticide that is effective against ticks and other pests, such as bed bugs (Singh et al., 2015, p. 225). Oosporein functions by suppressing the host's immune system and producing an antibacterial compound, thereby serving as a protection against *B. bassiana* (Chen et al., 2022, p. 2).

Current solutions against tick-borne diseases include checking clothing for ticks, showering after being outdoors, and treating clothes with off-the-shelf insect repellents (Preventing Tick, n.d.). However, these solutions are primarily preventative and do not effectively control *I. Scapularis* population. Our enzyme-based spray not only acts as a preventative repellant, but also eliminates *I. Scapularis* by rendering them unviable.

Our goal is to create a spray that repels *I. Scapularis* with the added effect of controlling tick populations. By exploiting naturally occurring fungal enzymes, coupled with a natural repellent, we devised a bio-friendly super-spray that people can use on their clothes, skin, or environments that are susceptible to ticks, such as grassy or heavily wooded areas (Figure 5). We genetically modify *Escherichia coli* to produce a host of fungal-specific enzymes that will readily digest the cuticles of *I. Scapularis* , increasing their susceptibility to environmental insults (such as pathogens), thereby increasing their mortality rate (Figure 5) To do this, we derive purified chitinase and lipase enzymes from *B. bassiana* fungi by cloning their respective genes into vectors containing compatible Histidine (His)-tags and introducing these vectors into *E. coli* for protein synthesis., Purified proteins will then be transferred into a water or oil medium. Subsequently, *Chamaecyparis nootkatensis* (Nootka tree) essential oil is added to serve as the natural tick repellent. While ticks have been difficult to control, our design uses biochemistry-based solutions that combat tick-borne diseases and control tick

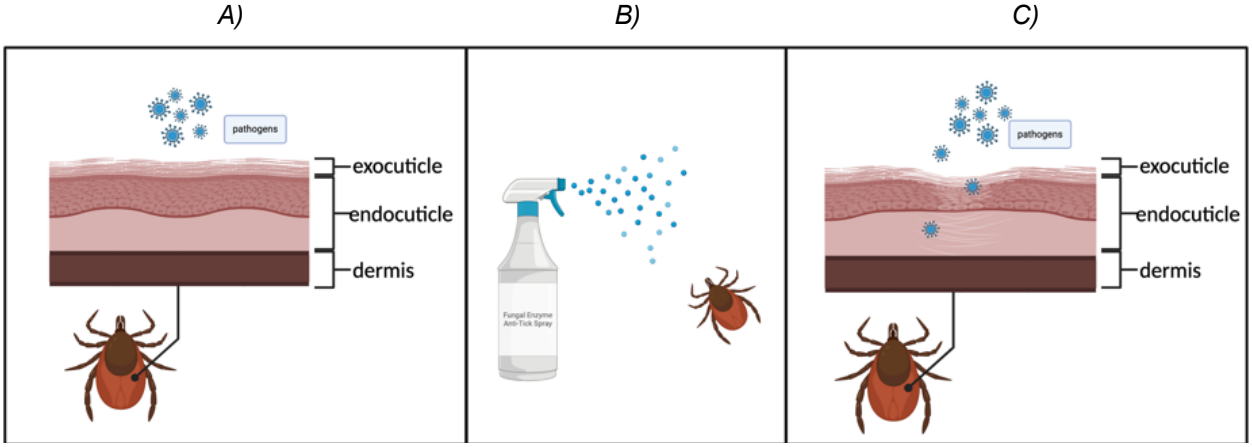

Figure 5. Depiction of how our enzymatic spray aims to increase mortality in black-legged ticks. (A) Ticks are protected from environmental factors such as pathogens by cuticle. (B) When in contact with ticks, our enzymatic spray will (C) degrade their protective cuticle and increase the ticks' chance of infection and fatality.

population.

Systems level

Through our design, our team intends to make a spray that repels and controls the tick population using enzymes chitinase and lipase from the *B. bassiana* fungi and natural essential oils. Genes encoding for chitinase Bbchit1 and Bbchit2, and newly discovered lipase BBL351, will be added to a chassis to produce the respective gene products- two types of chitinases and a lipase. The enzymes will be purified using His-tags (that are present in the cloned genes) and added to a spray at an optimum concentration. As aforementioned, the enzymes contribute to tick death by disintegrating their external cuticle layers. While the enzyme degradation of the cuticle alone does not directly cause tick death, the lack of a protective cuticle allows other environmental factors, such as pathogens or predators, to kill ticks more easily (de la Fuente et al., 2020). The essential oil derived from *C. nootkatensis* will also be added to the solution as a protective repellent against the ticks. Water will be used as a diluent and to assist enzyme function. Chitinases can remain active in oil containing medium. It was previously shown that chitinases can serve as a defense enzyme for plants in the leaves of an oil palm (Nahar et al., 2012, p. 333). Therefore, the enzymatic activities of chitinase are preserved in oil-rich environments. The lipase should not have a significant effect on the terpenes present in essential oils; however, future testing will have to verify this. *C. nootkatensis* oil is naturally produced by some trees and grapefruit (*Citrus x paradisi*) and is a natural tick and insect repellant (*Nootkatone: A New Active*, n.d.).

The genes encoding for the enzymes will be cloned using a transformed chassis, *E. coli* DH5α, a highly competent strain of *E. coli,* via the use of the vector backbonepBR322. However, the plasmid is unable to function if added directly into the spray. Therefore, *E. coli* is used to express the gene products from the plasmid before purifying them with the His-tags. We insert all three *B. bassiana* genes to form the pT0FF plasmid. The pT0FF plasmids generally comprise a pBR322 vector backbone which has ampicillin as the selection marker. DNA sequences of the Bbchit1 Bbchit2, and BBL351 genes will be manufactured separately and inserted into pBR322 plasmids (Fig 6 & 7). Each DNA construct contains a promoter, His-tag, ribosome binding site, gene encoding of the respective enzyme, and a terminator. After inserting the complete plasmid (containing all three enzyme-encoding genes) into *E. coli* DH5α, successfully transformed cells will be selected using ampicillin.

Device level

To produce *B. bassiana* enzymes, we must construct a plasmid containing all three genes and transform multiple copies of the plasmid into *E. coli* for protein production. The first part of plasmid construction requires using restriction endonuclease EcoRI, HindIII, and EcoRV to cut the DNA at specific cut-sites in the plasmid (Figure 6). Although the EcoRV cut site coincides with the tetracycline marker, the ampicillin selection marker will remain intact, allowing us to use it to identify bacterial clones containing the plasmid.

After pT0FF plasmid construction, the next step involves transforming the plasmids E. coli DH5α cells. The plasmid-containing cells will be purified using the selection

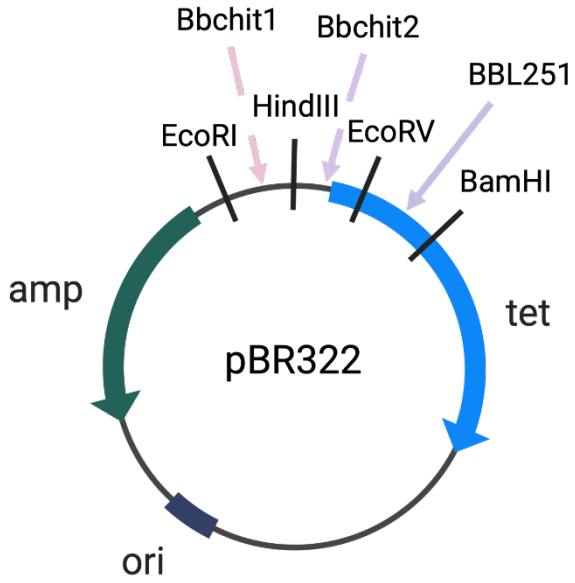

Figure 6. The pBR322 plasmid that will be used as a backbone and the cut-sites we will use to insert the enzyme sequences.

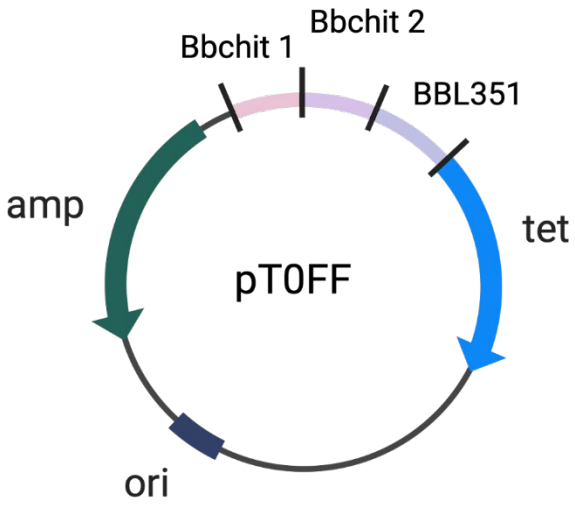

Figure 7. The pT0FF plasmid we will be creating by inserting the enzyme sequences into the pBR322 vector backbone.

marker ampicillin, then the cells will be grown in large quantities. Then the proteins produced by the cells will be purified through the His-tags. We then use low-pressure liquid chromatography to filter the purified enzymes with the His-tag for further testing. During the process, the His-tagged enzymes bind to nickel ions while passing through a nickel column, straining out miscellaneous cell proteins (Amos et al., 2021, pp. 3). After the low-pressure liquid chromatography with resin-bound Ni2+ passes, the His-tagged enzymes bind with liquid imidazole, competing against the nickel ion bonds and releasing the attached purified enzymes (His-Tag Purification, n.d.).

Parts level

There are several components to the plasmid construction that must be considered. When designing the three separate DNA constructs of the three enzyme genes, we included the same promoter, ribosome binding site, and terminator, and each gene will consist of individual b His-tags. Among the J23100 through J23119 family of constitutive promoters, the promoter BBa_J23119 is the strongest. Therefore, we decided to utilize it in our plasmid construction (Anderson & iGEM2006_Berkeley, 2006). For protein purification, His-tags must be added to the ends of the gene sequences. His-tags. Others have shown that *B. beauveria* chitinases can be produced through the BBa_K2718022 His-tag (*Experiments*, n.d.). However, *B. beauveria* lipases do not have a specific His-tag that have shown success (*Design*, n.d.). Therefore, we decided to use the general protein-purification tag BBa_K1223006 instead (Schlesinger, 2013). Next, we chose to use the BBa_J61100 ribosome binding site (RBS). This RBS is part of the Anderson

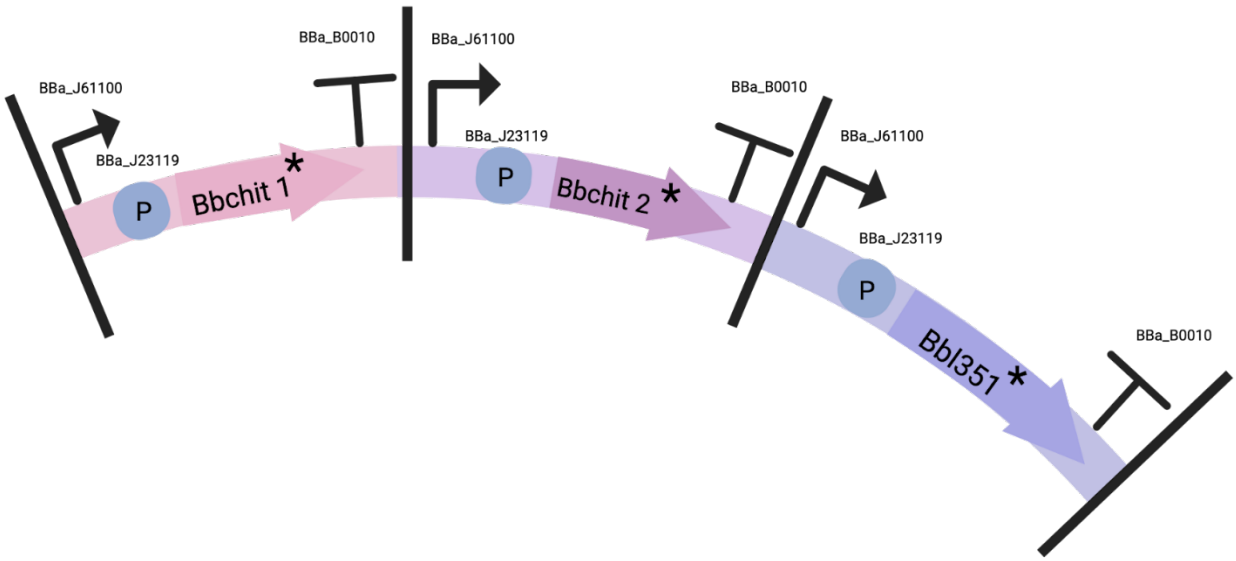

Figure 8. A close-up on the components of the three DNA sequences showing the ribosome binding site, promoter, genes encoding for the three enzymes and terminator. The asterisk represents the His-tags that is added to the 5' end of each of the gene sequences.

RBS family. Although not much is known about the relative activity of these RBS, it is known that they are roughly in decreasing order of activity -RBSs with low naming numbers are stronger RBSs than those with higher numbers. Therefore, we select BBa_J61100 with the assumption that it has the highest activity (*Ribosome Binding*, n.d.). Next, we included the genetic sequences ofBbchit1 (Figure 7), Bbchit2 (Figure 8), and BBL351 lipase (Figure 9). Finally, we selected the terminator BBa_B0010 as it did not exhibit a burden that was significantly greater than zero (Rettberg & Antiquity, 2003). Thus, we expect the DNA construct to remain stable for many bacterial cell divisions in large culture volumes. After completing the construction of the final pT0FF plasmid and protein purification process, the enzymes can then be transferred into a liquid medium where we can add our essential oil to the formulation to create the final spray product.

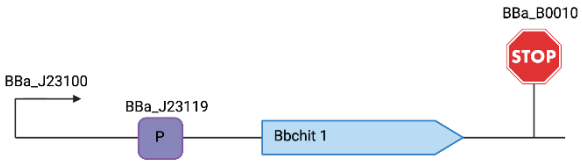

Figure 9. The DNA sequence map of the Bbchit1 gene.

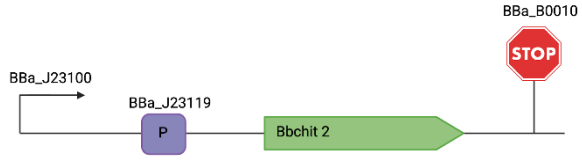

Figure 10. The DNA sequence map of the Bbchit2 gene.

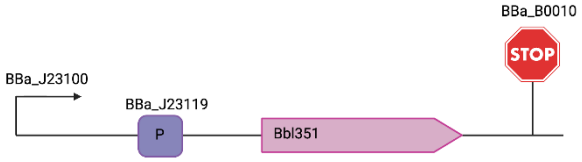

Figure 11. The DNA sequence map of the BBL351 gene.

Safety

We must ensure this spray would not significantly harm other non-tick arachnid or insect species to prevent significant impact on the ecosystem. Additionally, it is also possible that certain fungal-derived enzymes are toxic to animals and humans, and testing determines which enzymes are safe for human and pet use through skin contact, inhalation, or otherwise.

Although unlikely, there are concerns that our spray may pose harm to the environment. Chitinase emits nitrogen and carbon dioxide (Hamid et al., 2013, p. 21). On the other hand, lipase is currently undergoing research as an environmentally friendly catalyst and is considered environmentally safe (Sen, 2017, p. 1). Essential oil of *C. nootkatensis* is completely safe for humans and animals as the CDC has approved it as an ingredient in other insect repellents and even lotions or soaps. Only people allergic to *C.hamaecyparis nootkatensis* develop adverse reactions. Otherwise, since this oil is safe to consume and used for cooking, *C. nootkatensis* does not pose harm to humans, animals, or the environment (*Nootkatone: A New Active*, n.d.).

Insects beneficial to the environment, including bees and ladybugs, also have a cuticle made of chitin and lipids, and could likely be negatively affected by the chitinase and lipase enzymes present in our spray (*Beauveria Bassiana*, n.d.). There are some solutions to potentially limit the impact of our spray on them - limit the enzyme spray on clothing and objects and avoid directly spraying gardens or plants. The enzymes derived from *B. bassiana* have the potential to affect many other insect species but the impact may vary between species(*Beauveria Bassiana*, n.d.). Most, if not all mammals produce some form of lipase in their skin or hair follicles that acts as a natural antimicrobial, so skin contact with the spray and enzymes is likely harmless. Chitinase has been found in humans, specifically in the stomach (Paoletti et al., 2007, p. 244). However, the chitinase and lipase enzymes may cause minor irritations if directly inhaled or consumed by animals or humans, with the possibility of inducing an allergic reaction in certain individuals (Bussink et al., 2007, pp. 1, 2). Testing would be necessary to determine the appropriate concentrations of enzymes that are safe for humans but still effective against ticks.

Discussions

Our enzymatic spray against ticks is the first to utilize chitinase and lipase enzymes derived from *B. bassiana*. Our method is scalable, which means that we can efficiently produce large quantities of the enzymes in a controlled environment to facilitate the production of the spray. Additionally, our method of engineering three individual DNA constructs for each gene encoding for each enzyme and subsequently inserting them into a single plasmid, will allow for the production of all three enzymes simultaneously. This aims to increase the efficiency of enzyme production and circumvent the need to produce and add different enzymes separately into the final product. However, simultaneous cloning of genes for three enzymes may limits our ability to control and identify the production levels of each enzyme. Therefore, one of the first experiments that we plan to conduct is to start with inserting one DNA construct (for one of the enzymes) into the plasmid to determine efficiency of enzyme production and perform the necessary optimization steps.

Our method requires the production of purified fungal-derived enzymes without the use of the actual living fungal material, which could potentially have undesired effects on the environment. Although *B. bassiana* does not typically infect humans, individuals who are immuno-compromised may be at risk of acquiring fungal infection. Separating the enzymes from the original fungi removes the benefits of the mechanical process used by the fungus. This includes hyphal bodies from the conidia burying from the epicuticle to the hemolymph. This process allows the enzymes to reach the endocuticle resulting in tick death. Relating to this, *B. bassiana* also uses another enzyme, protease, to penetrate the endocuticle. Our design does not include protease production, which may hinder other enzymes' substantial effect on the tick.

At present, we are unable to determine if our chosen enzymes will affect other insects and cause adverse effects to the environment. Also, another potential shortcoming of our design is that we are unable to determine the efficiency of the selected promoter, ribosome binding site, and terminator in driving the production of the enzymes in cells. There may be a possibility that the chosen sequences may not be compatible with one another and no enzymes can be produced as a result. Before creating the spray, other testing would be required to see if the enzymes will work in an oil-based medium or if a water-based medium would be necessary for the enzymes to work.

Next steps

This spray aims to make I. *scapularis* more susceptible to external insults by degrading the ticks' protective cuticle. We must verify that the spray would effectively eradicate *I scapularis* while not significantly harming other non-tick arachnid or insect species that are crucial for our ecosystem. Testing may include a mix of spot-testing and a tick-repellant test on the *I. Scapularis* and other insects. Spot testing is a chemical test that uses analytic assays or the analysis of the few drops of a substance's composition and concentration on a subject or filter paper (*Tick Repellent*, n.d.). We recommend a spot test with the enzymes alone to determine a tick-specific effect. Adjustments to the enzymes after this step may have to be made depending on efficiency. Afterward, a retest with the essential oil must be done to see if combining enzymes and essential oils does not nullify their effect on insects. We identified *C. nootkatensis* as our essential oil; however, it may have to be changed depending on whether the enzymes cancel its repellency or whether another alternative is more compatible.

Spot testing with water as the controlled variable and the spray mixture will need to be tested on various types of insects and compared to see the effects and the amount of spray concentration necessary to conduct our solution. Before an enzymatic pesticide spray can be widely used, the efficacy against ticks and other organisms must be spot tested. Spot testing allows us to put each insect in a beaker and record their reactions to a drop or spray of the solution. In the event our formulation is found to not be specific against *I. Scapularis*, other chitinases or enzymes could be tested to determine which ones have

more specificity against *I. Scapularis*.

A tick-repellant test can be conducted in a couple of ways. We can test various tick repellents, including essential oils, enzymes, and our spray formulation, by placing a piece of fabric with ticks over a small organism that naturally attracts ticks due to their body heat within an enclosed space. We then compare treated fabric to untreated fabric over the organism. Repellence is then calculated by counting and comparing the remaining ticks on the two types of fabrics (Figure 12) (*Tick Repellent*, n.d.).

Another method of testing repellency is by using bug dorms. One recent study has shown the use of such a method to test the efficacy of their mosquito sprays (Barton et al., 2020, p. 3). In that study, a sugar solution enticed mosquitoes to a cotton ball saturated with the oil concentrations. Researchers then observed mosquito activity including how often they landed and how much they avoided the cotton ball. This method can be adjusted to fit *I. scapularis* and their reactions to the potency of natural repellants. Testing the spray's effectiveness and repellency against the appropriate controls is important in determining the final spray formuation.

Author contributions

A.C. and E.C. proposed the idea. A.C., E.C., N.O. and C.O. researched and wrote the background as well as researched *B. bassiana* and its enzymes. A.C. researched the plasmid. N.O. researched the essential oil. C.O. and N.O. wrote the systems level. A.C. and C.O. wrote device and parts level. C.O., A.C. and N.O. wrote safety. A.C. wrote discussion and next steps. All visuals were made by E.C. and A.C. C.O. worked on citations.

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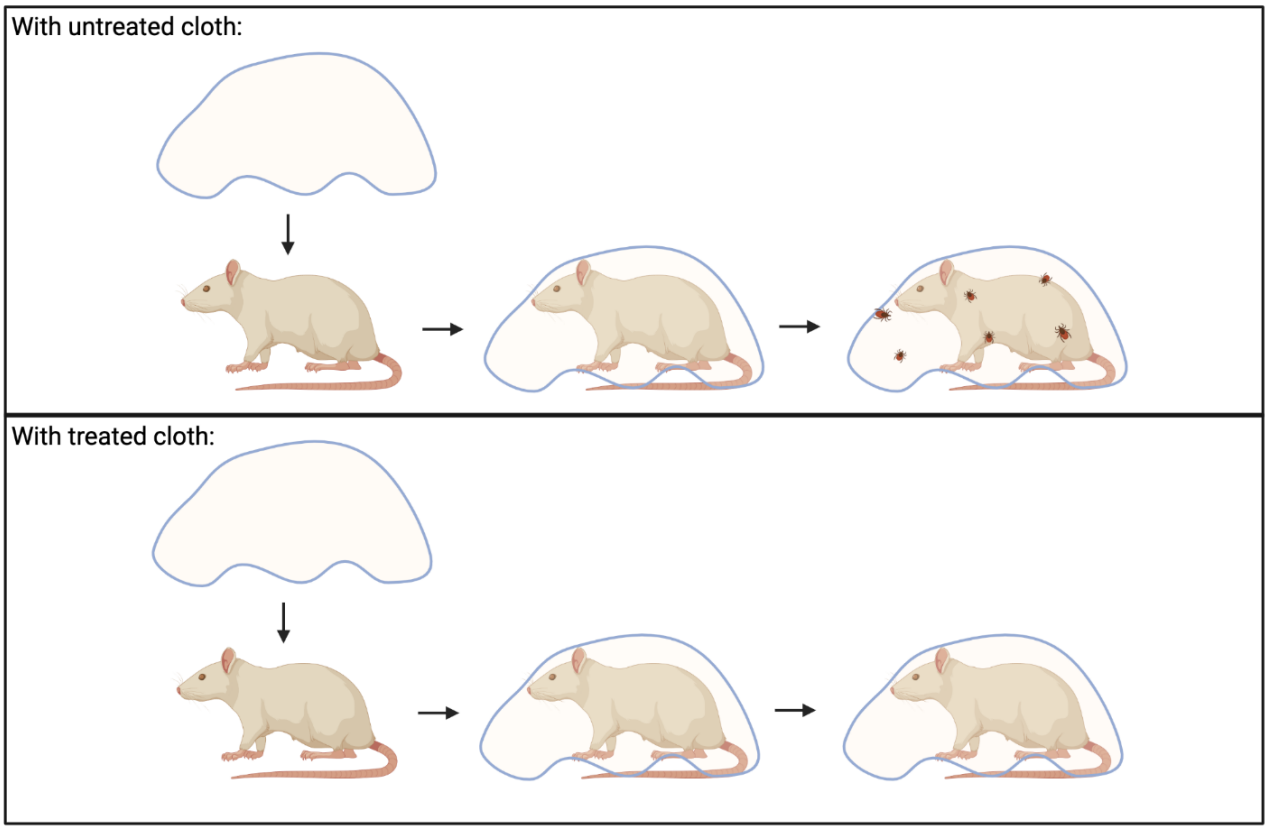

Figure 12. An example of how tick repellency test can be conducted.

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