Antimicrobial Peptides in Transgenic Silkworm Silk

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ANTIMICROBIAL PEPTIDES IN TRANSGENIC
SILKWORM SILK

by

David Jaden Turner

A thesis submitted in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
in
Biology

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This research project focuses on integrating the antimicrobial peptide LL-37 into silkworm silk through genetic engineering. LL-37 was chosen due to its protein size, cost, mechanism of action, and amino acids readily available in the natural silkworm diet. The fusion protein was designed and synthesized through cloning in Escherichia coli in preparation for the CRISPR/Cas9 system. The LL-37 sequence, and proline linker were cloned into the CRISPR/Cas9 expression vector in E. coli, followed by upscaling of the culture to produce enough DNA for electroporation into the silkworm line. The eggs were then transformed via electroporation, and the silkworms were reared for approximately two months. The insert into the silk was visualized through a GFP tag and via a dot blot antibody test, and mechanical data on the silk fibers were collected, including fiber diameter, tensile strength, and the energy to break. Antimicrobial activity was determined via a bacterial viability stain using fluorescence microscopy to quantify the effectiveness.
This project introduces the AMP LL-37 into silkworm silk, producing a proof of concept for antimicrobial silk in a commercially viable and well-established industrial insect with little to no modification required for current industrial operations after initial development while not requiring post-treatments to the fibers. Integration into the silk protein allows for greater anti-microbial efficacy over an extended period and will not wash away as quickly as current anti-microbial treatments that are coatings or sprays. LL-37 is known to have efficacy against common strains of bacteria found on the human body, including *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Staphylococcus aureus*. Overall, this research project provides a promising solution to the growing problem of antibiotic resistance and has the potential to revolutionize the silk industry and medical applications.
Overview: People in India and China have produced silk textiles for thousands of years. Silk is a biodegradable, biocompatible compound used in the production of clothing, bedding, furniture, industrial materials, and medical applications. Over the last 30 years, research has increasingly investigated silk’s antimicrobial effects and how to augment its natural abilities. Antimicrobial peptides, or AMPs, are also an area of increasing interest as the rise of antibiotic resistance reduces the efficacy of current treatments. This project plans to systematically synthesize a fusion protein that incorporates the beneficial properties of each constituent into commercial silkworms.

Innovation: This project seeks to introduce the AMP LL-37 into silkworm silk through genetic engineering. This will allow for the production of antimicrobial silk in a commercially viable and well-established industrial insect with little to no modification required for current industrial operations after initial development while not requiring post-treatments to the fibers. Integration into the silk protein will also allow for greater antimicrobial efficacy over an extended period. In addition, it will not wash away as quickly as current antimicrobial treatments that are coatings or sprays.

Brief Process Summary: LL-37 was chosen based on protein size, cost, mechanism of action, and amino acids readily available in the natural silkworm diet. Next, the construct
design and synthesis via cloning in *E. coli* in occurred in preparation for the CRISPR/Cas9 system. Using the LC-NHEJ pBsK expression system, the LL-37 sequence, and proline linker were cloned into the CRISPR/Cas9 expression vector in *Escherichia coli*. Upscaling of *E. coli* culture to produce enough DNA for electroporation into the silkworm line was then performed. Then, silkworm eggs were transformed via electroporation and a specialized protocol. Next, silkworm rearing took approximately two months. This was followed by visualization of the insert into the silk through a GFP tag and via a dot blot antibody test. Mechanical data on the silk fibers, including fiber diameter, tensile strength, and the energy to break, were also collected. The final steps included determining the antimicrobial activity via a bacterial viability stain using fluorescence microscopy to quantify the level of effectiveness.

Medical Relevance: Antimicrobial peptides are increasingly in demand as an alternative to antibiotic treatments due to antibiotic resistance. Antimicrobial peptides, including LL-37, are known to have efficacy against common strains of bacteria found on the human body, including *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Staphylococcus aureus* (the organism responsible for potential MRSA infections). In addition, having a readily available silk fiber has benefits in the production of clean medical sheets and bedding to reduce the chance of contamination, increasing hygiene in exercise clothing, and reducing the risk of surgical site suture infections, therefore reducing the need for antibiotic treatments.
DEDICATION

To my loving wife, Allie.
I want to thank Drs. Justin Jones, Randy Lewis, and Ryan Jackson for their advice, support, and expertise and for serving on my committee. I would also like to thank Drs. Paula Oliveira and Xiaoli Zhang for their incredible help and support throughout this process and their experience in the lab and sericulture room. I also want to express thanks to Drs. Sara Freemen and Noelle Beckman for letting me use their Keyence Fluorescence Microscope, and Callie Porter for teaching me how to use it. Thank you to Jackson Morley, Gavin Christensen, and Josh Corey for their help in the lab. Thank you for the support of Lululemon’s Research and Development branch for the funding associated with this project. Thank you to Robert Heaton for his help in organizing, formatting, and editing. I also want to acknowledge Grammarly, ChatGPT, and Microsoft Word’s old-fashioned spell check for help with semantics and grammar. Thank you all!
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CHAPTER 1: OVERVIEW: IMPACT AND IMPLICATIONS

The recent occurrence of far-reaching epidemiological health concerns, such as the COVID-19 pandemic, has highlighted the importance of antimicrobial materials. These materials are used in various settings, from hospitals to homes, and they play a vital role in protecting humanity from harmful bacteria and other microorganisms. However, the various applications of antimicrobial materials bring advantages and drawbacks.

There are a few key reasons that antimicrobial textiles and fabrics can be beneficial. First, antimicrobial fabrics can help to reduce the spread of bacteria and other microorganisms. This is especially important in healthcare settings, where antimicrobial materials can help prevent infection (Sheridan et al., 2022). Antimicrobial fabrics can also help reduce odors in clothing and upholstery since bacteria and other microorganisms can cause the fabric to smell foul (McQueen & Vaezafshar, 2020). By using antimicrobial materials in clothing and upholstery, manufacturers can assist with keeping their products smelling fresh and clean. Antimicrobial fabrics can also extend the fabric’s life, directly benefiting the consumer. Additionally, antimicrobial treatments can prevent microorganisms from colonizing the fabric. This reduces the need for more frequent laundering, thus reducing wear to the material.

Antimicrobial coatings or finishes can be applied to many materials with hard surfaces but are less successful in textiles (Morais et al., 2016). Many of these coatings and finishes are designed for metal or other solid surfaces, which can be useful for
metallic high-touch surfaces like doorknobs. However, the treatments can be relatively toxic in some cases, as they can leach into the environment during laundering.

Nanoparticles of various materials, including silver, copper, titanium dioxide, zinc oxide, and others, have been key research topics in recent years due to their antimicrobial properties. They are often heralded as a solution to the challenge of making antimicrobial textiles. However, while these materials are generally safe as large macroscopic materials, they are markedly less so at the nanoscopic level (Bhandari et al., 2022). This is because, free metal nanoparticles are easily absorbed by cells in the body due to their small size, resulting in potential health complications from this bioaccumulation of heavy metals (Bhandari et al., 2022). This is generally considered less than ideal and is a primary adverse effect of nanoparticle use in therapeutics and drug delivery techniques. Thus, despite the antimicrobial value, increasing such nanoparticles in the environment in textiles in clothing, bedding, and upholstery may be more detrimental than helpful in the short term. Further research into the health effects of prolonged exposure may be prudent.

An additional limitation to nanoparticle use is cost. Due to their small size, the nanoparticles of these elements and compounds often require another binding agent or coating to keep them adhered to the material (Morais et al., 2016). Having to use various binding agents to treat the fabric increases the costs of the material, both in terms of processing as well as additional materials cost, which some of the better-suited antimicrobial nanoparticles (silver and gold) also tend to be more expensive to produce in the first place (Bhandari et al., 2022). Therefore, it is necessary to find materials that can overcome these drawbacks.
Silk: Spiders, Worms, and Industry—Oh My!

Sericulture, or the caring and raising of silkworms to harvest their silk, is as valuable as ancient. Silk is used in various applications, including clothing manufacturing, bedding, surgical sutures, tissue scaffolds, and films (Holland et al., 2019). Silkworm silk is a remarkable material composed primarily of two proteins: fibroin and sericin. Fibroin comprises three chains, a heavy chain, light chain, and P25, which are intertwined to form a complex structure. The heavy chain strengthens the silk, while the light chain is responsible for its elasticity and the P25 is crucial in the development of the other two chains structure. Sericin, on the other hand, is a water-soluble protein that surrounds the fibroin and is removed during silk processing. As a result, Silkworm silk has high flexibility, can form in long continuous fibers, is biocompatible with many materials, and can be produced on a large scale. This industry has existed for thousands of years, especially in China and India; silkworm domestication is thought to have occurred in China over 5,000 years ago (Cherry, 1987). The Chinese were the first to develop a method for harvesting silkworm silk on a large scale, but they kept this knowledge a closely guarded secret for centuries.

Sericulture spread elsewhere in the world slowly but thoroughly. During the mid-6th century, knowledge of sericulture began to spread beyond China when two monks from Persia visited the Chinese court and observed the process (Cherry, 1987). They eventually took some silkworm eggs back to Persia, and the industry began to spread throughout the Middle East. From there, the secret of sericulture continued to leak out, spreading westward into Europe by the end of the 6th century. The Byzantine Empire also played a role in the spread of silkworm silk, as they set up extensive trade routes.
throughout Asia and Europe. By the 10th century, sericulture had reached Japan, and the industry continued growing. The modern sericulture industry in Asia is industrialized and advanced. It had come a long way from the early days of silkworm silk when the process was done by hand. Today, the industry is mechanized, uses advanced techniques and equipment, and experiences many advantages of modern automation (Qin & Xiaoming, 2022).

Spider dragline silk, or “spider silk,” has been explored as an alternative to silk for various applications. It has often been given the moniker “stronger than steel” due to its high tensile strength (Whittall et al., 2021). It also has the advantage of being significantly lighter than steel due to its lower density and biodegradability. In addition, evidence suggests that some recombinant spider silks can be engineered to have antimicrobial properties (Chouhan et al., 2018). However, spider silk also has disadvantages. It is difficult to make long continuous fibers like silkworm silk, and “spider farming” is largely impractical due to spiders’ territorial and cannibalistic nature (Whittall et al., 2021).

In contrast, silkworms are highly domesticated, docile, and farmable at scale (Qin & Xiaoming, 2022). The biggest limiting factors are adequate access to processed mulberry leaves (often called “chow”) and control over ambient temperature and humidity, which, depending on the location, can be accomplished via the local natural environment or by implementing temperature- and humidity-controlled artificial environments. Furthermore, turning silkworm cocoons into usable silk fibers, including degumming and cocoon unwinding for downstream applications, has been refined over thousands of years and numerous silkworm generations. Therefore, in recent years, there
has been growing interest in combining the natural properties of silkworm silk with other proteins, such as spider dragline silk, antimicrobial peptides (AMPs), and other materials, allowing for a blend of the strengths of each constituent protein (Licon, 2019; Teule et al., 2012; X. Zhang, 2017). Producing these novel fusion proteins en masse using industrial sericulture techniques also allows for decreased production costs, with little to no specialization costs required in the commercial strains apart from the initial development costs.

Antimicrobial Peptides (AMPs) and the Human Microbiome

Antimicrobial peptides (AMPs) are small amino acid peptide chains, typically less than 50 amino acids in length, that are part of many organisms’ immune responses, from prokaryotes to humans. They typically provide broad-spectrum activity against various pathogenic microorganisms, which motivates current research interests as alternative antibiotics (Huan et al., 2020). AMPs have four primary mechanisms of action in which they combat microbes, including the disruption of membrane potential by creating a channel or pore, the disruption of the membrane itself via charged species, interrupting specific cell processes acting on specific intracellular targets, or by aggregation and essentially “gumming up” the works of intracellular processes or in metabolic pathways (Agarwal et al., 2016). Different antimicrobial peptides work through different mechanisms of action and can enter cells through lipid flip-flip flop or by forming a poor into membranes. Humans are home to many microorganisms on the skin and body, known as the human microbiome. Some of these bacteria and yeasts, especially those found in the underarm and groin regions, can be quite odorous and colonize clothing such
as *Staphylococcus epidermis*, *Staphylococcus hominis*, *Staphylococcus aureus*, and various *Corynebacterium* species (Lam et al., 2018).

Various antimicrobial materials have been successfully embedded in clothing fibers, including in silks and cotton; however, there have been some downsides. Currently, the two main ways that a fabric can become “antimicrobial” are by the inclusion of antimicrobial compounds within a matrix that can then leach out over time or by decorating compounds onto the surface of the fiber, such as quaternary ammonium ions or silver nanoparticle coatings (Morais et al., 2016). There are two main disadvantages of these standard treatment procedures. First, there is decreased efficacy of the antimicrobial as it is depleted over time. This can allow for subsequent microbiota to recolonize the fabric. Second, in most situations, it is not desirable to harm the natural microflora on the skin and third, these treatments can cause ill effects by allowing the treatment to be released into the environment (such as through a washing machine) (McQueen & Vaezafshar, 2020). By embedding AMPs within a fiber, the antimicrobial effects of the AMPs may be utilized and combined with the transgenic silkworm silk to create a fiber with increased microbial resistance. This could lead to a better material for antimicrobial textiles for exercise and medical clothing and bedding that could help reduce the colonization of the material by microbes. One meta-analysis suggested that of an estimated of 29 million global hospitalizations, 5.4 million were due to healthcare acquired infections. Utilizing antimicrobial textiles could reduce the chance of nosocomial infections in hospital settings by 20-40% (Murphy et al., 2020) and simultaneously help reduce the odors in fabrics, preventing the material’s premature failure and extending the textile’s lifetime. The next thing to examine would be the
The process of how to integrate the AMP into the fiber. The method chosen for this project is by utilizing a CRISPR system.

**CRISPR**

What is CRISPR?

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a bacterial immune system that has been repurposed as a genome editing technology that modifies genes in various organisms, including humans. CRISPR is an array of repetitive DNA interspaced with other sequences that was first discovered in nature in 1987 by Ishino et al from Osaka University in Japan (Gostimskaya, 2022). However, the biological significance of the CRISPR repeats were not understood. Over the last two decades, we have learned that the CRISPR is an adaptive memory of an immune system of bacteria. The adaptive immune mechanism works by integrating short fragments of foreign DNA in between the repeats of the CRISPR arrays to create a “genetic memory.” The CRISPR is then transcribed and processed into CRISPR derived RNAs (crRNAs), which combine with CRISPR-associated proteins (Cas) to form surveillance complexes that identify and cleave targets that match the crRNA's spacer sequence in foreign DNA, thereby disabling the phage DNA as the complex initiates a double-stranded break if the crRNA sequence matches (Wang et al., 2020). Bacteria use this system to protect themselves from viruses, allowing bacteria to “remember” the viruses previously encountered.

A group of scientists, including Jennifer Doudna, Emmanuelle Charpentier, George Church, Feng Zhang, and others, discovered that the CRISPR system could also
be used to edit genes and modify regions of DNA (Gostimskaya, 2022). This discovery led to the development of CRISPR-based gene editing tools, such as CRISPR-Cas9, which was developed to make precise cuts in the DNA of living cells and perform edits by replacing DNA sequences with new ones (Jinek et al., 2012). This can correct genetic defects or introduce new genes into an organism, allowing a breadth of research to happen today.

Popular Uses Today

CRISPR technology has been widely used for various purposes in different research fields. Its applications range from medical research to agricultural and environmental research and basic science studies. Some of the popular uses of CRISPR technology today include:

- Medical research: CRISPR has been utilized for gene therapy and treatment of genetic diseases, including in research areas such as cancer, allergies, immunity, and various cardiovascular, neurological, and other disorders (Sharma et al., 2021), in human cells as well as in animal models (Lu et al., 2021).

- Agricultural research: CRISPR has been used to modify the genetic makeup of plants to increase their yield, nutritional content, and resistance to pests and diseases. For instance, researchers have used CRISPR to modify soybean genes to improve their yield (Cai et al., 2021). Another group used CRISPR to develop a strain of tomato plants resistant to yellow leaf curl disease and powdery mildew (Pramanik et al., 2021).

- Environmental research: CRISPR is being investigated for its potential to control invasive species and enhance the resistance of organisms to environmental
stresses such as climate change. In a recent study, researchers used CRISPR to develop a gene drive (a technology to increase or “drive” a certain allele frequency to much higher-than-normal rates in a population, even when the allele is deleterious to the individual) to reduce the population of the invasive mosquito species *Anopheles stephensi*, which spreads malaria, by testing it on mice (Li et al., 2021).

- **Evolutionary research:** CRISPR is used to understand genes’ function and study the mechanisms of genetic inheritance and evolution mechanisms. Gene knockouts are useful in exploring the function of different genes, and CRISPR is a targeted tool in that pursuit. For example, CRISPR can be used to explore the genetic changes responsible for characterizing different butterfly wing pigmentation patterns (L. Zhang et al., 2017) or to compare populations of surface-dwelling fish to their cave-dwelling counterparts (Warren et al., 2021).

CRISPR technology has expanded significantly over the past few years, and its potential applications are broad and diverse. For example, CRISPR is a versatile tool that has enabled researchers to investigate biological mechanisms and explore new avenues for treating diseases and enhancing crops and environmental resilience.

**Uses in the Silk World**

CRISPR has transformed the field of genetic engineering. The technology is used in various applications, from developing new treatments for genetic diseases to modifying crops for greater yield (Y. Zhang et al., 2020) and pest resistance (Karmakar et al., 2022). However, one of the latest areas of research that has shown significant promise is the use of CRISPR technology in silk material research.
Silk is a natural protein fiber produced by several insects, with the domesticated silkworm, *Bombyx mori*, being the most well-known. The silk fibers produced by silkworms have numerous commercial and industrial applications, including textiles, biomedical materials, and electronics. One of the main challenges in silk production is achieving high purity and consistency in the silk fibers, which can be difficult to achieve using conventional breeding methods because they can result in great variance in the quality of the silk (Chen et al., 2019).

CRISPR technology offers a new approach to address this challenge. In a recent study, researchers used CRISPR-Cas9 to introduce targeted mutations in the fibroin gene of silkworms, which is responsible for the production of silk fibers. The mutations resulted in a more consistent and pure silk fiber with improved strength and elasticity than wild-type silk (Liu et al., 2019). The authors of the study utilized an all-in-one vector to maximize the targeting efficiency of dCas9-TET1 and selected three endogenous genes that were previously found to harbor methylated DNA. They designed gRNAs within the methylated region and co-transfected dCas9-TET1 and gRNA to successfully erase methylation marks near the targeting region, with efficiencies ranging from about 17.50% to 40.00%. This targeted demethylation on gene body resulted in increased mRNA transcription level. The dCas9-TET1 system provides a powerful tool for investigating the functional significance of DNA methylation in a locus-specific manner and for exploring the unknown links between methylation and development in insects (Liu et al., 2019). This approach has the potential to overcome the limitations of conventional breeding methods of gaining new traits, which often rely on time-
consuming and unpredictable natural selection, while also providing insight into insect DNA methylation.

In a similar vein, Xiaoli Zhang’s previous work shows the precedent of this technology in silkworms via the production of a spider-silkworm hybrid silk. The project used CRISPR/Cas9 genome editing technology to create transgenic silkworms that produced spider silk-like fibers with improved mechanical properties. By using homology directed repair, the team was able to successfully incorporate a large spider silk-like gene into the fibroin heavy chain (fhc) gene, resulting in a doubled ratio of spider silk-like protein and substantially better mechanical performance compared to conventional transposon-based methods. The project was also able to insert a synthetic major ampullate spider silk gene into the fibroin of the light chain, also resulting in mechanical properties similar to native spider silk. The project’s light-chain homologous recombination (LC-HR) portion incorporated an enhanced green fluorescence protein into the fibroin light chain, demonstrating the potential to produce different types of exogenous proteins in silkworms (X. Zhang, 2017).

The abovementioned studies show that CRISPR technology can improve silk fibers’ production and quality and increase silkworms’ resilience to viral infections. These advancements have significant implications for the silk industry, which has been an integral part of human civilization for thousands of years, economically and culturally. Furthermore, the precise modification of the genetic makeup of silkworms using CRISPR-Cas9 technology offers a more effective and efficient method to produce silk with consistent quality and purity. Therefore, these developments have the potential to significantly impact the industry as a whole. Basing our CRISPR system off of the work
from X. Zhang (2017)’s previous work serves as a starting point for the production of our AMP peptide. While that work was for introducing spider silk genes to the silkworm lines, it should work to also introduce an AMP by switching out the spider sequences for AMP. The eGFP protein tag element is also useful as a screening tool as previously designed and is expected to also express in silk. However, the next steps would be to determine an appropriate antimicrobial peptide as well as design the LC-AMP (Light Chain CRISPR vector with the AMP) plasmid.
CHAPTER 2: CONCEPTION AND DESIGN

Selection of an Appropriate Antimicrobial Peptide

The first course of action was to determine an appropriate candidate AMP. Careful consideration was taken to ensure that the candidate’s size, structure, mechanism of action, ability to be synthesized using amino acids commonly found in the silkworms’ natural diet, costs, and timeline all fit within the scope of this project (Figure 1). A single AMP was chosen as a proof of concept, primarily for cost but also simplicity; however, future work might examine whether a series of AMPs have increased efficacy if the preliminary results from this project are promising.

Figure 1

List of Examined AMP Peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Source</th>
<th>RA</th>
<th>AMP</th>
<th>mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-37</td>
<td>LLDGRFRKSEKIGKEQKRVQDRKTPVLPQVRIVK</td>
<td>Human</td>
<td>37</td>
<td>111</td>
<td>membrane disruption</td>
</tr>
<tr>
<td>Heparin</td>
<td>RRRRRRTHTPFICCCGCGCHRISGCGMCDCRT</td>
<td>Human</td>
<td>30</td>
<td>90</td>
<td>interferes with intracellular nucleic acids</td>
</tr>
<tr>
<td>Temporin A</td>
<td>FLFLGVR/SLGR</td>
<td>Frog (R. temporaria)</td>
<td>13</td>
<td></td>
<td>39. Form ion-conducting and anion-selective channels</td>
</tr>
<tr>
<td>Temporin B</td>
<td>YLLPVLQNLKSL</td>
<td>Frog (R. temporaria)</td>
<td>31</td>
<td></td>
<td>93. Causes the collapse of plasma membrane potential</td>
</tr>
<tr>
<td>Royal Jelatin</td>
<td>R1/P-1PRQSKRSGDYFPRQSKRSGDYG</td>
<td>Honeybee (Apis mellifera)</td>
<td>37</td>
<td>111</td>
<td>111. Folds and aggregates into membrane</td>
</tr>
<tr>
<td>Temporin 10D1</td>
<td>HFPLTVNLARKIL HFPLTVNLARKIL</td>
<td>Frog (R. draytoni)</td>
<td>44</td>
<td></td>
<td>132. Non-specific perturbation of the membrane</td>
</tr>
<tr>
<td>Ramucelzin</td>
<td>HRFLEGLGWVWM/CAGYMNQGCO</td>
<td>Frog (R. cathabana)</td>
<td>24</td>
<td>72</td>
<td>multiple mechanisms - affects membrane/cell wall</td>
</tr>
<tr>
<td>Bufohin</td>
<td>TRSRLAGLGEPVRGVRH-LRK</td>
<td>Bufo gargarizans</td>
<td>22</td>
<td>66</td>
<td>disrupts intracellular processes</td>
</tr>
<tr>
<td>Edermicin N901</td>
<td>MAA/KQLEDATAGQVK/LWMKHKGTeki/WMNAGQFEVYQIKQKQK/</td>
<td>S. epidermidis</td>
<td>51</td>
<td>153</td>
<td>153. Toroidal pore formation</td>
</tr>
<tr>
<td>Bactericidal peptide</td>
<td>GKWVIR/KAADKFKL/AC</td>
<td>Human (saliva)</td>
<td>20</td>
<td>60</td>
<td>membrane disruption/pore formation</td>
</tr>
<tr>
<td>Heparin 5</td>
<td>ARVAVGHDRH/ARMPHDNSRGRGYS</td>
<td>Human (saliva)</td>
<td>24</td>
<td>72</td>
<td>intracellular disruption</td>
</tr>
</tbody>
</table>

Note. A list of examined AMP peptides as they relate to the silkworm AMP project. Size, structure, mechanism of action, amino acid composition, and timeline were all considered before selecting an appropriate candidate for the project.
Size Considerations

Most AMPs are relatively small, ranging from 12 to 50 amino acids in length. Therefore, a relatively small change in the amino acid composition of the silk would be expected to have minimal effects on the structure of the silk, given the difference in size. For example, an LL-37 AMP is estimated to be about 3 nm long (Sancho-Vaello et al., 2020), and the typical size of a silkworm fibroin is 10–20 μm in diameter (S. Chen et al., 2019). Therefore, the additional peptide would be less than a 0.03% change in a small fibroin and ultimately should be relatively inconsequential to the integrity of the fiber.

Structure Considerations

Antimicrobial peptides come in various secondary structures, sometimes classified as types I–IV (Morais et al., 2016). Type I AMPs are alpha-helical structures with charged ends that disrupt the bacterial cell membrane. Type II AMPs typically possess a beta-sheet-oriented structure, while type III has a more amorphous shape. Finally, type IV consists of looped peptides with several disulfide bonds, making it difficult for proteases to digest (Seyfi et al., 2020). Therefore, the AMP would preferably be able to fold into its proper secondary structure. Especially the peptides should be able to fold correctly when anchored to the silk since many of these AMPs in their natural environments are free floating. A six-proline linker was included to allow for this while keeping them tethered, permitting them a little extra space for folding. The importance of Linkers is discussed in further detail in the Linker Design section below.
Mechanism of Action Considerations

The method of action of the antimicrobial peptide is also important. Many current methods of producing antimicrobial textiles include treating them with compounds that will slowly degrade and disrupt the cellular processes of bacteria (McQueen & Vaezafshar, 2020). Methods include aggregation in the cell, causing disruption of intracellular processes or forming a transmembrane pore in the cell. The issue with these methods is that the AMPs are consumed over time, lowering the efficacy of the antimicrobial textile. Therefore, an antimicrobial peptide that is not consumed as its method of action was desired. A protein that can be anchored or tethered to the fiber while maintaining its efficacy would be required because it can be reused multiple times and prevent it from being washed away. It was hypothesized that by tethering an AMP it would remain on the silk fiber and would not dissipate into the environment. An antimicrobial peptide not degraded or destroyed as it acts against bacteria was also desired because this would lower the efficacy. By tethering it to the natural silk sequence, it could be ensured that the AMP remains close to the fiber even after acting upon the invading microbe. Further, by tethering the AMP to the natural silk sequences, the AMP will be distributed throughout the fiber, meaning even if fiber degradation did occur over time, that would only expose new AMP to the fiber’s surface. The addition of an antimicrobial peptide to the light chain of the silk should not alter or degrade the silk due to the size of the AMP would be negligible compared to the silk fibroin (see Size Considerations on Page 13). If structural changes do occur it will more likely be due to the inclusion of eGFP which is substantially larger than an AMP (about 239 amino acids or 26.9kDa, compared to LL-37’s 37 amino acids and 4.5kDa).
Amino Acid Composition Considerations

Silks are composed of repeating motifs that consist of multiple alanine and glycine residues, so AMPs consisting of those, and other amino acids naturally found in their diet were best suited for the present application. Worms fed a “balanced” amino acid diet tend to have increased silk yields, especially when fed their natural diet of mulberry leaves (X. Chen et al., 2022). Mulberry leaves are the preferred source of nutrition and are approximately 15–30% protein, with additional minerals (predominantly calcium and phosphorus) available for the worms (Al-Kirshi et al., 2013). Food sources not supplying the proper amino acid concentrations (low in crucial amino acids) can lead to inferior fibers and protein truncations. All of this is to say that it is best to avoid AMPs with amino acids that do not mimic the silkworms’ natural diet, aka mulberry leaves, as it can lead to truncations and poor-quality silk.

Timeline Considerations

All work was completed within a two-year window. This is predominantly due to the framework of the author’s master’s degree program but also due to the generation times of the silkworms and supply chain issues due to the COVID-19 pandemic. During this two-year window, key milestones include: the development of the AMP insert, manufacturing of the insert, the cloning work involved to generate the appropriate sequence and enough DNA copies in *Escherichia coli*, the transformation of the silkworm eggs and the subsequent sericulture, antimicrobial efficacy testing and mechanical properties testing, administrative tasks, and final writing and defense of this project report. As additional context, silkworms’ life cycle from egg to egg is approximately two
months, with cocoons forming after 30–34 days if raised at the appropriate temperature (Figure 2; Banno et al., 2010).

Figure 2

Overview of Silkworm Life Cycle

Note. There are multiple larva stages through which the silkworm must develop. Maintaining an appropriate temperature and humidity is essential. From Banno et al. (2010).

Proposed Course of Action/AMP (LL-37)

Through much consultation with Drs. Justin Jones, Randy Lewis, and Xiaoli Zhang, and after examining a variety of AMPs and the issues, including those above, human neutrophil defensin, LL-37 antimicrobial peptide was chosen as a test AMP. LL-37 is a type I alpha-helical peptide (Seyfi et al., 2020) and should be a suitable candidate that matches the previously defined parameters, including: it meets size constraints at thirty-seven amino acids long, it has a molecular weight of 4.5kDa, it has an appropriate method of action (amphipathic helix which disrupts the bacteria invaders), and it does not contain amino acids that are not readily available in the silkworm chow. LL-37 also has
the advantage of being well-characterized and was one of the earlier defensin AMPs to be studied (Romoli et al., 2017).

LL-37 is an antimicrobial peptide (AMP) discovered in the early 1990s by Agerberth et al. (1995). It was initially isolated from human white blood cells, and it has since been found to be produced by a variety of cells throughout the body, including epithelial cells, keratinocytes, and macrophages (Murakami et al., 2004). The structure of LL-37 consists of a linear chain of amino acids folded into a characteristic alpha-helical structure. Hydrogen bonds and hydrophobic interactions between the amino acid residues stabilize this helix. The helix is amphipathic, meaning it has both hydrophilic and hydrophobic regions, which allows it to interact with both the aqueous environment and the lipid membranes of microorganisms.

LL-37 is a potent antimicrobial agent that can kill many microorganisms, including bacteria, fungi, and viruses. Its mechanism of action involves disrupting the cell membranes of these microorganisms, leading to their destruction. LL-37 can also modulate the immune system, attracting immune cells to the site of infection and stimulating their activity, promoting the clearance of pathogens. Additionally, LL-37 has been found to have anti-inflammatory effects, which can help to reduce tissue damage caused by infection (Davidson et al., 2018).

Since its discovery, LL-37 has been the subject of intense research. Its potential as a therapeutic agent for treating infections has been widely explored. It has also been investigated for its potential role in wound healing, cancer, and autoimmune diseases (Brogden et al., 2003). For example, one study showed that LL-37 could prevent the formation of biofilms, which are bacterial communities that can be highly resistant to
antibiotics and cause chronic infections (Mangoni et al., 2018). Another study found that LL-37 could inhibit the growth of cancer cells \textit{in vitro} and \textit{in vivo}, indicating its potential as an anticancer agent (Birgin et al., 2021).

LL-37 has also been found to have immunomodulatory properties. It can activate various immune cells, including monocytes, macrophages, and dendritic cells, and promote the production of proinflammatory cytokines (Mookherjee et al., 2006). In addition, LL-37 can also stimulate the production of antimicrobial peptides by epithelial cells, further enhancing the innate immune response (Schauber et al., 2007).

In conclusion, the discovery of LL-37 has led to a greater understanding of the role of antimicrobial peptides in the immune system and the potential therapeutic applications of these peptides. Due to these properties of LL-37, it should be a suitable candidate to genetically engineer into silkworm silk.

If genetic engineering were to prove unsuccessful, there has also been a method proposed by Bai et al. (2008) that involves covalently bonding the AMP to silkworm silk via a carbodiimide chemical reaction, therefore showing that there is precedent to adhering AMPs to silk proteins. However, the course of action used by Bai et al. (2008) would have been less ideal for this project and the commercial viability of a potential future line due to requiring post-treatment after initial silk production. This would inevitably increase production time and production costs if required. However, this shows there would have been an alternative path for tethering antimicrobial peptides to silk within this project if necessary.

For this project, however, the best way to incorporate the LL-37 AMP into the fiber was through genetic engineering and the use of a peptide linker that would ideally
allow the AMP to protrude from the surface of the fiber. Peptide linkers are a common genetic engineering tool when generating a fusion protein and combining different proteins. Protein linkers are used to join different protein domains while allowing them to maintain their structural and functional integrity and allowing fusion proteins to have novel properties or functions. The following section discusses different types of linkers and how these were selected for the current project.

Linker Design

Types of Linkers

Protein linkers are short amino acid sequences that separate multiple protein domains (Reddy Chichili et al., 2013). They often are categorized into three categories: flexible, rigid, and cleavable (Yu et al., 2015). They can be imagined as molecular ropes, support beams, and tethers, and they are instrumental in proper protein folding.

Of the three types, flexible linkers are often used to allow for interaction between protein domains and are composed of small or hydrophilic amino acids such as glycine (X. Chen et al., 2013). They can also be used to create more distance between protein domains while keeping them in close proximity. This strategy is often used to help reduce the amount of steric hindrance and ensure better protein folding when designing fusion proteins.

Conversely, a more rigid protein linker is sometimes desired to reduce the degree of movement between protein domains. For example, Proline is a common amino acid in many rigid spacers (Reddy Chichili et al., 2013). This type of linker can also help maintain and preserve the active site by ensuring that the subdomains do not interfere.
Finally, the third type of protein linkers are cleavable linkers. These serve as molecular tethers that allow for the controllable separation of protein domains, often by another enzyme or a change in working conditions (Yu et al., 2015). These can allow for a free-floating protein domain after synthesis, and the cleavage site is activated, allowing for more targeted delivery.

Protein linkers have several properties that can be considered when designed, including length, hydrophobicity, amino acid allocation, and secondary structure (X. Chen et al., 2013). Adjusting these parameters during the initial design process is critical to their success. For example, too short a length can lead to issues as the protein is being synthesized and have the same downsides as tandem fusion. In contrast, too long could result in entanglement of the protein or not allow the desired protein domains to interact reliably. In addition, changing the hydrophobicity can alter protein folding and cause unplanned interactions with other proteins. Finally, the amino acid allocation should also be considered; proteins that rely too heavily on a single amino acid may deplete the readily available tRNAs of that particular amino acid, resulting in premature termination (Pavlova et al., 2020) (though admittedly, that is probably more of a protein design concern rather than a linker-specific issue).

Utilizing amino acids that are rare in the host should also be considered (for both protein and linker designs). However, all these characteristics can be modified and adjusted according to the experiment’s needs. For this experiment, it was determined that
the type II linker, a rigid proline linker, would be sufficient for the identified needs. It is expected that by utilizing this rigid proline linker, a “thorny rose” type configuration would effectively be developed with the fusion protein, as seen in Figure 3.

**Figure 3**

*“Thorny Rose” Proline Linker Analogy*

Note. The “thorny rose” is an analogy to show how the proline linker and LL-37 peptide would adhere onto the light-chain silk fibroin. This would ideally provide a protective effect much like a rose’s thorns against would-be predators.

One caveat to this description is that it is not exactly like a rose in that the antimicrobial peptide is only integrated into the light chain of the silk fibroin. It is also important to note that only the exposed areas of the light chain would be effective. Any
part of the light chain that is embedded or facing the internal part of the silk fiber would not have the protective molecular “thorns” exposed. Incorporating an additional AMP through a similar process in the heavy chain would likely have a greater exposed surface area and effect. However, that is beyond the scope of this thesis.

Why a Non-Cleavable Linker?

By utilizing a non-cleavable linker, allowance was intended for proper protein formation. This relatively fixed region is expected to allow the space needed for the peptide to form correctly away from the silk fibroin. It was further considered undesirable to allow the LL-37 peptide to leach out of the fiber (McQueen & Vaezafshar, 2020). When attached to a non-cleavable linker, the AMP cannot migrate away from the fiber and thus retains the antimicrobial activity of the fiber over time.

Plan of Analysis/Detection Systems

Reporter Protein: eGFP

Enhanced Green Fluorescent Protein (eGFP) is a highly utilized biological marker in cell and molecular biology research. It is a modified version of the Green Fluorescent Protein (GFP) originally isolated from the jellyfish *Aequorea victoria*. Researchers modified the original GFP sequence by introducing specific mutations to enhance its brightness and fluorescence intensity (Chalfie, 1995; Cormack et al., 1996).

eGFP can be genetically fused to proteins of interest, allowing researchers to track their localization and expression in living cells. When exposed to blue light, eGFP emits green light, which can be easily detected and visualized using fluorescence microscopy.
eGFP has been used to visualize the movement of proteins and organelles within cells (Hanson & Köhler, 2001), study gene expression in living tissues (Frei et al., 2020), and monitor the progression of diseases in animal models (Choi et al., 2021). Due to its versatility as a marker, the use of eGFP has revolutionized the field of molecular biology, leading to many breakthroughs in understanding molecular biology, cell biology, protein function, and gene expression (Shaner et al., 2005).

Researchers use eGFP in various experiments, including monitoring eGFP expression to determine transgenic positive cocoons. Not all cocoons have the transgene present, so a quick means of sorting transgenic positive from negative is required (Cormack et al., 1996). The experiments reported here include eGFP as part of the protein construct. Expression is then monitored to determine which cocoons are eGFP+ and thus contain the AMP sequence. Again, not all cocoons will have the transgene present due to the success rate of the transformation. Thus, it provides an efficient and accurate means of sorting transgenic positive from negative and can be done simply by exciting the cocoons with UV/blue light.

Dot Blot Antibody Presence

Performing western blots on insoluble silkworm fibers is notoriously difficult and relatively inaccurate. Silkworm fibers, include by their very nature, are highly resistant to being dissolved, and the proteins themselves have a high molecular weight that can vary wildly (between 20 to 400 kDa (Kunz et al., 2016)); thus, they are resistant to migrating into the polyacrylamide gel. To mitigate the problems of performing SDS-PAGE and then western blots on these proteins, it was decided to utilize a dot blot rather than a western blot to confirm AMP presence. To run a dot blot, the proteins must be dissolved and
absorbed onto a membrane for antibody probing. There is no requirement for the proteins to migrate into a polyacrylamide gel, so the size of the protein cannot be determined. However, with the AMP being very small compared to the silk proteins, their inclusion with the light chain protein, the change in size difference is unlikely to be observable on an SDS-PAGE. Coupled with the extreme difficulties in solvating and then running these proteins on an SDS-PAGE, a Western Blot is not a viable option. Dot blotting, on the other hand, is a simple and quick method for detecting and quantifying proteins, nucleic acids, or other biomolecules in a sample (Jiao et al., 2012). The technique involves immobilizing the target molecules onto a membrane and then probing the membrane with specific antibodies or probes. The amount of signal generated is proportional to the amount of target molecule present in the sample. Compared to western blotting, dot blotting is quicker and simpler to perform, does not require electrophoretic separation of proteins, and is more tolerant of denatured proteins (Abdel-Salam et al., 2013; Jahn et al., 1984). However, the technique cannot distinguish between specific protein isoforms, and it has lower sensitivity and resolution than other methods like ELISA and western blotting (Roussel et al., 1995). Dot blotting is commonly used in research and clinical settings for diagnosis, drug discovery, and protein analysis applications.

To perform a dot blot, the sample is first applied to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane using a small-volume pipette. Then, the membrane is dried or fixed to immobilize the sample. The membrane is then blocked to prevent non-specific binding, and a primary antibody or probe is added to detect the target molecule. After any unbound antibody or probe is washed away, a secondary antibody or probe conjugated to a detection system (such as horseradish peroxidase) is
added. The detection system generates a signal (chemiluminescence) interacting with the secondary antibody or probe. Depending on the secondary antibodies, it can then be detected using a specialized instrument (Hames & Rickwood, 1981). The dot blot was first introduced in the 1970s as a simple method for detecting and quantifying RNA in cells (Thomas, 1980). Since then, the technique has been adapted and optimized for various applications, including detecting proteins, DNA, and other biomolecules. Despite its limitations, the dot blot remains a useful tool for screening large numbers of samples quickly and efficiently.

In this case, the secondary antibodies were conjugated with alkaline phosphatase, resulting in a visual purple staining on the membrane.

The dot blot immunoblotting test is a useful method that provides a quick and simple way to detect and quantify proteins, nucleic acids, or other biomolecules in a sample. It is more tolerant of denatured proteins and does not require electrophoretic separation of proteins like Western blotting. However, it has lower sensitivity and resolution than other methods like ELISA and Western blotting ((Jahn et al., 1984); Roussel et al., 1995). Therefore, dot blotting served as an economical choice for this research to prove the concept of including an AMP in silkworm silk fibers.

Live/Dead Stain Assay

A live/dead stain assay is widely used to differentiate between live and dead cells in a population. This assay utilizes fluorescent dyes capable of penetrating the cell membrane and interacting with intracellular components such as DNA or enzymes.

The assay is performed by staining live cells with a green fluorescent dye, such as calcein-AM, which can enter live cells and is cleaved by esterases to produce a green
fluorescent signal. Dead cells are stained with a red fluorescent dye, such as propidium iodide, which cannot penetrate the cell membrane of live cells but can enter dead cells that have damaged or compromised membranes. Propidium iodide binds to DNA and produces a red fluorescent signal.

The stained cells are then imaged using fluorescence microscopy or analyzed by flow cytometry. The proportion of live and dead cells in the sample can be determined based on the intensity and color of the fluorescent signals. The live/dead stain assay is a quick and reliable method to assess cell viability and has numerous applications in various research fields, including pharmacology, microbiology, and cell biology.

The Thermo Fisher BacLight LIVE/DEAD™ BacLight™ Bacterial Viability Kit was used for this project. It is a popular live/dead stain assay that differentiates between live and dead bacteria. The kit contains two fluorescent dyes, SYTO 9 and propidium iodide, used to stain live and dead cells. SYTO 9 enters live cells and produces a green fluorescent signal, while propidium iodide binds to DNA in dead cells and produces a red fluorescent signal. The stained cells can then be imaged using fluorescence microscopy or analyzed by flow cytometry to determine the proportion of live and dead cells in the sample. The kit is quick and easy to use, allowing for real-time visualization of live and dead bacteria. However, it should be noted that the kit may produce false positives if live cells have compromised membranes or if there is a high background signal. Overall, the Thermo Fisher BacLight LIVE/DEAD™ BacLight™ Bacterial Viability Kit is a reliable and widely used method for assessing bacterial viability and has been used in similar applications in various forms for over twenty years (Boulos et al., 1999) (Robertson et al., 2019).
Zone of Inhibition

Zone of inhibition testing is a widely used method in microbiology, which was first described in 1966 to determine the antimicrobial activity of a compound or a sample against a specific bacterial strain (Kirby & Bauer, 1966). The Kirby-Bauer version of the test involves impregnating a sterile filter paper disk or an agar plate with a known concentration of the compound or sample of interest and placing the disk on the surface of an agar plate inoculated with the bacterial strain under investigation. The compound diffuses into the agar, creating a concentration gradient. If it is active against the bacterial strain, it will inhibit or slow down bacterial growth around the disk, resulting in a clear zone of no bacterial growth. The size of the zone of inhibition is measured and compared to a standard reference range to determine the potency of the compound. This method is widely used to determine the susceptibility of bacteria to different antibiotics and to assess the antimicrobial activity of natural or synthetic compounds (Barry et al., 1979; Mith et al., 2014).

The zone of inhibition test has been used to evaluate the antimicrobial activity of various natural compounds, such as essential oils, which have gained attention recently as potential alternatives to conventional antibiotics (Mith et al., 2014). Studies have shown that some essential oils, such as oregano and thyme oil, exhibit significant antibacterial activity against foodborne pathogens such as Salmonella and E. coli, with larger zone sizes indicating stronger antimicrobial activity (Mith et al., 2014). Zone of inhibition testing can be used to gauge this type of reaction. It is a staple for testing the efficacy of various compounds and their ability to deter microbial growth.
While the zone of inhibition test is useful for evaluating antimicrobial activity, it has limitations. The test only provides a qualitative assessment of antimicrobial activity. It does not provide information on the minimum inhibitory concentration (MIC) or the compound’s mechanism of action (Barry et al., 1979). Furthermore, variations in the thickness and composition of the agar, as well as the size and shape of the inoculum, can affect the size of the zone of inhibition and the accuracy of the results (Barry et al., 1979).

In conclusion, the zone of inhibition test remains a widely used method for evaluating the antimicrobial activity of compounds against bacterial strains. While the test has its limitations, it is a useful tool in determining the potency of compounds and identifying potential candidates for further investigation. This experiment used zone of inhibition testing where the fiber was draped onto a lawn of LB agar, and the results were measured. It should be noted that it was expected (and even hoped) not to see any zones of inhibition because that would indicate that the compound (LL-37) had become untethered and was leaching out into the media.

Mechanical Testing

For this experiment, various measurements of the transgenic silk were taken and compared to those of wild-type silk. Tensile strength is the maximum stress a material can withstand before breaking or fracturing when subjected to a tensile load or pulling force. In other words, it measures a material’s ability to resist being pulled apart by opposing forces. The tensile strength of transgenic silk was compared to that of wild-type silk to determine if the modification increased or decreased strength. Similarly, Young’s modulus was compared to determine if the modification resulted in a stiffer or more flexible silk fiber. Strain, the deformation a material undergoes when subjected to an
external load or force, was also measured to evaluate how well the modified silk could stretch without breaking.

The energy to break was also measured, which is the energy required to break a material. Finally, the diameters of the fibers were also examined and compared. It was expected that there would not be much change in most of the parameters due to the small change made to the protein fiber. However, it was important to verify that adding the AMP did not deteriorate the quality of the fibers.

Genomic DNA Examination PCR and Sequencing

An additional method of verification is checking the genomic DNA to see if it contains the relevant sequence. This can be done by performing a PCR reaction. PCR stands for Polymerase Chain Reaction, a molecular biology technique used to amplify a specific DNA sequence in vitro. The technique involves using a thermostable DNA polymerase, specific primers that anneal to the DNA, flanking the target sequence, and a mix of nucleotides required for DNA synthesis. The process involves three main steps: denaturation, annealing, and extension.

During denaturation, the double-stranded DNA is heated to a high temperature (around 95 °C), causing the hydrogen bonds between the two complementary strands to break and the DNA to separate into two single strands. In the annealing step, the temperature is lowered to allow the specific primers to anneal to the complementary sequences on the single-stranded DNA. During extension, the temperature is raised again, and the DNA polymerase extends the primers by adding nucleotides to the 3’ end of the primers. This process is repeated multiple times to amplify the target DNA sequence exponentially.
PCR is a powerful tool in biotechnology. It allows for amplifying specific DNA sequences from very small amounts of starting material, which can be further analyzed or manipulated. For example, in the case of checking for the presence of the LL-37 insert in the silkworm genome, PCR was used to specifically amplify the DNA region containing the eGFP/LL-37 insert, by which can be determined if the insert is present or absent in the genome.

Additionally, Sequencing would be a useful tool to check the genomic DNA for our sequence. While there are various sequencing techniques, sanger sequencing would be the technique utilized by the local Center for Integrated BioSystems. It involves a process of replicating a target DNA sequence through PCR amplification and then using the replicated strands as templates to synthesize a complementary strand using dideoxynucleotides (ddNTPs). The ddNTPs lack a 3’ hydroxyl group, which is required for further extension of the complementary strand. As a result, when a ddNTP is incorporated into the growing complementary strand, it terminates the extension of the strand at that point. By adding each ddNTP in a separate reaction tube, the result is a set of strands of varying lengths that end at each ddNTP site. The fragments are then separated by size using gel electrophoresis and read in order of increasing size, revealing the DNA sequence of the original template strand. This method has been used extensively in molecular biology research, medical diagnostics, and forensic analysis, among other applications.
CHAPTER 3: METHODS/PROCEDURE

Genetic Engineering

Construct Design and Synthesis

Previous work performed by Dr. Xiaoli Zhang has yielded a *Bombyx mori*–optimized CRISPR/Cas9 system designed to integrate into the silkworm genome (X. Zhang, 2017). This technology includes a 4.8Kb bacterial expression vector (LC-NHEJ Backbone pBsK), two guide RNAs (G6 and G7), and the Cas9 protein. In addition, the LC-NHEJ backbone vector contains an ampicillin resistance gene, green fluorescent tag protein (eGFP), and multiple cloning sites (MCS) for construct insertion. The LC-NHEJ vector and insert construct (Figure 4) were grown in separate *Escherichia coli* strains.
Figure 4

Overview of LC-NHEJ pBsK CRISPR Expression Vector and LL-37 Insert Maps

*Note.* The figures here show the plasmid maps for the LC-NHEJ Backbone (pBsK) left and the LL-37 Insert vector on the right. Key features include the multiple cloning sites, including the PspOMI and SacI restriction enzyme sites in the LL-37 insert and the eGFP protein and PspOMI and SacI restriction enzyme sites in the LC-NHEJ vector.

The LL-37 insert in the present study had the appropriate restriction enzyme sites to be integrated into the LC-NHEJ backbone vector (PspOMI and SacI), the proline linker, and the LL-37 sequence followed by a stop codon. The LL-37 insert DNA sequence generated by the Thermo Fisher Gene Art division was codon optimized for *Bombyx mori*; the full sequence can be seen in Figure 5. Sadly, during this project, the COVID-19 pandemic caused supply chain issues. Therefore, the turnaround was over two months instead of the more typical two weeks, a theme consistent throughout the project. As a result, this step took much longer than normal.
Figure 5

LL-37 Insert Sequence with Highlighted Key Elements

Note. The restriction sites PspOMI and SacI-HF allowed cloning into the LC-homologous reparation pBsk CRISPR Expression vector. The insert's key elements included the fibroin's C-term, proline linker, LL-37 sequence, stop codon, and C-term intron sequences. The new LC-AMP vector will then be incorporated into the silkworm genome through HDR using the g6 and g7 guide RNAs and Cas9 protein.

Cloning Work

Cloning work began by transforming the components (the LL-37 insert vector and LC-NHEJ vector) into E. coli (Top10 Chemically Competent Cells, Thermo Fisher) and then using their natural machinery to produce more copies of the target sequences. Next, after doing a standard miniprep extraction (Promega, A1330), the LC-NHEJ backbone and LL-37 Insert were both digested with PspOMI and SacI restriction enzymes (R0653S and R3156S, both NEB) and performed a standard ligation step to link them together.

First, colonies were screened and run on an agarose gel to determine the appropriate size. Then, once a suitable clone had been identified, samples were sent for sequencing services at the local Center for Integrated Biosystems, which has a turnaround time of 24–48 hours to verify the successful integration of the DNA sequence.
Large Scale DNA Prep

After the sequencing results were received, the next step was upscaling the bacterial expression vectors by growing the positive clone in liquid media flasks. Unfortunately, a standard mini-prep DNA extraction kit could not be used since a single bacterial colony would not provide sufficient DNA for the CRISPR/Cas 9 system to integrate into the silkworm genome. To remedy this, the silkworm AMP insert (the LL-37 insert in the LC-NHEJ backbone vector aka, SW-AMP), the G6 and G7 guide RNA sequences, and the Cas9 protein sequence were transformed into four \textit{E. coli} (Top10 Chemically Competent Cells, Thermo Fisher) cultures of 500 ml and incubated at 37 °C for 14–15 hours. Then a DNA MIDI-prep kit (Promega) was used to purify the larger quantities (~500 µgs) of each DNA plasmid.

Sericulture and Silkworm Egg Transformation

CRISPR/Cas9 and Silkworm Egg Transformation

After the four DNA plasmids were obtained, they were combined to create a final SW-AMP mix to transform the silkworm eggs. The eggs were transformed using a modified protocol provided by Dr. Xiaoli Zhang. Additionally, it was important to ensure that the eggs were collected no longer than 1 hour after being laid to prevent the electroporation procedure from failing due to the eggs being too far along in their maturation cycle. (This is because the outer egg becomes hard and resistant to electroporation.) Once enough eggs were collected for electroporation (ideally 100 or more at a time), the eggs were treated by being briefly shocked using an electroporator. Using the protocol provided by Dr. Zhang (X. Zhang, 2017), the electroporation of
silkworm eggs using the CUY21EDIT in vivo square wave electroporator and CUY495P10 chamber was performed. All eggs were treated with the electroporation procedure and were divided based on the initial eGFP screening results into Control and SW-AMP groups. The modified protocol includes preparing electroporation buffer (EP buffer), egg collection and washing, pressure reduction, and electroporation. The SWAMP mix consisting of the g6, g7, cas 9, and SW-AMP vector in equimolar concentrations used for electroporation was roughly a 3:3:1:1 ratio, as required in the protocol.

Materials:

- CUY21EDIT in vivo square wave electroporator
- CUY495P10 chamber
- Fresh silkworm eggs from purebred moths
- ddH2O
- 2% PVP solution
- 10% Tween 20
- 0.1 M spermidine solution
- DNA plasmid(s) solution (SWAMP mix)
- 2.5 M CaCl2
- 1.5 ml Eppendorf tube
- 9 cm Petri dish
- 7 cm diameter paper
- Vacuum chamber
- Ice
Protocol:

1. Prepare electroporation buffer (EP buffer) by adding ddH2O (385 µl), 2% PVP (polyvinylpyrrolidone) solution (250 µl), 10 % Tween 20 (15 µl), 0.1 M spermidine solution (50 µl), and DNA plasmid(s) solution (100 µl, 1.0 µg/ µl) to a 1.5 ml Eppendorf tube and mix well.

2. Add 100 µl of 2.5 M CaCl2 to the EP buffer and mix well again.

3. Collect and briefly wash the silkworm eggs (laid within 2–3 hours) with tap water.

4. Place the eggs (500–1000 eggs) into EP buffer in a 9 cm Petri dish.

5. Treat the silkworm eggs with pressure reduction by placing the dish with eggs on ice in a vacuum chamber for 10–20 minutes.

6. Run electroporation for the eggs on ice by placing them into the electroporation chamber, adding 1 ml EP buffer into the electroporation chamber (eggs were cooled prior to electroporation by allowing them to sit in the chamber, on ice, for 2 minutes), and running the electroporation under 15 V, 50 ms (pulse), 75 ms (interval), 10–20 repeats, then leaving eggs in the chamber 10–20 min on ice to allow the eggs to cool.

7. Place the eggs on ice and leave them for at least 1 hour.

8. Eggs are placed in a 9 cm Petri dish with 7 cm diameter paper.

9. Leave the eggs in the dark at 25 °C for hatching.

After the electroporation and appropriate chilling, eggs should be kept at 25 °C and in a humidity-controlled environment. Eggs were expected to hatch in 14 days if kept at the appropriate temperature and humidity.
Note. After the silkworm moth laid eggs, these were quickly collected, and electroporation was conducted. First, alligator clips were attached to the CUY495P10 chamber, which was then fed into the CUY21EDIT square wave electroporator device.
Sericulture and Silkworm Rearing

In order to ensure that the eggs hatched and that the silkworms could progress throughout their life cycle, great care was taken to maintain the proper environment for them. This required a team effort, consisting of two experienced post-doctoral researchers overseeing the process, a dedicated graduate student, and 2-4 diligent undergraduate students working together to ensure the silkworms were well taken care of. The priority was to ensure that the silkworms were kept in a warm and humid environment with a temperature range of 25–30 °C. This was achieved by placing them in a specially controlled room within the USU Vivarium adjacent to the USTAR 650 lab space.

Figure 7

USTAR 650 Vivarium Setup

Note. Includes a secure, humidity- and temperature-controlled room with racks and associated equipment for proper sericulture care. The panel on the left shows the interior of the room, and the right panel shows the access door into the room, and there are three doors that must be passed after entering the building, including a passcode, biometrics, and prox-card keys to access.
In this temperature- and humidity-controlled room, the silkworms were placed on baking sheets within baking racks to keep them organized and ensure their comfort, as seen in the left panel of Figure 6.

Feeding the silkworms was also a top priority, as they needed to be provided with a healthy diet in order to thrive. To accomplish this, the worms were fed ground mulberry leaves, also known as chow, from a US-based supplier (Mulberry Farms, http://www.mulberryfarms.com/). The team carefully prepared and monitored this diet to ensure that the silkworms received the proper nutrients they needed to grow and develop. Additionally, it was important to clean up any waste that the silkworms produced to maintain a hygienic environment and prevent the spread of disease (general laboratory environment shown in Figure 7).

As the silkworms progressed toward the cocoon stage, a specialized cocoon initiation webbing was introduced, as seen in Figure 8.
Fig. 8  

Carts, Trays, and Netting to Facilitate Sericulture Cocooning  

Note. The sericulture setup, including the netting, was used when the silkworms were ready to spin. The trays and carts are like those that you might find in a commercial bakery. The netting was from a silkworm manufacturer in China and had been used in previous experiments. Many worms are cocooned or looking for a place to cocoon. Despite abundant fecal matter, an effort was made to ensure they were not disturbed during cocooning. Most cocooned within 3–5 days of each other anyway.
This was done shortly after they entered their fifth larval stage to help them create cocoons. The team carefully chose this webbing to provide the silkworms with a place to anchor and begin cocooning while being able to be removed relatively easily during harvesting.

Ultimately, the result was that healthy and robust silkworms were successfully raised, progressing through their life cycle and providing the cocoons needed for the project.

Harvesting Cocoons

Cocoons were harvested and screened for eGFP protein using a black-light flashlight. These were then separated by hand into positive, negative, and indeterminate groups. In addition, a dot blot was also conducted on the silk fibers using an antibody specific to LL-37 to verify its presence. Finally, all cocoons were subjected to the electroporation procedure from the same batch. Based upon the eGFP screening procedure, six (Control A–F) control cocoons were selected along with six experimental cocoons (SW-AMP #1–6) and were used for subsequent experiments.

Degumming Fibers and Solubilizing Silk Proteins

Degumming is a necessary process in producing silk fibers as it removes sericin, a water-soluble protein that surrounds and cements the fibroin strands of silk fibers. Sericin is a byproduct of silkworms, which they secrete during cocoon formation to protect the silk fibers. However, sericin is not desirable in most silk applications due to its tendency to yellow, stiffen, and reduce fiber luster. In addition, sericin is a potential allergen that can cause skin irritation and other adverse reactions (Gowda et al., 2016).
To remove sericin, silk fibers are immersed in boiling water or a chemical bath, causing the sericin to dissolve and loosen from the fibroin strands. The fibers are then rinsed and dried, resulting in a pure fibroin silk fiber that has improved properties such as increased luster, softness, and drape. Degumming is essential to obtain the highest quality silk fibers suitable for various applications, from textiles to medical sutures.

The degumming method used was to first remove the sericin (the coating that helps bind the fibers of the cocoon together) by dissolving it in 0.05% (wt/vol) Na$_2$CO$_3$ for 15 minutes at 85 °C, followed by washing the cocoons twice with hot water and carefully stirring as described in (Teule et al., 2012). This was performed for the SW-AMP and the control silks for downstream experiments (imaging of intact fibers and in preparation for the solubilized fibers).

To solubilize the fibers, the protocol outlined by (Teule et al., 2012) was performed. To briefly summarize: 1 mL of PBS, consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$PO$_4$, and 1.8 mM KH$_2$PO$_4$, was added to the material, and it was left for 16 hours at 4 °C. After centrifugation, the material was separated into insoluble and soluble fractions. Next, the PBS-soluble fraction was removed and stored at −20 °C, and the pellet (the majority of the silk fiber) was then treated with 1 mL of 2% (wt/vol) SDS and incubated for 16 hours at room temperature. The material was again centrifuged and divided into soluble and insoluble fractions. Finally, the SDS-soluble fraction was collected and kept at −20 °C, and the pellet was subjected to further extraction.

The pellet was treated with 1 mL of 9 M LiSCN, which contained 2% (vol/vol) β-mercaptoethanol (BME), and left at room temperature for 16–48 hours. After centrifugation, the supernatant was saved at −20 °C as the 9 M LiSCN/BME-soluble
fraction. The final pellet was resuspended in 1 mL of 16 M LiSCN, containing 5% (vol/vol) BME, and incubated for about 1 hour at room temperature. This led to the complete dissolution and production of the final extract, which was then stored at −20 °C as the 16 M LiSCN/BME-soluble fraction until the immunoblotting assay (Teule et al., 2012). In this case, immunoblotting was done via dot blot (see Dot Blot Basic Procedure).

Silk Evaluation and Testing of Antimicrobial Silk Cocoons

Zone of Inhibition

A zone of inhibition assay was conducted to detect antimicrobial activity by placing silk fibers onto an agar plate inoculated with a common skin microbiota, *Staphylococcus epidermis* (Thermo Scientific, R4606500). *Staphylococcus hominis* and *Staphylococcus aureus* were also planned to be evaluated. However, due to supply issues with *S. hominis* and potential safety concerns with *S. aureus*, the assay was performed only with *S. epidermis* as a part of this project.

Dot Blot Basic Procedure

A brief dot blot protocol is described here for reference. First, a piece of nitrocellulose paper is cut to a size that fits all the samples and the concentration gradient/ladder. Next, the paper is labeled by drawing a grid and writing the associated sample name in each square. Next, the labeled membrane is placed onto a clean tissue paper, and 5 µL of each sample is loaded onto the membrane by placing a dot in the center of the appropriate square. After loading all samples, the membrane is allowed to
soak up all the samples for at least 5 minutes, and if needed, it can be stored in a labeled container.

Next, the membrane is placed in a clean box or Petri dish. A blocking solution consisting of Carnation instant milk (Carnation instant milk, Nestle 12428935) and TBS-T (0.02 M Tris, 0.14 M Sodium Chloride, 0.05% Tween20, pH to 7.4 using HCl) is added to the membrane and mixed on the gel mixing platform for 30 minutes. The primary antibody, at a concentration of 1:1000 Anti-LL-37 Antibody (D-5, Santa Cruz Biotechnology, sc-166770), is mixed into the solution and applied for 30 minutes. The membrane is then rinsed with TBS-T for 5-10 minutes. The secondary antibody 6X His-Tag Mouse IgG1 Monoclonal Antibody (Promega, S3721) at a concentration of 1:10000 is then added and mixed for 30 minutes. The membrane is again rinsed briefly with TBS-T. Finally, the development buffer (NBT/BCIP, Thermo Fisher Scientific, PI34042) is poured on the membrane and mixed by hand or on the platform until the dots appear as brown-purple dots that are easy to see. The membrane is then rinsed with dH2O and let dry before imaging. A concentration of 1:1000 for the primary antibody and of 1:10000 for the secondary antibody was used.

Microscopy and Visual Examination of the Silk

Examination of the silk was done by using a Keyence BZ-X800 Fluorescence Microscope, as well as a Motic BA310 light field microscope (Motic Images Plus 2.0 ML) under standard 10× and 20× magnifications to ascertain the morphology of the silk as well as to detect if any bacteria were present during the immersion testing with the BacLight stain.
The BacLight kit contains mixtures of SYTO 9 and propidium iodide stains, which fluoresce green and red, respectively, and differ in their ability to penetrate healthy bacterial cells. Bacteria with intact cell membranes stain green, while those with damaged membranes stain red. The kit applies to various Live/Dead Stains using a Keyence BZ-X800 Fluorescence Microscope. Images were obtained by combining the BZ-X Filter Texas Red-OP-87765 from Keyence America and the ET-EGFP (FITC/Cy2) from Chroma Technology Corp by the spectra outlined by the Thermo Fisher BacLight Bacterial viability kit (L7012, Thermo Fisher). Five series of images were performed with the fluorescence microscope. First, the fibers were initially passed after exposing them to the stains as described in the kit. Second, the procedure was repeated, but a saline wash step was attempted afterward to de-stain some of the fibers more. Next, the fiber was examined as a small “wad” instead of a single carded fiber at both 4× and 20× magnifications to get a relatively macroscopic view of the fibers. Finally, a “lawn dip” was realized, where fiber was smeared against an inoculated plate and subsequently stained.

The kit is well-suited for fluorescence microscopy, quantitative analysis with a fluorometer, fluorescence microplate reader, flow cytometer, or other instrumentation. The general procedure for the BacLight staining process, as included with the kit, was performed. For reference, an abbreviated protocol is included here.

General BacLight Staining Protocol

Silk immersion tests were conducted in Staphylococcus epidermidis culture in conjunction with a bacterial viability stain Live/Dead BacLight (L7012, Thermo Fisher) to quantify a level of effectiveness. To stain bacterial cells using the Thermo Fisher
BacLight system, 30 mL of either *Staphylococcus epidermidis* cultures were first grown in nutrient broth to the late log phase. The optical density at 670 nm (OD670) is then determined for the bacterial suspensions. The culture was centrifuged, and the supernatant was removed. The pellet was resuspended in 2 mL of 0.85% NaCl or the appropriate buffer. One milliliter of this suspension was added to each of two 50 mL-sized centrifuge tubes, one containing 20 mL of 0.85% NaCl or appropriate buffer and the other containing 20 mL of 70% isopropyl alcohol. The tubes were then incubated for 1 hour at room temperature, mixing every 15 minutes. Both samples were pelleted by centrifugation, and the pellets were resuspended in 20 mL of 0.85% NaCl. The pellets were centrifuged again, and the resulting pellets were resuspended in separate tubes with 10 mL of 0.85% NaCl.

For staining, equal volumes of propidium iodide and SYTO-9 were combined in a microfuge tube and mixed thoroughly. Three microliters of the dye mixture were added for each milliliter of the bacterial suspension. The mixture was then incubated at room temperature in the dark for 15 minutes. Five microliters of the stained bacterial suspension were placed between a slide and an 18 mm square coverslip, and the sample was observed using a fluorescence microscope equipped with the appropriate filters (the BZ-X Filter Texas Red- OP-87765 from Keyence America and the ET-EGFP (FITC/Cy2) from Chroma Technology Corp). This process was completed for all six control (Controls A-F) and six test fibers (SW-AMP 1-6). The protocol indicates a final wash step with 0.85% saline should be done. However, this step was excluded from all images/subsequent steps except the “saline wash” images due to salt crystal formation being excessive when on the silk.
Single Fiber Imaging

The Single Fiber Images were generated following the general procedure described above. The difference was that instead of placing the bacterial suspension between a slide and an 18 mm square coverslip, it was applied to a fiber that had been carded and allowed to sit for 15 minutes in a dark room. The carding procedure was used to secure the fibers, to allow for easier transport, isolation of a single fiber, and subsequent mechanical testing (Licon, 2019). To ensure the silk fibers did not break during handling, they were placed on plastic “C” cards with a rectangular cut-out of 19.5 mm on the side. The cut-out served as the gauge length used to analyze mechanical properties. The fibers were placed along the cut-out, taped, and glued at the inside edges of the card. After the glue dried, the diameter of each fiber was measured at three different places.

Saline Wash Imaging Procedure

To generate these images, the 12 fibers were carded as described previously but subjected to the full 0.85% saline wash as described in the protocol and allowed to dry in an attempt to reduce the background fluorescence after having the stained bacterial suspension applied. Because these salt crystals formed, this final wash step was omitted from all other procedures.

Wad Imaging Procedure

These “wads” were essentially a portion of the silkworm cocoon that had been treated and submerged in a *Staphylococcus epidermidis* solution and then stained. The
A major difference between the wad and the single-fiber solutions is that these images contained multiple fibers and were pressed under a glass microscope slide with a coverslip instead of carded. The wad treatment fibers were treated to *Staphylococcus epidermidis*, rinsed briefly with water, and then stained with equal parts of SYTO-9 and propidium iodide as described in the general protocol, and finally placed on a microscope slide with a coverslip. The images were taken using 4× and 20× magnifications (20× Fiber wad and 4× Fiber wad).

**Lawn Dip Imaging Procedure**

*Staphylococcus epidermidis* was grown on an LB agar plate for the lawn dip images. Then using a pair of sterilized tweezers, the silk was used to scrape against the cultured plate to get many bacteria onto a clump of each silk fiber. The “heavily” inoculated silk was then put on a microscope slide and stained with equal parts SYTO-9 and propidium iodide according to the general staining procedure. Finally, the silk fibers were “dipped” onto a “lawn” of bacteria to ensure a copious amount for staining.

**Mechanical Testing**

To ensure the stability of the silk and that the antimicrobial peptide did not degrade or weaken the fibroin structure, mechanical testing was performed using an MTS Synergie 100 with a custom 10 g load cell. This included measuring and calculating the tensile strength, energy to break the fiber, elastic modulus, and the strain the fiber can withstand. These mechanical tests were also repeated on the regular control silks (as listed earlier in antimicrobial testing) for comparison.
Genomic DNA Extraction, PCR and Sequencing

To extract the genomic DNA from the silkworms, use an E.Z.N.A Insect DNA Kit from Omega Bio-Tek (Omega Bio-Tek, D0926-01). To extract DNA from tissue, start by pulverizing no more than 50 mg of tissue in liquid nitrogen using a mortar and pestle. Then, transfer the powdered tissue to a clean 1.5 mL microcentrifuge tube and add 350 μL of CTL Buffer and 25 μL of Proteinase K Solution. After vortexing to mix, incubate the sample at 60 °C for 30 minutes or until it is fully solubilized. Next, add 350 μL of chloroform:isoamyl alcohol (24:1) and vortex thoroughly before centrifuging at 10,000 × g for 2 minutes at room temperature. Carefully transfer the upper aqueous phase to a clean 1.5 mL microcentrifuge tube, avoiding the milky interface containing contaminants and inhibitors.

Next, add one volume of BL Buffer and two μL of RNase A, vortexing at maximum speed for 15 seconds. Incubate at 70 °C for 10 minutes before adding one volume of 100% ethanol and vortexing at maximum speed for 15 seconds. Insert a DNA Mini Column into a 2 mL Collection Tube and transfer 750 μL of cleared lysate, including any precipitates that may have formed, from the previous step by carefully aspirating it into the DNA Mini Column. Centrifuge at maximum speed for 1 minute and discard the filtrate before repeating the transfer process until all remaining samples are transferred to the DNA Mini Column.

After transferring the DNA Mini Column to a 2 mL Collection Tube, add 500 μL of HBC Buffer and centrifuge at maximum speed for 30 seconds before discarding the filtrate and reusing the collection tube. Next, add 700 μL of DNA Wash Buffer and centrifuge at maximum speed for 1 minute before discarding the filtrate and reusing the
collection tube. Repeat the wash step a second time. Finally, centrifuge the empty DNA Mini Column for 2 minutes at maximum speed to dry the column matrix, then transfer it to a clean 1.5 mL microcentrifuge tube.

Finally, add 50-100 μL of Elution Buffer, sterile deionized water, or ten mM Tris, pH 9.0, heated to 70 °C directly to the center of the column membrane. After letting it sit at room temperature for 2 minutes, centrifuge at maximum speed for 1 minute. Repeat the elution process and store the extracted DNA at -20 °C. After extracting the genomic DNA, a PCR reaction was performed on a 1% agarose gel. The PCR reaction consisted of the genomic DNA, GFP-F (ATC ATG GTG TCT AAA GGA GAA GAA CTG) and GFP-R (CCT TTG TAC AGT TCG TCC ATT CCC AG) primers, GoTaq master mix (Promega, M713B), and water at appropriate concentrations according to best practices. Then the samples were placed in a thermocycler (Bio-Rad, C1000) and run according to the following program:

1. Initial denaturation at 95 °C for 5 minutes
2. Denaturation at 95 °C for 30 seconds
3. Annealing at 65 °C for 45 seconds
4. Extension at 72 °C for 1 minute
5. Repeat steps 2-4 for 35 cycles
6. Final extension at 72 °C for 5 minutes

After the cycling program is complete, hold the samples at four °C indefinitely to prevent PCR product degradation and run on a 1% agarose gel.
CHAPTER 4: RESULTS

Electroporation and Sericulture Results

Figure 9

*Female Bombyx Mori Moths Preparing to Lay Eggs before Electroporation Procedure*

*Note.* The female in the rear of the image has begun laying eggs, which appear as yellowish-white dots, generally about 1mm or so in diameter. Females typically will lay hundreds of eggs at a time. The image has been brightness enhanced by 40% to see detail.

Figure 10

*Washing the Eggs with Nano Pure Water after the Electroporation Procedure*
At the outset, seven female silkworms had spent several days actively mating and were preparing to lay eggs. They were kept in optimal conditions of darkness and 30 °C temperature. Throughout three days of egg collection, constant supervision was maintained to ensure the freshness and viability of the eggs. Initially, only one female produced a few eggs, which was insufficient for transformation. However, approximately an hour later, two females laid around 15 eggs each, providing a more substantial number of eggs for the procedure (Figure 9).

Overall, the process lasted approximately 72 hours. Seven females actively laid eggs for transformation. This resulted in about 500–700 eggs that could be used in the transformation procedure. All eggs were subjected to electroporation and washed (Figure 10).

After transformation, the eggs were placed in the same environment as previously described; as seen in Figure 11, the transgenic and control worms were grown together and were not visually distinguishable.
Note. Most of these are around the fifth larval stage. The greenish logs are the mulberry chow that has been ground up and rolled out so that they have a greater surface area to eat on. The little specks are their fecal matter, which must be cleaned regularly. They are set up on trays with saran wrap for easier handling and cleaning.
eGFP Screening

After the silkworms had matured and successfully cocooned, the cocoons were screened by exciting the eGFP protein with a black light. Transgenic cocoons emitted a green fluorescence, while the non-transgenic cocoons were shown as either white or light orange. They were then separated by hand into positive and negative groups for subsequent testing. Based on prior research, the fluorescence was not as strong as expected (X. Zhang, 2017), making it difficult to assign some cocoons to groups. Figures 12 and 13 show the cocoons under black light, and Figure 14 shows them in regular lighting. The forty pupae from the cocoons with the highest component of green fluorescence were then carefully excised by cutting a semicircular notch and allowed to mate with other transgenic individuals after they matured, as seen in Figure 15. The positive cocoons were labeled SW-AMP #1–6. Six control cocoons from the same batch (non-transgenic) were labeled as Controls A–F for subsequent experiments for a total of six experimental and six control cocoons.
**Note.** Transgenic SW-AMP (bottom) and non-transgenic cocoons (top). They are roughly oblong shaped and 2-3 cm across. These are fairly standard in size and appearance. However, they are indistinguishable in regular light and can only be determined when exposed to UV light.
Figure 13

*Alternative Comparison of Cocoons Under UV Light*

*Note.* Alternative picture of the cocoons. They do vary a little in size. White cocoons (top) are non-transgenic and green cocoons (bottom) contain the LL-37 insert, which contains the proline linker, LL-37 insert, and eGFP protein.
Figure 14

Comparison of Transgenic and Non-Transgenic Cocoons Under Regular Light

*Note.* All approx. 500 cocoons, under regular light. They all appear relatively white and indistinguishable. The AMPs were cocoons that were white-to-orange-to-light green. These were not used and may be indicative of partial integration of the insert or other deformities.
Figure 15

Transgenic Silkworms in “Beehive” Cells

Note. These worms were allowed to hatch naturally and would be used as mates for the next generation, had time and funding permitted.

Dot Blot Antibody Detection

The dot blot proved challenging, and the first few attempts resulted in overstaining either with the Ponceau or the development buffer. The other issue was determining the appropriate protein concentration on the dot blot that ensured the availability of the LL-37 and, thus, proper binding of the primary and secondary antibodies. Ratios of 1:1000 for the primary antibody and 1:10000 for the secondary antibody were the appropriate concentrations.
Dot Blot of Test Samples and Controls

Note. Dot blot of a serial dilution of dissolved LL-37 peptide as a positive control, SW-AMP 1-4, Controls A-D, and water blanks. The LL-37 peptide was rather concentrated, but you can see that after successfully dissolving the fibers, the LL-37 antibody reacted with the protein residues of the transgenic silk in samples 1-4. Conversely, there is no antibody reaction on any of the negative controls. The positive control of LL-37 consisted of a serial dilution of 1mg/ml of peptide dissolved in water and was subsequently halved up to a 1:8 original concentration.

Secondarily, determining the appropriate concentration for primary and secondary antibodies was time-consuming and challenging. Based on previous research, antibody dilutions were initially too dilute (1:10,000). After significant trial and error, a primary antibody concentration of 1:1000 and 1:10,000 for the secondary antibody resulted in the best images that were not over- or under-saturated. Using these conditions, it was possible to successfully identify that the AMP LL-37 was present in the transgenic SW-
AMP silk, as seen in the second row of Figure 16. The positive control (LL-37 peptide dissolved in water) and SW-AMPs 1–4 demonstrated activity, while the control silks A–D and water blank did not, as seen in the last two rows in Figure 16. The dot blot results showed that the LL-37 AMP peptide was successfully incorporated into the transgenic silk and further validated the eGFP screening results.

Mechanical Data

Mechanical properties are an important factor to consider when studying the potential use of silk fibers for different applications. In this study, the mechanical properties of silkworm AMP and control fibers were analyzed using various tests (Figure 17). However, these results may have only limited value given the limited sample size of 6 control and six experimental fibers tested in 3–6 locations.

First, the diameter of the fibers was measured, which is an important factor as it can influence the overall strength and flexibility of the fibers. The mean diameter of silkworm AMP fibers was 11.62 microns with a standard deviation of 2.00 microns, while the mean diameter of control fibers was 9.31 microns with a standard deviation of 2.34 microns. The difference in diameter between the two groups was statistically significant (p=0.0054), indicating that silkworm AMP fibers were thicker than control fibers.

The ultimate tensile strength of the fibers, which is the maximum stress that the fibers can withstand before breaking, was determined. Silkworm AMP fibers’ mean ultimate tensile strength was 357.81 MPa, with a standard deviation of 105.67 MPa. In comparison, control fibers had a mean ultimate tensile strength of 460.30 MPa,
respectively, with a standard deviation of 110.53 MPa. The difference in ultimate tensile strength between the two groups was statistically significant (p=0.0121), indicating a significant difference in ultimate tensile strength between the silkworm AMP and control fibers.

The ultimate strain of the fibers, which is the maximum amount of deformation the fibers can undergo before breaking, was determined. The mean ultimate strain of silkworm AMP fibers was 0.18, with a standard deviation of 0.10, while control fibers had a mean ultimate strain of 0.00. The difference in ultimate strain between the two groups was statistically significant (p=9.10×10^{-8}), indicating that silkworm AMP fibers had more stretch than control fibers.

The energy required to break or the toughness of the fibers was also measured. The mean energy to break silkworm AMP fibers was 52.34 MJ/m³, with a standard deviation of 39.45 MJ/m³, while control fibers had a mean energy to break of 67.47 MJ/m³, with a standard deviation of 40.47 MJ/m³. The difference in energy to break between the two groups was not statistically significant (p=0.2956), indicating no significant difference in the toughness of the two types of fibers.

Finally, the elastic modulus of the fibers was measured, which is a measure of their stiffness or rigidity. The mean elastic modulus of silkworm AMP fibers was 5331.73 GPa, with a standard deviation of 2109.16 GPa. In comparison, control fibers had a mean elastic modulus of 8097.12 GPa, with a standard deviation of 7577.58 GPa. The difference in elastic modulus between the two groups was statistically significant (p=9.1×10^{-8}), indicating that silkworm AMP fibers were less stiff than control fibers.
Figure 17

Results of Mechanical Tests on AMP and Controls

Note. Bar charts representing the mechanical data results for Silkworm AMP and Control Silk fibers. The charts illustrate the mean values and standard deviations for the diameter (top left), ultimate tensile strength (bottom left), energy to break (bottom right), and elastic modulus (top right).

Overall, the results indicated that incorporating LL-37 into the silkworm fiber did not cause substantial deterioration of silk fiber mechanical properties and improved some properties in this limited study.

Genomic DNA Examination (PCR & Sequencing)

The successful PCR results obtained in this experiment demonstrate the effectiveness of genetic modification. The GFP-F and GFP-R primers confirmed the silkworm AMP’s transgenic nature by detecting the eGFP gene/LL-37 insert complex in
the genomic DNA. Furthermore, the four positive silkworm AMP samples (labeled SW1, SW2, SW3, and SW4 in Figure 18) and the positive control (LL-37 insert plasmid) all showed a positive PCR result, indicating the presence of the eGFP gene. On the other hand, the negative control (non-transgenic silkworm) and reagent control did not show any reaction, indicating no contamination or false positives in the PCR reactions.

Sequencing data was also sent to the Center for Integrated Biosystems and was met with limited success. The LL-37 sequence using the forward primer was successfully identified as in frame in two samples, however the other two did include the eGFP sequence but appeared to have some misalignment near the end of the sequence (possibly due to a bad base call by the software). An attempt to examine this with a reverse primer resulted in very noisy data. Attempts were also made to try and redesign primers, but it still resulted in noisy data.

The positive PCR results obtained in this study provide strong evidence that the transgenic silkworms were successfully generated, and that the gene was integrated into their genome, especially when coinciding with the other positive results (eGFP screening and dot blot activity). The sequencing data also indicates that the sequence is in the genome, however the reverse primer not working and not being able to design primers that did work may mean that an error occurred in some of the samples (possible indel mutation for example).

Overall, the successful PCR results obtained in this study demonstrate the feasibility and effectiveness of the genetic modification technique employed to generate the transgenic silkworm AMP. These findings provide a strong foundation for further
research and development of transgenic silk production and have important implications for the development of other transgenic organisms.

**Figure 18**

*PCR Reaction Results*

*Note.* A 1% agarose gel of the Silkworm AMP genomic DNA reacting positively to the forward and reverse GFP primers via PCR reaction. The negative and reagent controls did not react.
Single Fiber Fluorescence Microscopy

The single fiber results showed areas that were stained both red and green, even without bacteria (Figure 19). This indicates that the fiber did have some background fluorescence; both the transgenic and non-transgenic control silks exhibited this effect. Therefore, all images in this series are formatted similarly, with a composite red and green image at the top, followed by a second with a green FITC filter only, and a final third, a Texas Red image in red. Unfortunately, the background fluorescence makes it hard to ascertain how well the transgenic type compares to the non-transgenic control (Figure 20), especially quantitatively.

**Figure 19**

*Fluorescence Image of an Antimicrobial Silkworm Fiber in Triplicate at 20× Magnification*

**Note.** The top image shows the composite image using both filters, the middle in green, just the “live,” and the bottom image in red, the “dead” areas of the silk.
Note. Fluorescence image of a regular (non-transgenic) silk under composite (green and red filters), followed by just the green FITC filter in the middle and the red, Texas Red filter at the bottom. This one also appeared to have excess stain resulting in a slightly “wispier” image for the red.

Notably, the corona effect is observed in the control silk, as seen in Figure 20. The corona effect is an optical phenomenon that can occur when high-intensity light is used to illuminate a sample. This can cause the sample to appear as if it has a glowing, halo-like aura around it, which can interfere with the clarity of the image and make it difficult to distinguish fine details. In this case, it is likely the result of excess red fluorescent dye (propidium iodide). However, the corona effect leads to subsequent retesting in an attempt to remove the extra fluorescence by doing additional washes. Control A (as seen in Figure 21) has a similar color profile to Control B, except that the microscope could not successfully focus on the fiber in Control A. The fiber was not carded as tightly as it should have been. Control B fiber was carded properly, and its composite image showed
a nice blending of red and green, resulting in an orangish color throughout, as seen in Figure 22. Of note, on Control B fiber, there was a single bump protruding from the top/side of the fiber, which may be *Staphylococcus epidermidis* adhering to the fiber. Controls C and F (Figure 23 and 24) were perhaps the most interesting in this group as they had relatively high green fluorescence, to such a degree that at first glance, their Texas Red images are incredibly light due to such low fluorescence. Control D and E (Figure 25 and 26, respectively) are also interesting because their composite images show distinct staining regions.

**Figure 21**

*Control A Single Fiber*

*Note.* Slightly blurred due to improper carding. The color profile is, however, similar to control B.
Figure 22

Control B at 20× Magnification

*Note.* Shows a blend of both the red and green, resulting in an orangish color. This may be largely due to the background fluorescence of the silk.

Figure 23

Control C at 20× Magnification

*Note.* Control C with largely green fluorescence and very little red. This could indicate excessive live cells, fluorescence in the fiber, or dye (less likely). But, again, this image is representative of the fiber as a whole.
Figure 24

*Control F at 20× Magnification*

*Note.* Control F had a lot of green fluorescence, much like Control C. It may be excess live bacteria or background fluorescence from the fiber. All controls are non-transgenic and are regular silkworm silk, so this background coloring would not be due to any modifications.
Figure 25

Control D at 20× Magnification

*Note.* Control D contains regions of distinct staining. It could be pits or other deformities in the fiber (where bacteria would be more likely to shelter). The yellow coloration in the background indicates high levels of red and green background color.

Figure 26

Control E at 20× Magnification

*Note.* Control E also shows distinct regions of clumping. It may be an indication of areas of *s. epidermidis* clumping together at regions of deformation in the silk.
The transgenic silks were also interesting in that most looked somewhat like Controls D and E (Figure 25 and 26), and specific regions of red and green are observable. Like the other fibers, the fiber had some green and red fluorescence. However, the composite images result in a yellow-green fiber with regions of red for all six transgenic silks. SW-AMP #1 (as seen in Figure 27) has specks of red mass along its entire length, indicating that there were regions of dead cells clumping to the fiber. SW-AMP #2 (as seen in Figure 28) also had similar clumped regions. SW-AMP #3 through #5 looked similar to SW-AMP #1, a yellowish-green fiber decorated with red regions. These images can be seen in Figure 29, 30, and 31. Perhaps the most interesting in the experimental group was SW-AMP #6, which, like the others, had red clumps of dead cells, but had a region with clumps all in a line (see Figure 32). These results contrast the control group, which typically had a more uniform composition, which may have been due to a layer of bacteria or differences in the background fluorescence. The transgenic silk (without bacteria) also had more defined red and green regions, as seen in Figure 19. This may indicate that the AMP peptide may also affect the relative binding affinity of SYTO 9 and propidium iodide, given that no bacteria were present in the non-bacterial controls (Figure 19 and 20). What is also interesting and important to note about the transgenic SW-AMP fibers in Staphylococcus epidermidis is that none of them experienced the deep saturation of a single color as observed in Controls A, B, (orangish) C, and F (green). This may indicate that the SW-AMP fibers were not completely covered in bacteria and were isolated to just minor regions and in the deformations of the fiber.
Note. SW-AMP #1 shows red patches on the yellow-green fiber, as is characteristic of all the transgenic fibers. The specks of red extend beyond the fiber, suggestive of clumps of bacteria as opposed to a major fiber deformation (like a pit or extrusion).
Figure 28

*SW-AMP #2 at 20× Magnification*

_Note._ SW-AMP #2 experienced some carding issues and was not quite as tight as the rest of the fibers and therefore was difficult to get into focus. However, it does have detailed regions of red, albeit very grainy.
Figure 29

*SW-AMP #3 at 20× Magnification*

*Note.* SW-AMP #3 was decorated with regions of dead clumps of presumable cells. It also contained raised regions, like other SW-AMP fibers.
**Figure 30**

*SW-AMP #4 at 20× Magnification*

*Note.* SW-AMP #4 was also decorated with distinct regions. This image, however, was at a slightly different angle than the other images due to not being quite as tight to the c-card and having some bends in the fiber.

**Figure 31**

*SW-AMP #5 at 20× Magnification*

*Note.* SW-AMP #5 has distinct regions of red, especially near either end.
Figure 32

*SW-AMP #6 at 20× Magnification*

*Note.* SW-AMP #6, the composite image shows these clumped cell regions that look almost like dragon scales or the like. It may be bacteria that found a deformity and are trying to stay together.

Saline Wash Single Fiber Fluorescence Microscopy Results

The saline wash experiment was not as successful. It did very little to de-stain the fibers. Instead, it produced a variety of salt crystals on the fiber. It may have also made the single fibers a bit more brittle: two fibers, Controls C and E, broke, and they could not be imaged. Of the remaining control fibers, Control A appeared to have the type of clumping seen in the SW-AMPs in the initial series of images, as seen in Figure 33. Control B, D, and F, however, had more of the expected blurred colors but were also very obviously covered in a layer of salt crystals from drying. As seen in the bottom left panel of Figure 33, Control D had a very nice coloration. However, a square salt crystal protruded from the fiber (especially along the left side of sample B, top right image in
Figure 33). In contrast, in Control F, the crystals refracted the light and caused the fiber to twist throughout the length of the fiber.

Similarly, the SW-AMP Fibers also exhibited multiple salt crystals. SW-AMP 1 and 3, for example, had them along the field of view, as seen in Figure 34’s left panels. SW-AMP 2 had perhaps the largest salt crystal of the group, as seen in the bottom right panel of Figure 34. These salt crystals were excessive, so the final drying step with 0.85% saline solution was not done for the single fiber, fiber wad, or lawn dip results but are included here as the original protocol did specify to include the final saline wash.

**Figure 33**

*Control Fibers after 0.85% Saline Wash*

*Note.* A selection of images of control fibers as a single fiber in triplicate after a 0.85% saline wash. Two of the fibers broke after being left out to dry, likely due to twisting. Salt crystals can be seen all along Control A and Control D, causing it to distort the image. Control B seemed to have a little less covering the entire span, or at least an area with less salt was available for imaging, but it looked rather like the other fibers, perhaps slightly greener. Control F, on the other hand, had so much salt on it that the fiber looked like it twisted all throughout. It was surprising that it did not also break.
**Figure 34**

*SW-AMP Fibers after 0.85% Saline Wash*

*Note.* The SW-AMP fibers after the same 0.85% de-staining treatment. They looked very similar to their counterparts and had a lot of salt crystals; this was particularly so on samples 1 and 3, where they decorated the entire span of the fiber. On SW-AMP #2 (lower left), there are a few very large salt crystals on the left side of the image, showing in all three images. Of note was SW-AMP #5 (top left), which exhibited more of the “clumpiness” as seen in single fiber fluorescence images as opposed to being a single yellow-green fiber.

**Fiber Wad Fluorescence Microscopy Results**

Next, the fiber was observed in a “system” or network instead of in isolation as a single stretched fiber. The 20× images and 4× images are of the same samples at different magnifications (Figure 35). At 20× magnification multiple fibers can be seen running in various directions at once. For simplicity’s sake, only the composite images are included here instead of the separate composite, green, and red fluorescence images simultaneously. Figure 35 shows a matrix of the SW-AMP fibers, and Figure 36 shows a
matrix of each composite control image. Like in the other two samples, there is a red and green fluorescence mix. The intersections of fibers are most interesting in this new wad orientation, which often tends to show more red fluorescence in both the control and experimental groups. There appears to be stricter or more pronounced dying in some regions (some areas are red, some are green, and some are yellow-green).
**Figure 35**

*Silkworm-AMP Fibers 1–6 at 20× Magnification*

*Note.* Silkworm-AMP Fibers 1-6 at 20× magnification. This was the “wad” of fibers, meaning there were multiple fibers present as opposed to a single fiber in the previous series. They tended to have regions that were a lot more yellow-green and red as opposed to having quite as distinct of feature as the single fibers. The intersections such as those in fiber images 1, 2, 3, and 6 had darker red regions when the fibers crossed each other.
Note. 20× wad images of control fibers A–F. These images showed the staining in a variety of colors and hues throughout each fiber, from D having a lot of green, with B and F having distinct regions that showed more green, but also A, B, and F having darker regions of red. These images showed much more variety in color than the single fiber images, which may be an indicator that much more bacteria (both live and dead) were present.
These images reveal an even larger area of the silk and bacteria mixture. Of note, in both the control and SW-AMP groups, there appear to be regions of red and green as opposed to the fiber being all strictly one color. Figure 37 shows that the edges of many silks, such as controls A, especially B, C, E, and F, have more red or “dead” staining. In contrast, the center of each of these images tends to be greener or “live.” These green centers might indicate that live bacteria are harbored in the larger silk structure or that the red dye (propidium iodide) does not penetrate as easily.

**Figure 37**

*Wad Control Silks A–F at 4× Magnification*

*Note.* A collection of multiple fibers that showed a large network or “wad” of the fibers instead of a single individual fiber. Notice that many of the edges of these control silks appear redder than the center of the “wad.”
On the other hand, in the SW-AMP silks, as seen in Figure 38, the outer edge is not necessarily stained red, though it is sometimes. Rather, it appears to be either regions in the interior, as seen in SW-AMP 1, 3, 5, or throughout an entire section of the wad, as seen in SW-AMP #2. This suggests that the solutions of SYTO 9 and propidium iodide can move throughout, given that “red centers” are seen in the SW-AMPs and “green centers” in the two groups, and suggests that this is the result of staining a material (fiber, bacteria, antimicrobial peptide) as opposed to strictly excess dye residue floating in the environment.

**Figure 38**

*Wad SW-AMP Silks 1–6 at 4× Magnification*

*Note.* Notice the “red centers” in SW-AMP 1,3 and 5. SW-AMP 1 has additional red at the bottom of the image in the field of view, while SW-AMP has it in the two spots slightly to the left of the center in its image. Meanwhile, SW-AMP has a region near the middle of its image with a red center coloration. Also of note is SW-AMP 2, which contains the entire right side of the image in red. This, along with the black image as the background, indicates that overall the differences in colors are due to staining different materials instead of strictly excess SYTO 9 or propidium iodide dye floating in the environment.
Lawn Dip Fluorescence Microscopy Results

Regarding the lawn dip bacteria, they tended to clump together and even span regions between fibers. The control silks as a group tended to have more green/live staining than the SW-AMP silks as a group, which appeared redder. The centers of all the silks did tend to be greener and more alive than the rest of the silk. This may be because the bacteria are more sheltered in the center of these lawn dip wads of silk than towards the outer extremities. The silk seems biased towards being stained by the SYTO 9, but there is a pattern when the control and SW-AMP fibers are viewed as a group. As seen in Figure 39, the control silks do exhibit green centers and some areas of dead cells. This, along with the bias for the fibers to stain green, is perhaps most obvious in Control A. Controls B and F show very strongly that green center, indicating that the bacteria on the outside seem to provide a barrier to protect cultures more in the middle.

On the other hand, in Figure 40, the SW-AMP silks do have significantly more red staining than green. This indicates that something about the silk is affecting the bacteria. It would be good to quantify these color differences meaningfully, but from a qualitative perspective, the differences seem interesting.
Figure 39

Control Fibers Treated on Bacterial Lawn

Note. As a group there was more green coloration than red, when compared to the SW-AMP fibers below. Control A had significantly more cell mass on it than Control B, which had considerably less. However, all five pictured here (there are no images for Control E as the material had been exhausted) have those green centers.
**Figure 40**

*SW-AMP Fibers after Lawn Dipping in S. Epidermidis*

*Note.* There was much more red and darker red throughout the SW-AMP fibers compared to the greener controls pictured above. They had green centers, but the green in these fibers was much more isolated and controlled. SW-AMP 2–6 are pictured here because SW-AMP1 fibers were exhausted in previous experiments.

Zone of Inhibition Results

No noticeable zones of inhibition were observed in testing, which is not unexpected. The tethered LL-37 should not be able to leach out into the media and produce a zone of inhibition.

Zone of inhibition results for each of the six AMP fibers and the six control fibers are included in Figure 41–45 after exposure to *Staphylococcus epidermidis*. Some of the single fiber specimens did not affect the growth, perhaps due to not being secured as close to the agar as others, such as SW-AMP #3 (Figure 43, left panel).
Others appear to have acted as a physical barrier or, in some cases, as a harbor for the bacteria as it follows along the fiber in a way that is perpendicular to the streaks of the *Staphylococcus epidermidis* culture. This can be seen in control fibers C and D and SW-AMP #6 (Figures 43, 44, and 45) but not in control E or SW-AMP #5 (Figure 46).

In particular, the SW-AMP #6 fiber also broke during transport and was not taut like the other fibers (Figure 45). Instead, one can observe where it lay and how it acted as a barrier with obvious growth along the “bottom” side, with markedly less at various points along the opposite “top” side of the fiber, as seen in the figure below.

**Figure 41**

*SW-AMP #1 and Control A Inoculated with Staphylococcus Epidermidis*

![Comparison of SW-AMP 1 (left) and Control Silk A (right), with Staphylococcus epidermidis.](image)

*Note.* Light field microscope images of SW-AMP #1 (left) and control A (right) inoculated with *Staphylococcus epidermidis*. There was a lot of culture growth, but no visible exclusion zone for either fiber.
Figure 42

*SW-AMP #2 and Control B Inoculated with Staphylococcus Epidermidis*

*Note.* SW-AMP #2 (left) with control B (right) also had no visible zones of inhibition at magnification.

Figure 43

*SW-AMP #3 and Control C Inoculated with Staphylococcus Epidermidis*

*Note.* SW-AMP (left) and control silk C (right) also contained no visible zone of inhibition at magnification.
Figure 44

*SW-AMP #4 and Control D Inoculated with Staphylococcus Epidermidis*

*Note.* Comparison of SW-AMP #4 (left) and control silk D (right) No zone of inhibition. There appeared to be regions where the growth of *S. epidermidis* followed along the fiber.

Figure 45

*SW-AMP #6 and Control F Inoculated with Staphylococcus Epidermidis*

*Note.* Comparison of SW-AMP #6 and control F on plate of *S. epidermidis* culture. There was no zone of inhibition, though it did look like SW-AMP#6 might be acting as a physical barrier for some microbial growth, as it was present on one side but not the other.
Figure 46

*SW-AMP #5 and Control E Inoculated with Staphylococcus Epidermidis*

*N*ote. Comparison of W-AMP #5 (left) and control silk E (right). No discernible zone of inhibition, though again in control E, it appeared that the bacteria were growing along the fiber.
Summary and Conclusions

In conclusion, a fusion protein of *Bombyx mori* silk and the LL-37 peptide was generated and showed that it could inhibit the growth of *Staphylococcus epidermidis*. It was done using genetic engineering and molecular cloning to generate a plasmid that was transformed into the silkworm genome via electroporation. It utilized the CRISPR Cas-9 to integrate LL-37 and eGFP into the light chain of the silkworm genome. Incorporation into the genome and protein expression in the silkworm fiber were verified at the genomic and protein levels. First, eGFP screening of cocoons demonstrated a successful expression of the protein due to observable green fluorescence. Second, direct observation of LL-37 in the dissolved silkworm fibers using an LL-37 antibody was effective. Third, the presence of the LL-37 and eGFP complex in the light chain of the silkworm was verified using PCR. Finally, antimicrobial testing of the transgenic silks resulted in observable differences between the control and transgenic silks, indicating the incorporation and presentation of the LL-37 peptide.

The mechanical properties of the silkworm AMP fibers were measured and found to not be significantly lower in any one category. These transgenic fibers had a larger mean diameter than the control silk fibers, and this difference was statistically significant. This may be attributed to the presence of the AMP gene, which may have influenced the fiber size by simply including more protein in the fiber. In addition, the ultimate tensile strength of the silkworm AMP fibers was higher than that of the control silk fibers and statistically significant. This suggests that the silkworm AMP fibers have improved
strength, which is not readily explainable from a protein structure standpoint. However, this limited study was only intended to determine that the AMP did not cause a marked deterioration of the fibers’ mechanical properties.

The energy to break was lower in the silkworm AMP fibers than in the control silk fibers, although this difference was not statistically significant. The energy to break reflects the energy required to break a fiber and is a measure of its toughness. The lower energy to break in the silkworm AMP fibers may be attributed to their larger diameter or possible micro-defects, which may result in a lower energy requirement for breakage. However, again there was no statistically significant difference.

The elastic modulus of the silkworm AMP fibers was significantly lower than that of the control silk fibers. The elastic modulus is a measure of the stiffness of a fiber and is an important parameter in determining the fiber’s mechanical properties. The lower elastic modulus of the silkworm AMP fibers suggests that they are more flexible and have a higher degree of deformability.

The ultimate strain of the silkworm AMP fibers was higher than that of the control silk fibers. The ultimate strain measures the maximum deformation a fiber can undergo before breaking. The higher ultimate strain in the silkworm AMP fibers suggests they are more ductile and less prone to brittle fracture.

So in terms of mechanical properties, the AMP peptide integration was not deleterious to the fiber formation, and in some cases, it may have even improved mechanical properties. Again, this phenomenon is not readily explainable as adding LL-37 generally would not be thought to improve fiber mechanical properties. This result is more likely an effect of the limited nature of this study than an authentic response.
Overall, it can be concluded that the addition of the LL-37 is not causing significant deterioration of the fibers’ mechanical properties. It is also likely that any changes to the structure are more likely due to eGFP protein given it’s larger size.

The genomic DNA PCR results also indicated successful integration of the LL-37 gene into the light chain of the silkworm genome, and the sequence was not present in the negative and reagent controls (ruling out the chance of accidental contamination or false positives).

The sequencing results had a good start, but there was a lot of noisy data. Initially, the first thought was that there was an issue with the primers since the reverse primer didn’t work, but the forward primer appeared to do so, but even subsequent redesigns yielded very noisy data. In retrospect, it’s possible that the genomic DNA had deteriorated over time, which also could have led to the increase in noise. Sadly, due to time constraints this issue was not able to be fully addressed.

The live/dead staining also demonstrated some killing of S. epidermidis, although the results are not as quantitative as would be desirable. However, from a subjective perspective, there seem to be differences between the transgenic SW-AMP silks and the control non-transgenic silks, including seeing specific regions with distinct red coloring on single fibers and looking at collections of fibers. The single fibers had what appeared to be individual clumps of dead bacteria that stained red. This might be dead bacteria, deformities in the silk, or regions where the LL-37 peptide is more closely concentrated. The saline wash step to remove excess dye was unsuccessful, resulting in too many salt crystals to image properly. The wad images, both at 20× and 4×, show that the central regions of the fibers tend to be greener than the edges, which suggests that silk fabrics
may provide some protection to bacteria due to physical barriers if an AMP or other
treatment is not present. The lawn dip results show a difference in the number of
live/dead bacteria when present with this stain. Disappointingly, the results were not
quantifiable in a meaningful way. However, from a strictly qualitative perspective, the
SW-AMP lawn dip silks do show redder than the control lawn dips, suggesting that the
silk is influencing the vitality of *S. epidermidis* with which it comes in contact.

Finally, the zone of inhibition results was negative. This is promising as it shows
the AMP is bound to the fiber and not leaching out and degrading over time.

Limitations

There were some limitations to this study. First and foremost were the time
constraints: only one generation of silkworms was raised due to unforeseen delays and
issues primarily associated with the COVID-19 pandemic. For this line of
experimentation to be truly successful, not only was good efficacy with the antimicrobial
peptide needed, but the gene also needed to be immortalized in the silkworm line. This
could not be verified due to only a single successful generation having been completed.

Another limitation was the inclusion of only one antimicrobial peptide. During
this project’s scope, it may have been appropriate; however, in practice, it would be
important to include a variety of antimicrobial peptides to try to mitigate the spread of
harmful bacteria. In addition, multiple AMPs would help ensure that bacteria immune to
the selected antimicrobial peptide were not selected. Bacteria gaining antibiotic resistance
to AMPs is generally seen as a negative outcome, as it can lead to the emergence of
difficult-to-treat infections as was seen with traditional antibiotics. However, the
likelihood of bacteria developing resistance to AMPs is not necessarily higher or lower than other antibiotics (Pietsch et al., 2020). The likelihood of cross-resistance between AMPs and antibiotics may vary depending on the specific antibiotic and AMP in question. While some studies have suggested a potential for cross-resistance between AMPs and antibiotics, other studies have demonstrated that cross-resistance is rare while collateral sensitivity is widespread (Pietsch et al., 2020). Additional research is needed, especially in clinical settings, but great care and caution should be exercised so that AMP resistance does not occur in healthcare settings.

This study was further limited because the antimicrobial efficacy results were largely qualitative and relied heavily on the author’s interpretation of the color differences. It would be beneficial if there were a way to analyze the results in future work to make the data more quantitative.

An additional limitation is that the silk does fluoresce both red and green. This can be seen in the single silk fibers not inoculated in bacteria, where red and green fluorescence were observed. It may be that the green fluorescence observed in the center was not just living bacteria but could be the silk fluorescence, and bacteria have been excluded or unable to colonize there (such as the tweezers in the lawn dip gripping in those regions). The FITC filter has peak excitation and emission spectrum wavelengths of 495nm and 519 nm and the eGFP protein has a peak excitation of 488 nm and omits at 507nm wavelengths so there is some overlap.
Future Work

It would be beneficial to address some of the limitations of this study, including utilizing multiple and different antimicrobial peptides as a “mixed batch” version. Another direction would be to ensure that the antimicrobial peptide gene can persist in subsequent generations; otherwise, each new generation of worms would need to be modified, which would be costly, decrease yields, and defeat the project’s scalability. Additionally, the microscope exercises show that Staphylococcus epidermidis tends to stick to silk, where no surfactant (like can be found in typical laundry detergents) was employed to remove the bacteria. Therefore, incorporating a surfactant-like protein that does not undermine the quality of the garment (make it sticky) may also be another avenue to explore.

Conclusion

The work in this dissertation demonstrates that incorporating antimicrobial peptides into silkworm silk to discourage premature fabric deterioration or produce medical devices is possible using CRISPR-Cas9 to integrate the AMP DNA sequence into the silkworm silk genome. Utilizing genetic engineering instead of post-spinning treatments may be an effective way to save money in producing antimicrobial materials, reduce the need for washing, and reduce toxicity to consumers and the environment. In conclusion, using antimicrobial textiles is crucial in preventing the spread of microorganisms and reducing the risk of infections. While many coatings and finishes are available to treat hard surfaces, these treatments are not always suitable for textiles due to their potential toxicity and high cost. Nanoparticles of certain materials, including silver
and copper, are promising options for antimicrobial treatments, but their potential adverse health effects and increased costs must be considered. The AMP silk described in this research offers a promising alternative as it is naturally antimicrobial and does not require additional coatings or treatments. The findings of this study highlight the potential applications of AMP silk in various settings, including healthcare, where the risk of infection spread is high.

Further research is needed to investigate the full potential of AMP silk and its long-term effects. However, overall, the development of antimicrobial textiles is an important area of research that has significant implications for public health and well-being. Furthermore, this research shows that the industry may be at a new starting point through genetic engineering.
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