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EVALUATION OF MELITTIN AS A NOVEL THERAPEUTIC FOR TREATING STAPHYLOCOCCUS AUREUS INFECTIONS

Christina Louise Russell

Evaluation of melittin as a novel therapeutic for treating *Staphylococcus aureus* infections

A Thesis

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Master's Degree

Christina Louise Russell

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I am submitting herewith a thesis written by Christina Louise Russell entitled "Evaluation of melittin as a novel therapeutic for treating *Staphylococcus aureus* infections". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

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Abstract

Novel therapies to combat multidrug resistant *Staphylococcus aureus* (*Sa*)infection is of increasing interest as more people become ill with these infections in hospitals and communities. To this end, a 26 amino acid peptide derived from honey bee venom, melittin, was evaluated for its bactericidal efficacy against *Sa*. Experimentation revealed that melittin has a half-life of approximately two days at room temperature and pH tolerance range of approximately >4 and <10. Also, 10.1μg could kill of $1x10^6$ *Sa* bacteria in 15 minutes *in vitro*. Mice that were cutaneously infected with *Sa* showed significantly greater zones of inflammation compared to infected-mice treated with a melittin gel after 24 hours indicating possible therapeutic uses *in vivo*. Most interesting is that *Sa* serially passed in the presence of melittin never showed enhanced resistance while other *Sa* cultures become resistant to many other antibiotics, such as streptomycin, indicating possible longer utility in medical settings.

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Introduction

An Overview of *Staphylococcus aureus*

Staphylococcus aureus was first described in 1880 by Sir Alexander Ogston while aiding the French military as a surgeon during war time. During microscopic examinations of pus recovered from wounded soldiers, he observed small clusters of spherical bacteria (45). Later, Sir Ogston was also able to grow *S. aureus* in artificial cultures using hen's eggs (46). Since its discovery, *S. aureus* has gained recognition as a notable human pathogen linked with human ailments such as pimples, impetigo, carbuncles, and toxic shock syndrome, just to name a few. Serendipity and *S. aureus* collided in the late-1920's when Alexander Fleming made a notable observation about a particular fungal contaminant growing on his *S. aureus*-inoculated agar plates. He noticed that the growth of *S. aureus* appeared limited to a couple of centimeters away from a *Penicillium* fungus. It would not be until years later that the potential of his finding would be completely understood.

As the story goes, Fleming left plates that he had been working on out on a table for two weeks while he was out of town. On his return he discovered that there was a fungus growing surrounded by a clear ring where bacteria were unable to grow. After culturing the mold and speaking with fungus expert, C. J. La Touche, he discovered that he had *Pencillium* fungus (54). Fleming continued to run numerous experiments to determine the effect of the fungus on other harmful bacteria. Surprisingly, the fungus killed a large number of them. Fleming

then ran further tests and found the fungus to be non-toxic to animals and fit for use human use. Though he saw its potential, Fleming was not a chemist and thus was unable to isolate the active antibacterial substance, penicillin, and could not keep the substance active long enough to be used in humans. In 1929, Fleming wrote a paper on his findings, which did not garner any scientific interest (3, 54). As the war with Germany continued to drain industrial and government resources, the British scientists could not produce the quantities of penicillin needed for clinical trials on humans and turned to the United States for help. They were quickly referred to the Peoria Lab where scientists were already working on fermentation methods to increase the growth rate of fungal cultures. One July 9, 1941, Howard Florey and Norman Heatley, Oxford University scientists came to the U.S. with a small but valuable package containing a small amount of penicillin to begin work (3). By November 26, 1941, Andrew J. Moyer, the lab's expert on the nutrition of fungi, had succeeded, with the assistance of Dr. Heatley, in increasing the yields of penicillin 10 fold. In 1943, the required clinical trials were performed and penicillin was shown to be the most effective antibacterial agent to date. Unfortunately only four years after drug companies began mass-producing penicillin, microbes began appearing that could resist the treatment.

Not surprisingly the first "super-bug" to battle penicillin and gain a resistance was *S. aureus* (3, 54). From studying the antimicrobial effects and the atomic structure of penicillin, scientists were able to develop other antibiotics (4). However, these new antibiotics designed to fight infections rapidly lost their

ability to combat such infections as the microbes acquired new strategies to circumvent the antibiotics. The problem was antibiotic resistance - the capability of bacteria or other microbes to adapt to the effects of antibiotics meant to kill or weaken them (7, 61). Antibiotic resistance occurs when a random mutational change in the bacterial results in a resistance to antibiotics and in the presence of antibiotics those organisms which are not resistant, die. Over a relatively short time compared to other evolutionary adaptations for organisms that have long reproduction cycles, bacteria quickly gain traits which allow them to survive in the presence of many antibiotics. The surviving bacteria continue to multiply, passing on the resistance to the next generation, causing more potential harm as the resistant forms begin to dominate the environment (7). More and more, strains of bacteria are still becoming resistant to current antibiotic treatments (61). The prevailing notion is that antibiotics have been overused by doctors, misuse by patients, the availability of antimicrobial products, and livestock being fed antibiotics, has given bacteria, such as *S. aureus*, ample opportunities to build up resistances (19, 61). Novel therapies to combat multi-drug resistant *S. aureus* and other microbes are at the forefront of the minds of researchers across the globe.

While staphylococci bacteria can be found normally in the nose and on the skin, but also likes areas that have skin folds like joints and armpits. *S. aureus* prefers an environment that is moist and is most likely to be found around the mouth, nose, eyes, and genitals (41, 64, 66). Studies have suggested that among the population, 20% are persistently colonized, 60% of the population

intermittently carry the bacteria and 20% are never colonized (11). Damage to the skin or other injuries may allow the bacteria usually kept outside the body to overcome the natural protective mechanisms, leading to infection (64). Because most cases of infection are limited to an opening in the body, weakened immune system or other infections, *S. aureus* is labeled an opportunistic pathogen. Simply put, *S. aureus* takes the opportunity given by circumstance/environment to be infectious to the host. *S. aureus* is known for a wide range of ailments including suppurative infections, food poisoning, toxic shock syndrome, minor boils or abscesses and may progress to severe infections involving muscle or bone and can also disseminate to the lungs or heart valves (41, 66). Worldwide, *S. aureus* is the most commonly identified agent responsible for skin and soft tissue infections (41).

With *S. aureus* being the most common bacteria found on skin, its ability to build up a resistance to antibiotics has posed a major health concern and has created many treatment obstacles for healthcare facilities. Resistant forms of *S. aureus* are not novel and have been reported in the United States for over 30 years (18). Initially, Methicillin Resistant *Staphylococcus aureus* (MRSA) infections were primarily a problem of hospitals and nursing homes; but by 1997, 50% of health-care-acquired *S. aureus* isolates in the United States were methicillin resistant (18). MRSA infections that are acquired by persons who have not been recently (within the past year) hospitalized or had a medical procedure (such as dialysis, surgery, catheters) are known as Community Associated-MRSA infections. Staph or MRSA infections in the community are

usually manifested as skin infections, such as pimples and boils, and occur in otherwise healthy people (8). The difficulty in treating MRSA strains is its resistance to the majority of antibiotics available. These antibiotics can include Methicillin and other more common antibiotics such as oxacillin, penicillin and amoxicillin (67). MRSA has also been labeled as multiple resistant *Staphylococcus aureus*; essentially these names mean the same thing. Methicillin resistant Staphylococcus aureus refers to strains that are resistant to the action of methicillin, and related beta-lactam antibiotics, which includes multiple drugs (66).

The resistance to antibiotics occurs by different mechanisms depending on the bacteria. Staphylococcal resistance to penicillin is mediated by penicillinase (a form of beta-lactamase) production: an enzyme which breaks down the beta-lactam ring of the penicillin molecule (5, 67). This same action can be demonstrated on methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin by staphylococcal to resist the microbiocidal effects (5). Aminoglycosides have also been used to treat infections, such antibiotics as kanamycin, gentamicin, and streptomycin but *S. aureus* bacteria have also had resistance shown to these. Aminoglycosides action stops the bacteria's ability to synthesize proteins by interacting with the RNA of the bacterial 30S ribosome, and thus cannot survive (22). Resistance is also mediated by PBP2a, a bacterial cell wall synthetic penicillin-binding protein (PBP) with low-affinity binding to ß-lactam antibiotics. PBP2a is encoded by *mecA*, which is located on a mobile element, staphylococcal chromosomal cassette *mec* (SCC*mec*), which is

horizontally transferable among staphylococcal species (33) *MecA* gene is carried on a mobile genetic element, the staphylococcal chromosome cassette mec (SCCmec). Loss or deletion of the *mecA* gene rarely occurs, mainly due to factors affecting the stability of SCCmec. However, vancomycin may induce deletion of the mecA gene in *S. aureus*. One study indicated a case of an implant-associated infection due to a methicillin-resistant *Staphylococcus epidermidis* which lost the mecA gene after prolonged treatment with glycopeptides (59).

Some clinically important antibiotics			
Antibiotic	**Producer organism**	**Activity**	**Site or mode of action**
Penicillin	*Penicillium chrysogenum*	Gram-positive bacteria	Wall synthesis
Cephalosporin	*Cephalosporium acremonium*	Broad spectrum	Wall synthesis
Griseofulvin	*Penicillium griseofulvum*	Dermatophytic fungi	Microtubules
Bacitracin	*Bacillus subtilis*	Gram-positive bacteria	Wall synthesis
Polymyxin B	*Bacillus polymyxa*	Gram-negative bacteria	Cell membrane
Amphotericin B	*Streptomyces nodosus*	Fungi	Cell membrane
Erythromycin	*Streptomyces erythreus*	Gram-positive bacteria	Protein synthesis
Neomycin	*Streptomyces fradiae*	Broad spectrum	Protein synthesis
Streptomycin	*Streptomyces griseus*	Gram-negative bacteria	Protein synthesis
Tetracycline	*Streptomyces rimosus*	Broad spectrum	Protein synthesis
Vancomycin	*Streptomyces orientalis*	Gram-positive bacteria	Protein synthesis
Gentamicin	*Micromonospora purpurea*	Broad spectrum	Protein synthesis
Rifamycin	*Streptomyces mediterranei*	Tuberculosis	Protein synthesis

Tabel 1. List of important antibiotics the type of bacteria they effect and the mode of action the antibiotics use against the bacteria.

The major problem associated with these highly aggressive strains of *S. aureus*, such as MRSA which does not respond well to antibiotic treatments, is their ability to produce several toxins which are harmful to individuals (47). The strains of MRSA that are causing the biggest problem in the United States right now are Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) and Healthcare-Acquired Methicillin-Resistant *Staphylococcus aureus* (HA-MRSA). Those that seem to be at the highest risk for infection include the elderly and prematurely born infants (2). Therefore, possible new drug therapies to treat these strains would be invaluable (56).

There has been more effort put into finding natural, home-remedy approaches to combat bacterial infection, including MRSA. Research completed with essential oils has shown much promise in their ability to clear MRSA infections. (42) Tea tree essential oil, in particular, appears a promising candidate, either alone or in combination with other essential oils. Two controlled studies have shown that use of tea tree oil in nasal ointments, body washes and creams was as effective as routine care in the elimination of MRSA (15). In another study, combinations of patchouli, tea tree, geranium, lavender essential oils and grapefruit seed extract were found to be effective against MRSA (17). The same group of researchers has also developed a blend of essential oils, which includes tea tree oil, encased in a shell of dead yeast cells, which attacks and kills MRSA. Clinical trials of this new treatment, which can be included in wound dressings, are about to start on 40 burns patients who have been diagnosed as having MRSA on their skin (40). There have also been many

naturally occurring antimicrobial proteins identified in many different organisms a few of which include the dermal tissues of frogs and bee venom (30, 65). Interestingly, bee venom has been used for a few decades to treat arthritis although the mechanism for relieving the symptoms is not well understood (30, 48, 51).

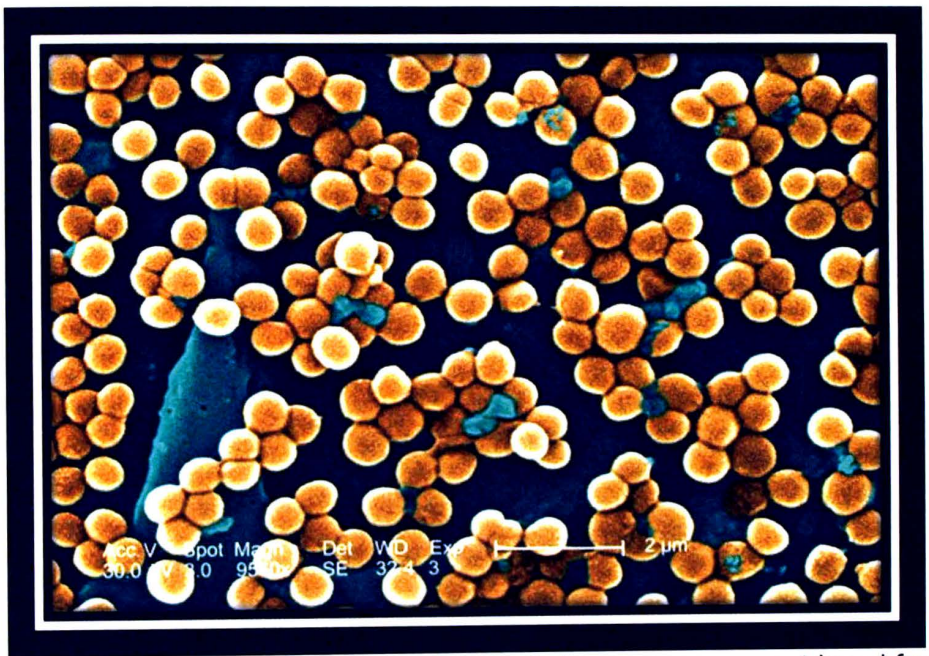

Figure 1. Illustration of the bacteria *Staphylococcus aureus* retrieved from http://www.biology4kids.com/extras/dtop_micro/7821_580.jpg

Treatment

Because MRSA infections are so difficult to treat and the numbers of cases are becoming more abundant, MRSA has developed into a significant health concern (66). Antibiotic treatments for *S. aureus*/MRSA usually are made up of a variety of antibiotic drugs including vancomycin, in hopes that the bacteria strain is susceptible to at least one of the antibiotics. The antibiotic vancomycin

tends to be the last resort of medication used in clearing the infection before more invasive measures are used (14). While vancomycin is effective, it is only available in intravenous form and has renal and bone marrow toxicities (67). Once antibiotic treatments have been exhausted, the only other options left include debridement of the area and/or amputation (62). Fatal cases of MRSA have also become more abundant since many treatment options fail to clear the infection. In 2005, it was found that more people die now from MRSA infections than from AIDS (69). MRSA was responsible for an estimated 94,000 life threatening infections and 18,650 deaths in 2005 as was reported by the CDC in the October 17, 2007 issue of the Journal of the American Medical Association (69).

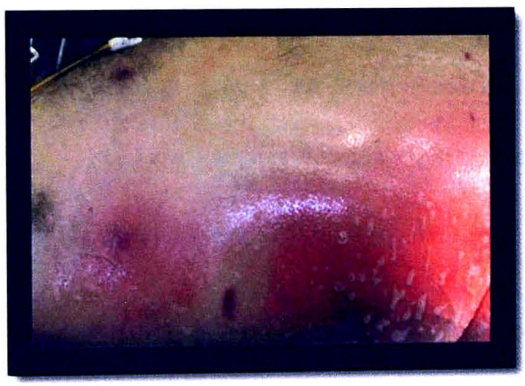

Figure 2. Illustration of *Staphylococcus aureus* infection in soft tissue. Google images

Opportunistic Pathogen

S. aureus is an opportunistic pathogen, which does not normally cause disease in individuals with healthy immune systems (16). It can however affect individuals with suppressed immune systems that have openings in the skin

created by lacerations, punctures, surgical wounds, or skin abrasions. These openings in the skin offer the bacteria an "opportunity" to become infectious. Once infected, *S. aureus* like many bacteria can be pathogenic, but there are different degrees at which *S. aureus* can cause infections. This degree of infection is known as virulence. Bacteria can be pathogenic but have a low virulence like some *S. aureus* strains that rarely causes any noticeable problems. Bacteria can also be pathogenic and have a high virulence like MRSA which can cause major infections. Some strains of *S. aureus* are capable of producing *staphyloxanthin* - a carotenoid pigment that acts as a virulence factor. It has an antioxidant action that helps the microbe to evade killing with reactive oxygen used by the host immune system. It is thought that staphyloxanthin is responsible for *S. aureus* characteristic golden color (10). When comparing a wildtype strain of *S. aureus* with a strain modified to lack the yellow coloration, the pigmented strain (wildtype) was more likely to survive dousing with an oxidizing chemical such as hydrogen peroxide than the mutant strain (10).

Colonies of the two strains were also exposed to human neutrophils. The mutant colonies quickly succumbed to the neutrophils while many of the pigmented colonies survived. Wounds on mice were swiped with the two strains. The pigmented strains created lingering abscesses. Wounds with the unpigmented strains healed quickly. These tests suggest that the yellow pigment may be key to the ability of *S. aureus* to survive immune system attacks. Drugs designed to inhibit the bacterium's production of the staphyloxanthin may weaken it and renew its susceptibility to antibiotics (38). In fact, because of similarities in

the pathways for biosynthesis of staphyloxanthin and human cholesterol, a drug developed in the context of cholesterol-lowering therapy was shown to block *S. aureus* pigmentation and disease progression in a mouse infection model (36).

Mechanics used by *Staphylococcus aureus* for infection

To obtain the energy from our cells needed for survival, *S. aureus* produces certain extracellular enzymes that break down our cell and gives *S. aureus* its infectious nature (39). These enzymes include coagulase, which is used as a defense mechanism by *S. aureus*. The coagulase enzyme produces coagulation of plasma around the surface of the *S. aureus* and fibrin deposition around the lesions of infection (39). This action allows the *S. aureus* to evade the natural immune response produced by the body when it is under attack. Other enzymes include, hemolysins which is produced by *S. aureus* to cause lysis of red blood cells (47). Exfoliative toxin is also produced by *S. aureus* and is responsible for scaled skin syndrome and causes desquamation especially in children (47). Enterotoxins are responsible for causing staphylococcal food poisoning. Other enzymes that are produced include catalase, leucocidine, hyaluronidase, staphylokinase, proteinase, and lipase (39). All of these enzymes/toxins are designed to break down eukaryotic cells and their components which enhances invading *S. aureus*. Without the production of these enzymes, *S. aureus* would have limited virulence to infect the host cells and cause infection (39). Adding to the concern of infection from *S. aureus* is its

survival in the environment. *S. aureus* has the ability to survive in the environment (not associated with humans/animals) for days, weeks, and even months depending on environmental factors. Because *S. aureus* can live without human/animal interactions for an extended period of time transmission of the bacteria is more possible (9). Because of transmission factors like this it makes the spread of *S. aureus* and MRSA somewhat unpredictable.

Epidemiology of *S. aureus* and MRSA

Humans are a naturally carrier for *S. aureus*, which tends to thrive better when it is exposed to or near a mucus membrane (9). For the majority of *S. aureus* strains to be infectious, they have to be able to enter the body through a break in the skin or an opening in the body, making piercings and surgical wounds at high risk for *S. aureus* infections. There are, however, a few strains of *S. aureus* that do not need an open entry point; they have the ability to penetrate directly through the skin. These strains such as are more commonly known as Necrotizing fasciitis, flesh eating *S. aureus* or flesh eating MRSA (26).

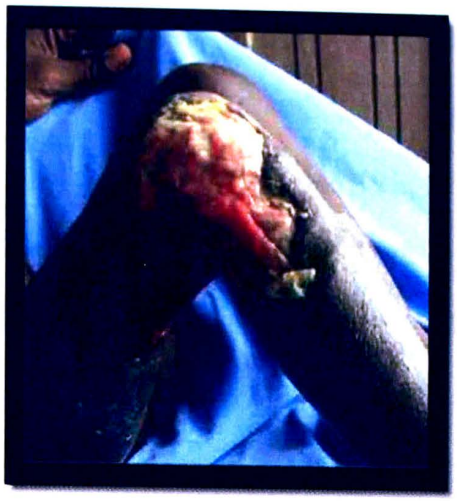

Figure 3. Illustration of Necrotizing fasciitis or flesh eating MRSA infection. http://www.thesahara.info/mrsa/mrsa_flesh_eating.jpg

Resistance

Just as *S. aureus* changed in the 1940 to show resistance to penicillin, *S. aureus* is once again changing and producing MRSA strains resistance to multiple antibiotics (9). The overuse and misuse of antibiotics has been tied to the resistance problem seen in *S. aureus*. Drug resistance can be considered a natural response to the selective pressure of the drug by the bacteria. However, it is exacerbated by several factors, including abuse, underuse or misuse of antimicrobials, poor patient compliance, and poor quality of available drugs (69). Several studies have been suggested for explaining why this misuse and overuse of antibiotics has created resistance in *S. aureus* bacteria. One study indicated that cell-wall thickness was a possible mechanism for resistance in *S. aureus*. They were able to show that a strain of MRSA (MRSA KT24) has a significantly thickened cell wall, and that the cell wall continued to increase in

thickness as the bacteria grew (32). For bacteria to have resistance, it is well known that the DNA of the bacteria has to mutate in some way which generates a novel protein mechanism of resistance. To show this, one study isolated DNA from different strains of MRSA that were only affecting children with Cystic Fibrosis (52). Their findings indicated that new isolates of MRSA in Cystic Fibrosis patients was being selected due to antibiotic pressure, this pressure put on the bacteria was able to cause lateral gene transfer (52). In this study, they were able to conduct an analysis on all *S. aureus* isolated from 2002 to 2007 in Cystic Fibrosis patients from their institution (52). They found a strain (CF-Marseille) was common among these patients. Another study indicated that new chromosomal factors influenced the resistance of *S. aureus*; examples included promoter and binding regions (18). This study, however, did not go on the state why these chromosomal changes occurred, simply stated that they did occur.

Genomic mapping and sequencing possibilities

Understanding genetic relatedness is easier to identify if the strain being studied is on a small scale in regards to geographic distribution, however this becomes more challenging when the strain population is larger, separated by time, and recovered from a larger geographic area (63). Since MRSA has become a significant concern across the world, special monitoring genetics have been formed in efforts to track specific strains and outbreaks, started by many different organizations both within the health care system and by individual researchers. In addition to tracking outbreaks, genotyping is used to distinguish

between contaminating and infecting isolates and between separate episodes of infection and relapse of disease (63). There are many advantages to being able to track *S. aureus* infection by the genome, one of which is possibly being able to predict future changes in the genomes. Genotyping is also able to link specific *S. aureus* clonal types with disease syndromes, such as in cases of food poisoning and toxic-shock syndrome. The present challenge is to continue to build bacterial database linking genetic markers and clinical presentation so that important correlates of disease can be identified (63). The CDC has preformed extensive research on *S. aureus* and MRSA outbreaks and identified factors of the bacteria that could possible contribute to the outbreaks. The CDC has typed *S. aureus*, specifically MRSA isolates. This binary typing is more objective than specific when looking at certain strains. The only comparisons made are in the presence or absence of 12 different targets in the *S. aureus* genome. Specific changes in the genome are not looked for with binary typing (63). The purpose of trying to identify genomic differences is to possibly identify relatedness between strains. With this information it might be easier to develop treatments for strains of *S. aureus*. This could possibly allow treatments to target specific genes and reproduction abilities certain strains of S. aureus. However, binary typing fails to provide information on genetic relatedness between strain types (63). The CDC has also proposed doing DNA sequence analysis as an objective genotyping method.

> "Sequencing the same DNA targets from disparate isolates and then cataloging mutation patterns constitute an

> approach termed comparative sequencing. Two different strategies have been used to provide genotyping data: multilocus sequence typing (MLST), which compares sequence variation in numerous housekeeping gene targets, and single-locus sequence typing, which compares sequence typing to a single gene. The other type of sequencing that has been developed is the multilocus enzyme electrophoresis (63)".

Like many approaches that have been tried by the heath care systems and by organizations, money and resources tend to be deciding factors. MLST has run into complications such as being too labor-intensive, time consuming, and too costly to use in a clinical setting. More than 2,500 bp must be compared for each isolate for MLST. In addition, for certain recent subpopulations, such as MRSA, genetic variability in the housekeeping targets will likely be limited and discrimination will be restricted (63). Housekeeping genes are genes that tend to be highly conserved from generation to generation and are responsible for things such as cell function. This study is stating that if they can find some variability among these highly conserved genes they could possibly isolate new strains of MRSA (63). Understanding these genomic changes of *S. aureus* is an important key into understanding the infectious nature of the bacteria and also gives us more information to establish possible treatments.

At any given time in the United States between 20% - 60% of the population is carrying *S. aureus* strains. Most of these infections will require no medical attention. However for a small percentage these infectious can turn into life threatening conditions (30, 35, 11). In 1997, one study reported the 50% of all cases of *S. aureus* that were reported were methicillin resistant, this number reached 64.4% in 2003 (30, 35). To watch and record data like these, organizations have been developed such as the Active Bacterial Core surveillance system (ABCs) which is an ongoing, population-based, active laboratory surveillance system and is a component of the Emerging Infectious Program (EIP) of the US Centers for Disease Control and Prevention (CDC) (35). In a report that was put out by the American Medical Association, it showed that there were 8987 observed cases of invasive MRSA. This did not list, however, the number of all MRSA cases (35).

		Invasive MRSA Infections	Invasive MRSA Deaths
Demographic			
Sex		Number of cases	Number of Cases
	Male	3066	571
	Female	2220	417
Age			
	<1	60	5
	1	9	0
	2-4	18	1
	5-17	47	3
	18-34	434	31
	35-49	1082	92
	50-64	1327	224
	≥65	2308	632
Totals		5287	988 (19%)

_Table 2. Estimated incidences of only invasive MRSA in the United Stated in 2005 (source: CDC).

What is Melittin?

Natural antimicrobials are being researched more for possible treatments for MRSA infections. Some of these antimicrobials include essential oils, proteins from the dermal tissue of frogs, and bee venom. This research study took a closer look at the antimicrobial effects of melittin on *S. aureus* and

hypothesized that melittin could be used as a novel drug therapy to treat other bacterial infections such as *S. aureus* and subsequently, MRSA.

Melittin, a 26 amino acid antimicrobial peptide found in European bee venom (*Apis mellifera*), is one of the most well studied antimicrobial peptides (1, 25, 27, 50, 68). Melittin's mechanism of action is to disrupt the cell membrane by protein insertion leading to pore formation then lysis of the cell (50, 68). Melittin, like many other antimicrobial peptides, are being studied because of the increasing number of antibiotic-resistant bacteria (44). This mode of action has been observed in other bacteria including *Borrelia burgdorferi*, the causative agent of Lyme disease which can lead to several aliments in infected people (30, 48, 51).

The direct mode of action used by melittin is not completely understood, however there have been few proposed mechanisms through which melittin is thought to be able to disrupt the cell membrane by forming a pore. These proposed mechanisms include: the toroidal mechanism, the barrel-stave mechanism, the peptide aggregate model, and the carpet-like mechanism (44, 68). The mechanism used by melittin can depend on the characteristics of the cell membrane (1). Melittin appears to be effective against both gram-positive and gram-negative bacteria despite the structural differences in the ultra structures (12, 68). Antimicrobial peptides have been shown to have highly variable effects on gram-positive bacteria when compared with gram-negative bacteria (60).

Melittin is well known to have hemolytic activity and can cause an immune response in humans, which could be a potential drawback for studies developed on humans. Hybrids of melittin, however can be made by separating the antimicrobial domain from the domains associated with hemolysis and hypersensitivity (1).

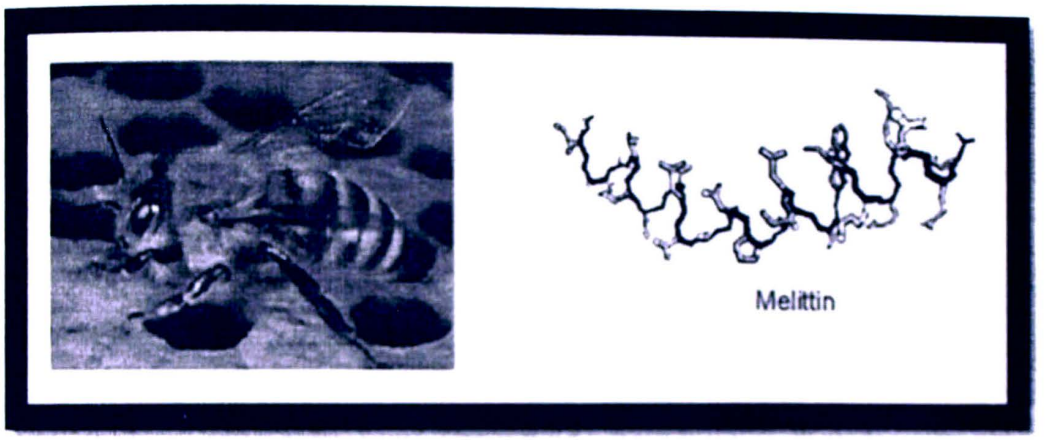

_Figure 4. Illustration of melittin, 26 amino acid peptide.

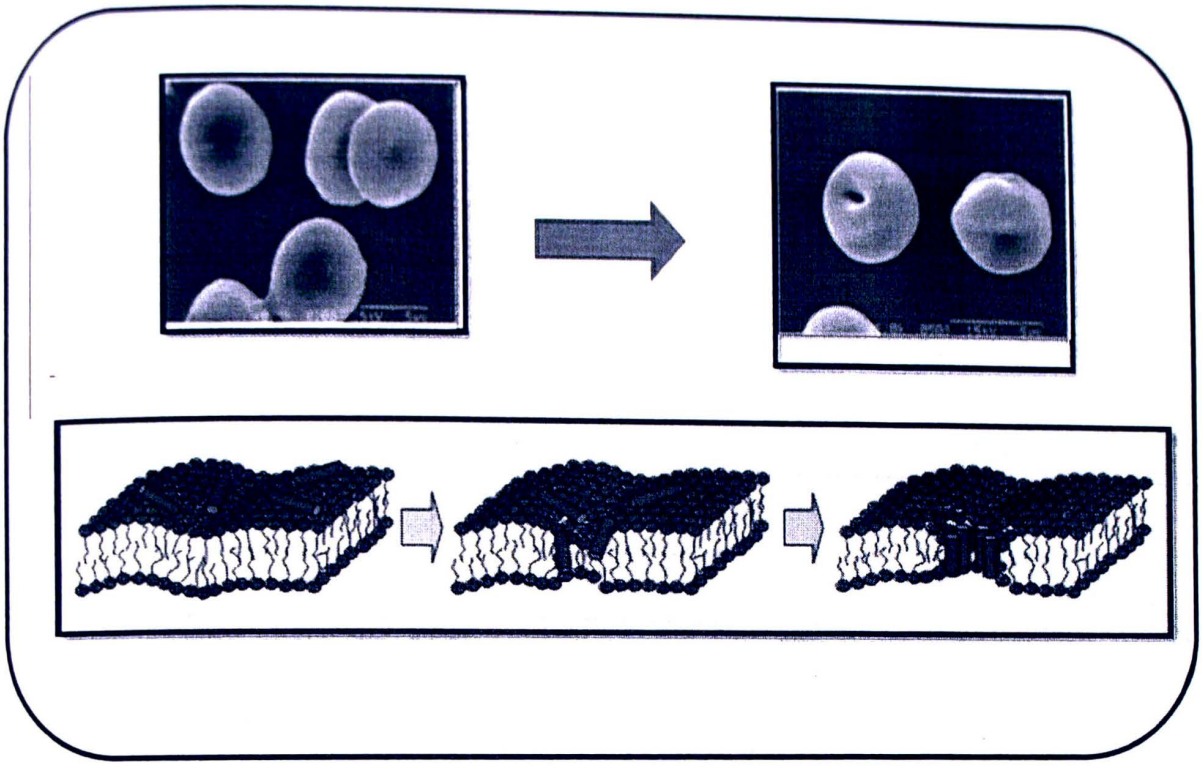

Figure 5. Illustration of the mechanim used by melittin to lysis the cell, pore forming molecule.

Materials and Methods

Cultivation of Staphylococcus aureus

S. aureus strain ATCC 25923 was used in this study. This strain was used because it does not have any resistances to antibiotics. The *S. aureus* pellet was re-suspended in nutrient broth (NB) that was made from Nutrient broth powder and de-ionized water then autoclaved to produce sterile NB media. The pellet was vortexed at max speed until completely dissolved in the NB. The *S. aureus* and NB solution were then incubated at 37° Celsius over night to allow for growth. New NB was added to the *S. aureus* solution every two to three days to ensure the survival of the bacteria. . Aseptic techniques were important in resurrection the *S. aureus* and to prevent contamination.

Determination of countable colonies

Spectrophotometer Spectronic Genesys2 was used to estimate the amount of *S. aureus* which would produce countable colonies on nutrient agar plates. Through preliminary data, a wavelength of 400nm and an ABS at 0.500 would produce countable colonies when performing 1:10 serial dilutions down to the $1:10^6$ dilution. This information was then used throughout the study as a reference to ensure that approximately the same amount of bacteria was used in the control and experimental groups. A comparison of experimental and control plated are shown in figure 8.

Determining the LD50

This experiment determined the lethal dose needed of melittin to kill 50% of *S. aureus* bacteria and was performed in triplicate. The control tube included 170 µL of NB, $4x10^5$ of *S. aureus* organisms and 10 µl of molecular water for a consistent volume. The experimental tube contained 170 µL of NB, approximately $4x10^5$ of *S. aureus* organisms and 100 pg of melittin solution. The effective concentration of melittin was determined by taking varying concentrations of melittin and constant *S. aureus* concentration. The *S. aureus* control and experimental tubes were then serial diluted from the original tube to a dilution of $1:1x10^{10}$. From each of the serial diluted tubes, approximately $8x10^5$ *S. aureus* organisms was placed on nutrient agar plates and spread over the

surface using a spin plate and sterilized glass rod. The plates were then placed in an incubator overnight at 37° C. After incubation time, the colonies on each plate were counted and recorded. The results were averaged together to produce standard curve seen in figure 6.

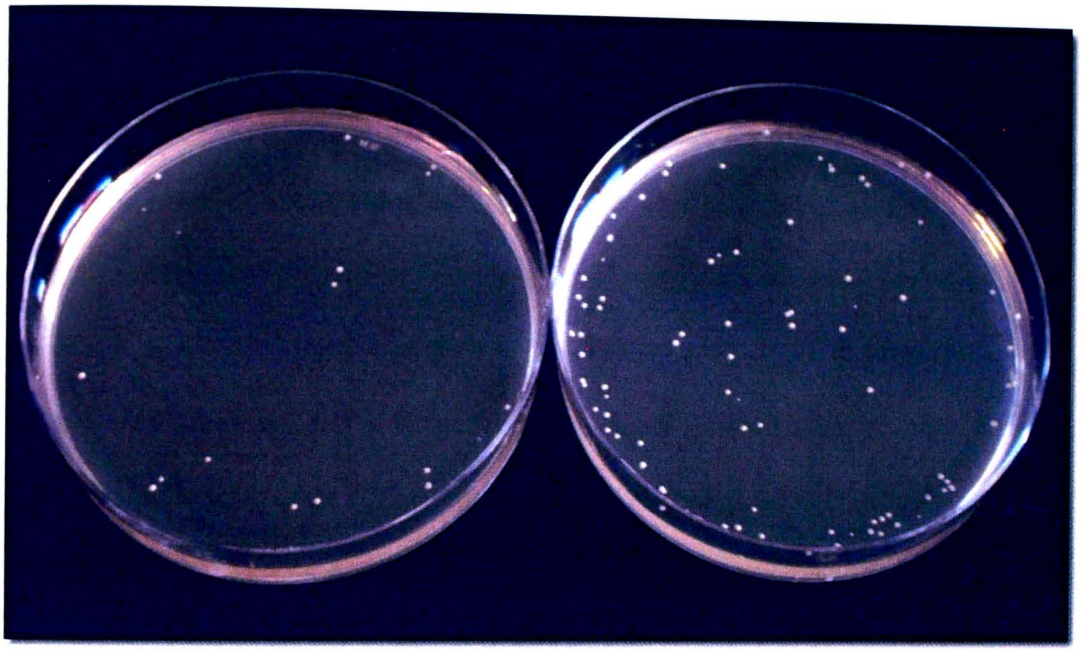

Figure 6. Example using *Staphylococcus aureus* of an experimental plate with melittin (left) verses control plate (right).

Determining if melittin had a killing effect on *S. aureus* after plating

To determine if melittin had a continued killing effect on *S. aureus* after being placed on nutrient agar plates an experiment was set up to centrifuge melittin out of solution. The experiment contained a control, which had no melittin, and two experimental groups. The experimental groups were: group1 that was plated with melittin and group 2 had melittin centrifuged out of the solution before being plated. All of the groups had approximately $4x10^5$ *S.*

aureus organisms and melittin was diluted to 1:100. The control tube contained 170µL of NB, approximately $4x10^5$ of *S. aureus* and 10µl of molecular water for a consistent volume. The experimental tubes contained 170 µL of NB, approximately $4x10^5$ of *S. aureus* organisms and 100 pg of melittin solution. All tubes were allowed to incubate for 15 minutes, at that time the second experimental tube was centrifuged briefly. This allowed the *S. aureus* to be centrifuged out of solution but the unbounded melittin remained in solution. The heavier S. aureus would centrifuge to the bottom and form a pellet while the lighter melittin would stay in solution. The aqueous layer was then pulled off and discarded. The *S. aureus* pellet was then re-suspended in an equal volume of NB. The control and experimental tubes were then serial diluted down to the $1:10^6$. Each dilution tube then had approximately $8x10^5$ of *S. aureus* organisms plated on nutrient agar plates and incubated at 37° Celsius overnight. The colonies were then compared to evaluate a difference.

Shelf-Life of melittin

To determine the half-life of melittin at real time, melittin was left at room temperature before being used in experimentation. A tube containing approximately $4x10^5$ of *S. aureus* organisms, 170 µL of NB, and 100 pg of melittin was used for each experiment. The melittin used for each tube varied only in the amount of time it had been left at room temperature. Initially, the melittin was pulled directly out of the 4°C freezer, allowed to thaw and then

added to the *S. aureus* and NB solution. The melittin was then left out at room temperature to be used the next day. The *S. aureus* +NB + melittin solution was serial diluted to $1x10^6$ dilution. From the standard curve it was determined that countable colonies would be observed from $1x10^4 - 1x10^6$ dilutions. With this information only dilutions $1:1x10^4 - 1:1x10^6$ were plated and counted after being incubated overnight at 37° C. This process was then repeated again the following day substituting the melittin for the melittin that had been left out at room temperature overnight. This was repeated for four consecutive days and then was repeated on a weekly basis until a leveling out was seen between the control and the experimental countable colonies. The colonies from each experiment were compared to the control from the first experiment. These results are shown in graph 9.

Effects of pH on melittin activity

Three pH concentrations were selected for testing if melittin can be denatured at any point, pH 4, pH 7, and pH 10. For each pH experiment, a control, experimental and a standard were produced in quadruplicate. Each experimental tube contained 170 µL of pH solution and 100 pg of melittin. The tube was then allowed to incubate for 15 minutes before approximately $4x10^5$ of *S. aureus* organisms was added to the solution. The solution was then allowed to incubate for 15 minutes before being serial diluted down to $1x10^7$ dilution. Approximately $8x10^5$ of *S. aureus* organisms from each tube was then plated on nutrient agar plates and allowed to incubate overnight. The control tubes followed tube contained 170 µL of molecular water and 100 pg of melittin. The

tube was then allowed to incubate for 15 minutes before approximately $4x10^5$ of *S. aureus* organisms was added to the solution. The solution was then allowed to incubate for 15 minutes before being serial diluted down to $1x10^7$ dilution. From each tube Approximately $8x10^5$ of *S. aureus* organisms from was placed on nutrient agar plates and allowed to incubate overnight. The standard tubes contained 170 µL of NB, approximately $4x10^5$ of *S. aureus* organisms and 10 µL of molecular water. All plates were incubated at 37°C overnight. The colonies for each experiment were then counted and the results are seen shown for pH 4 in graph 10 and for pH 10 in graph 11.

Most effective volume (*S. aureus* to melittin ratio)

This experiment was designed to determine the most effective volume needed to kill *S. aureus* bacteria in solution. The amounts of *S. aureus* and melittin were kept constant but the volume varied for each experiment. Volumes that were used were 50µL, 100 µL, 200 µL, and 500 µL. Each experimental tube contained the specified volume (50µL, 100 µL, 200 µL, or 500 µL) of NB, approximately $4x10^5$ of *S. aureus* organisms and 100 pg of melittin. The control tubes for each volume contained the specified volume (50µL, 100 µL, 200 µL, or 500 µL) of NB, approximately $4x10^5$ of *S. aureus* organisms and 10µL of molecular water to keep the volume constant. The tubes were allowed to incubate for 15 minutes before being serial diluted down to the $1:1x10^8$ dilution. This was completed for each volume in quadruplicate. The results for 50 µL is in

graph 12, the results for 100 µL are seen in graph 13, the results of 200 µL are seen in graph 14 and the results for 500 µL are seen in graph 15.

Evaluation of therapeutic effects of melittin *in vivo*

Based on these prior experiments, a melittin-gel was manufactured to determine the efficacious nature of melittin in the treatment of *S. aureus*-infected mice, *in vivo*. The gel was produced from 90 µL of glycerol and 10 pg melittin. This concentration was obtained from the experiments that were done *in vitro*. The C3H/HeJ mouse model that was selected for this experiment allows greater data precession due to all mice being highly inbred clones (4). Initially a colony of *S. aureus* was re-suspended in 200µL of NB solution (this gave us a 0.903 optical density at 400nm wavelength which is about $8x10^5$ *S. aureus* organisms). Each group will contain a total of five mice which has been historically used as the minimum number needed for statistical significance (34). A total of 15 mice were used and separated into groups including: control 1 (uninfected/untreated), control 2 (infected/untreated), and experimental (infected/treated). Ear punches were used to open a 2mm hole in the tissue of the mice's ear, giving the *S. aureus* bacteria a portal of entry. The control 1 group was used to show if the punch by itself had any noticeable inflammatory response when compared to the other groups which had S. aureus added. The mice were then inoculated with approximately 5,000 *S. aureus* organisms in an inoculum of 1.25 µL. The zone of inflammation on the ear was measured 16 hours later. A melittin gel that was produced to treat the bacteria *in vivo* had a concentration of 10µg/µL (90 µL glycerol and 10 µL of 100 µg/ml concentration of melittin). After the initial 16

hour incubation time the mice were treated with 2 µL of the melittin gel, which was placed on the infected ear. The zone of inflammation on the ear was again measured 16 hours later. These results are seen in graph 16.

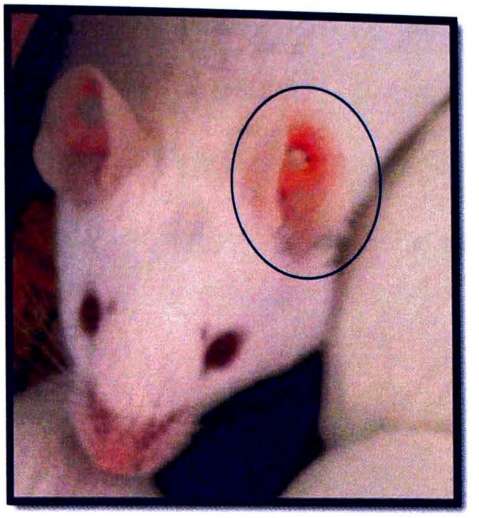

Figure 7. Photograph illustrating the hole punch made in the ear of the mice, and the zone of inflammation measured.

Resistance Testing

Several generations of *S. aureus* was exposed to melittin and compared to *S. aureus* exposed to ampicillin, streptomycin, and tetracycline to determine if *S. aureus* was able to build up a resistance as typically seen resulting from other antibiotics. A desired absorbency for the *S. aureus* solution was measured at wavelength of 400nm at an ABS of 0.500. Each antibiotic experiment was completed in quadruplicate. The concentration of the melittin and the antibiotics used were kept constant and 10 µg/mL. Approximately $8x10^5$ *S. aureus* organisms were plated on nutrient agar. A blank sterile paper disc, 6mm in diameter was pushed into the nutrient agar to produce a depression in the agar.

15µL of antibiotic or melittin was placed into the depression. The plates were then left at room temperature to incubate overnight. The zone of inhibition was then measured and recorded. The *S. aureus* that had grown closest to the zone was pulled off the plate using a sterile inoculating loop and placed in 200 µL of NB solution to produce a second generation of *S. aureus*. The *S. aureus* was the vortexed to break colonies apart. The experiment was then repeated over several generations. The comparison between antibiotic and melittin can be seen in graph 17.

Results

In an attempt to conserve resources, a standard curve was produced using Spectrophotometer Spectronic Genesys2. Experimental and control group contain *S. aureus* at a wavelength of 400nm at an ABS of 0.500 and melittin. The results indicated that when *S. aureus* has an ABS of 0.500 countable colonies can be found on dilution plates $1{:}10^4 - 1{:}10^7$. This information was also used throughout the study to determine the amounts of *S. aureus* organisms that were worked with.

The same experiment was used to determine the LD50 of melittin. A 15 min incubation time with melittin was determined to have approximately 90% killing effect on *S. aureus in vitro*. The control tube included 170 µL of NB, Approximately $4x10^5$ of *S. aureus* organisms and 10 µl of molecular water for a consistent volume. The experimental tube contained 170 µL of NB, 20 µL of *S.*

aureus and 100 pg of melittin. The effective concentration of melittin was determined by taking varying concentrations of melittin and constant *S. aureus* concentration. The *S. aureus* control and experimental tubes were then serial diluted from the original tube to a dilution of $1{:}1x10^{10}$. From each of the serial diluted tubes, approximately $8x10^5$ of *S. aureus* organisms was placed on nutrient agar plates and spread over the surface using a spin plate and sterilized glass rod. The plates were then placed in an incubator overnight at 37° C. After incubation time, the colonies on each plate were counted and recorded. This was shown by comparing the experiment tube which contained *S. aureus* and melittin to the control tube which only contained *S. aureus*. This indicated that melittin is effective at clearing *S. aureus in vitro*. The results showed that after 15 minute incubation time, approximately 90% of *S. aureus* was killed.

A concern was that melittin was able to kill *S. aureus* organisms after being plated on nutrient agar. The experiment contained a control, which had no melittin, and two experimental groups. The experimental groups were: group1 that was plated with melittin and group 2 had melittin centrifuged out of the solution before being plated. All of the groups had approximately $4x10^5$ *S. aureus* organisms and 100 pg of melittin. The control tube contained 170 µL of NB, $4x10^5$of *S. aureus* organisms and 10 µl of molecular water for a consistent volume. The experimental tubes contained 170 µL of NB, approximately $4x10^5$ of *S. aureus* and 100 pg of melittin. All tubes were allowed to incubate for 15 minutes, at that time the second experimental tube was centrifuged briefly. This allowed the *S. aureus* to be pelletted out of solution but the unbounded melittin

remained in solution. The aqueous layer was then pulled off and discarded. The *S. aureus* pellet was then re-suspended in an equal volume of NB. The control and experimental tubes were then serial diluted down to the $1:10^6$. Each dilution tube then had 40 µL plated on nutrient agar plates and incubated at 37° C overnight. The colonies were then compared to evaluate a difference. The findings indicated that there was no difference between centrifuging melittin out of solution before plating or leaving the melittin in solution.

For melittin to be used as a novel drug treatment for *S. aureus*, it has to be able keep its killing ability. To test the shelf-life of melittin a tube containing approximately $4x10^5$ of *S. aureus* organisms, 170 µL of NB, and 100 pg of melittin was made for each experiment. The melittin used for each tube varied only in the amount of time it had been left at room temperature. Initially, the melittin was pulled directly out of the 4°C freezer, allowed to thaw and then added to the *S. aureus* and NB solution. The melittin was then left out at room temperature to be used the next day. The *S. aureus* +NB + melittin solution was serial diluted to $1x10^6$ dilution. From the Standard curve it was determined that countable colonies would be observed from $1x10^4 - 1x10^6$ dilutions. With this information only dilutions $1:1x10^4 - 1:1x10^6$ were plated and counted after being incubated overnight at 37° C. This process was then repeated again the following day substituting the melittin for the melittin that had been left out at room temperature overnight. This was repeated for four consecutive days and then was repeated on a weekly basis until a leveling out was seen between the control and the experimental countable colonies. The colonies from each

experiment were compared to the control from the first experiment. Melittin showed a decrease in function after only being exposed to room temperature for two days. This indicates that melittin has a short shelf-life when left at room temperature. After a week of exposure to room temperature melittin had completely lost its killing ability on *S. aureus*.

Three pH concentrations were selected for testing if melittin can be denatured at any point, pH 4, pH 7, and pH 10. For each pH experiment a control, experimental and a standard were produced in quadruplicate. Each experimental tube contained 170 µL of pH solution and 100 pg of melittin. The tube was then allowed to incubate for 15 minutes before approximately $4x10^5$ µL of *S. aureus* organisms were added to the solution. The solution was then allowed to incubate for 15 minutes before being serial diluted down to $1x10^7$ dilution. Approximately $8x10^5$ *S. aureus* organisms from each tube were then plated on nutrient agar plates and allowed to incubate overnight. The control tubes followed tube contained 170 µL of molecular water and 100 pg of melittin. The tube was then allowed to incubate for 15 minutes before approximately $4x10^5$ µL of *S. aureus* organisms was added to the solution. The solution was then allowed to incubate for 15 minutes before being serial diluted down to $1x10^7$ dilution. From each tube approximately $8x10^5$ *S. aureus* organisms were placed on nutrient agar plates and allowed to incubate overnight. The standard tubes contained 170 µL of NB, approximately $4x10^5$ *S. aureus* organisms, and 10 µL of molecular water. All plates were incubated at 37°C overnight. At the varying pH concentration different results were observed. While there was no statistical

difference between the different pH concentration, the graphed results showed unpredictability when melittin is exposed to pH 4 and pH 10. The pH concentration that was selected for this study was pH 7, based on observed graphical data and not statistical data.

Varying volumes were tested to find the most effective conditions for melittin to encounter *S. aureus*. The amounts of *S. aureus* and melittin were kept constant but the volume varied for each experiment. Volumes that were used included: 50 µL, 100 µL, 200 µL, and 500 µL. Each experimental tube contained the specified volume (50 µL, 100 µL, 200 µL, or 500 µL) of NB, approximately $4x10^5$ µL of *S. aureus* organisms, 100 pg of melittin. The control tubes for each volume contained the specified volume (50 µL, 100 µL, 200 µL, or 500 µL) of NB, approximately $4x10^5$ *S. aureus* organisms, and 10 µL of molecular water to keep the volume constant. The tubes were allowed to incubate for 15 minutes before being serial diluted down to the $1:1x10^8$ dilution. Statistical data indicated no difference between any of the volumes used. However, there was a great variation and unpredictability in 50 µL, 100 µL, and 500 µL volumes. Volume was shown to play a crucial role in the occurrence of *S. aureus* and melittin interacting in solution. The volume that yielded the most predictable and consistent results, was the 200 µL volume. This was the volume that was chosen for use during the study.

Based on these prior experiments, a melittin-gel was manufactured to determine the efficacious nature of melittin in the treatment of *S. aureus*-infected mice, *in vivo*. The gel was produced from 90 µl of glycerol and 100 pg of melittin.

This concentration was obtained from the experiments that were done *in vitro*. The C3H/HeJ mouse model that was selected for this experiment allows greater data precession due to all mice being highly inbred clones (4). Initially a colony of *S. aureus* was re-suspended in 200µL of NB solution (this generated a 0.903 optical density at 400nm wavelength which is about $8x10^5$ *S. aureus* organisms). A total of 15 mice were used and separated into groups including: control 1 (uninfected/untreated), control 2 (infected/untreated), and experimental (infected/treated). Ear punches were used to open a 2mm hole in the tissue of the mice's ear, giving the *S. aureus* bacteria a portal of entry. The control 1 group was used to show if the punch by itself had any noticeable inflammatory response when compared to the other groups which had S. aureus added. The mice were then inoculated with 5,000 S. aureus organisms in an inoculum of 1.25 µL. The zone of inflammation on the ear was measured 16 hours later. A melittin gel that was produced to treat the bacteria *in vivo* had a concentration of 10µg/µL (90 µL glycerol and 10 µL of 100 µg/ml concentration of melittin). After the initial 16 hour incubation time the mice were treated with 2 µL of the melittin gel, which was placed on the infected ear. The zone of inflammation on the ear was again measured 16 hours later. The results showed a strong statistical difference between treated and untreated mice. Melittin was effective at clearing S. aureus in vivo. The zone of inflammation measured 16 hours after inoculation was 32mm in both treated and untreated mice. After 16 hour post-treatment was applied to the treated mice, the zones measured 34 mm in untreated and 7 mm

in treated mice. This gives promise to melittin's use as a topical treatment for *S. aureus* infections.

Several generations of *S. aureus* exposed to melittin and compared to *S. aureus* exposed to ampicillin, streptomycin, and tetracycline to determine if *S. aureus* was able to build up a resistance as typically observed resulting from exposure to other antibiotics. A desired absorbency for the *S. aureus* solution was measured at wavelength of 400nm at an ABS of 0.500. Each antibiotic experiment was completed in quadruplicate. The concentration of the melittin and the antibiotics used were kept constant and 10 µg/mL. Approximately $8x10^5$ *S. aureus* organisms were plated on nutrient agar. A blank sterile paper disc, 6 mm in diameter was pushed into the agar plate to produce a depression in the agar. To be consistent, 15 µL of antibiotic or melittin was placed into the depression. The plates were then left at room temperature to incubate overnight. The zone of inhibition was then measured and recorded. The *S. aureus* that had grown closest to the zone was pulled off the plate using a sterile inoculating loop and placed in 200 µL of NB solution to produce a second generation of *S. aureus*. The *S. aureus* was the vortexed to break colonies apart. The experiment was then repeated over several generations. *S. aureus* was able to show a level of resistance to the antibiotics streptomycin, ampicillin, and tetracycline after only the second generation. Importance of this experiment was to show that *S. aureus* had no ability to build up a resistance to melittin. After four generations *S. aureus* had no resistance to the melittin used.

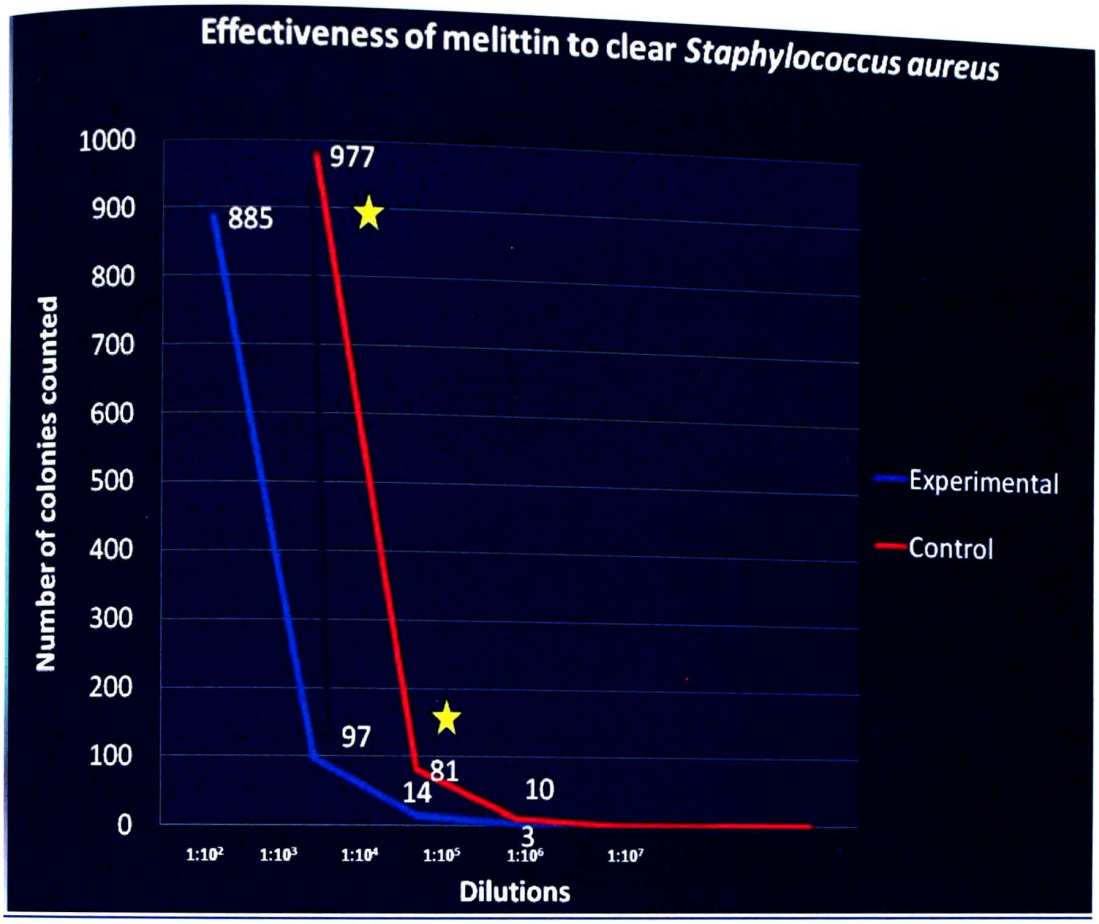

Figure 8. A melittin concentration of 10 pg/ul significantly killed *S. aureus* in 15 minutes *in vitro*. The experimental group contained S. aureus organisms at a wavelength of 400nm at an ABS of 0.500 and had 100 pg of melittin added. The control group contained approximately $4x10^5$ *S. aureus* organisms and molecular water for a consistent volume. Both groups were incubated for 15 minutes to allow melittin to bind to the surface of S. aureus. The tubes from each group were then serial diluted down to $1:10^7$ dilution and plated on nutrient agar. The plates were then incubated over night after which colonies were counted. The results can be seen above. Stars identify significance (p=0.05). This is also the figure that was used to show pH7, since the nutrient broth used for all experiments was at a pH 7.

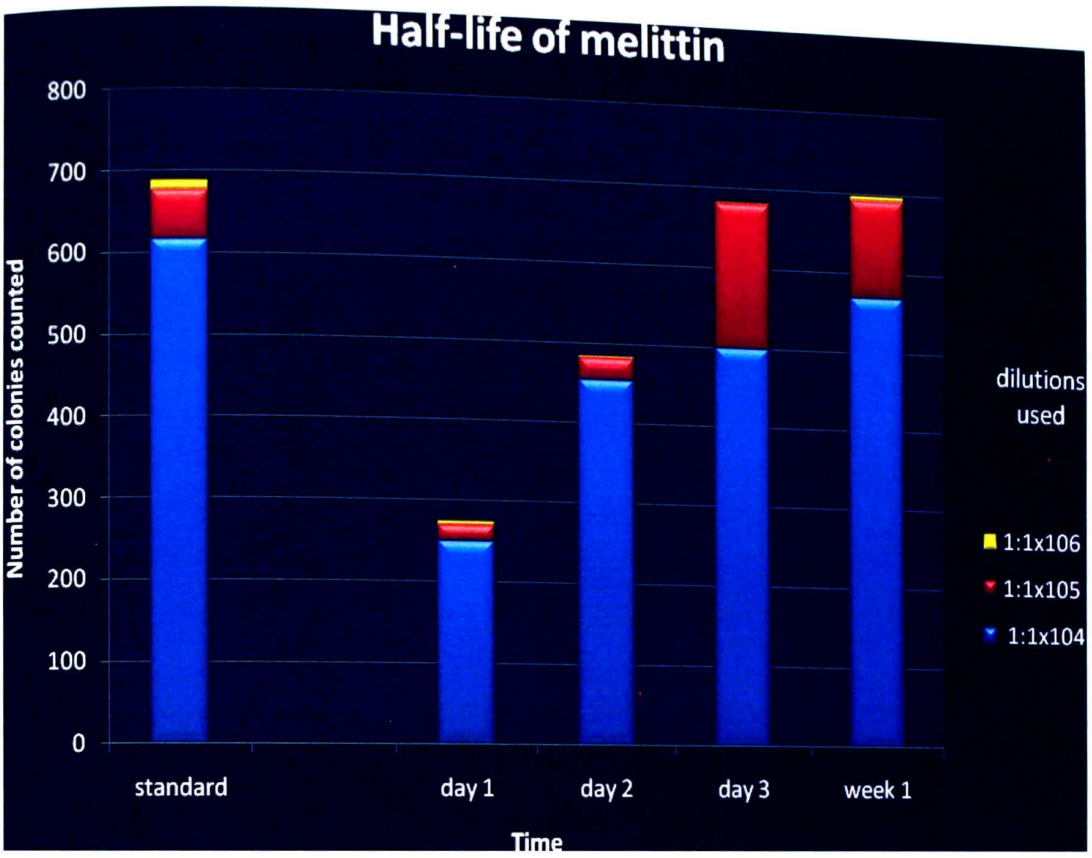

Figure 9. Melittin uses approximately half of its activity after 2 days at real time. The dilutions that were selected for this experiment ($1:10^4 - 1:10^6$) were done using the standard curve. This allowed for plates containing countable colonies to be determined. Self life of melittin was determined by adding melittin that had been left at room temperature to *S. aureus*. The days indicate the specific amount of time that melittin was left out at room temperature before being used for experimentation. The colonies from each plated were counted and the results can be seen above, showing that melittin loses its killing ability when left at room temperature for an extended amount of time. Standard deviation of the control at $1:1x10^4$ on day one is 614.25$\pm$ 205.89, control $1:1x10^5$ on day one is 61.25 $\pm$14.80, control $1:1x10^6$ on day one is 10.5 $\pm$ 3.32. Standard deviation for experimental on day one at $1:1x10^4$ is 248.5 $\pm$ 33.21, experimental $1:1x10^5$ on day one is 20.75 $\pm$11.59, experimental $1:1x10^6$ on day one is 3.75 $\pm$ 1.26. Standard deviation for control on day two at $1:1x10^4$ is 614.25$\pm$ 205.89, control $1:1x10^5$ on day two is 61.25 $\pm$14.80, control $1:1x10^6$ on day two is 10.5 $\pm$ 3.32. Standard deviation on day two for experimental at $1:1x10^4$ is 453.25 $\pm$ 66.22, $1:1x10^5$ is 27.25 $\pm$ 8.54, and $1:1x10^6$ is 1.75 $\pm$ 0.5. Standard deviation on day three for control at $1:1x10^4$ is 614.25 $\pm$ 205.89, control $1:1x10^5$ on week one is 61.25 $\pm$14.80, control $1:1x10^6$ on week one is 10.5 $\pm$ 3.32. Standard deviation on day three for experimental at $1:1x10^4$ is 489.75 $\pm$ 28.12, experimental $1:1x10^5$ on day three is 104.25 $\pm$52.94, experimental $1:1x10^6$ on day three is 1.75 $\pm$ 1.71. Standard deviation on week one at for control at $1:1x10^4$ is 248.5$\pm$ 205.89, control $1:1x10^5$ on week one is 20.75 $\pm$14.80, control $1:1x10^6$ on week one is 3.75 $\pm$ 3.32. Standard deviation on week one for experimental at $1:1x10^4$ is 576.25 $\pm$ 181.83, experimental $1:1x10^5$ on week one is 124 $\pm$17.94, experimental $1:1x10^6$ on week one is 5 $\pm$ 3.65.

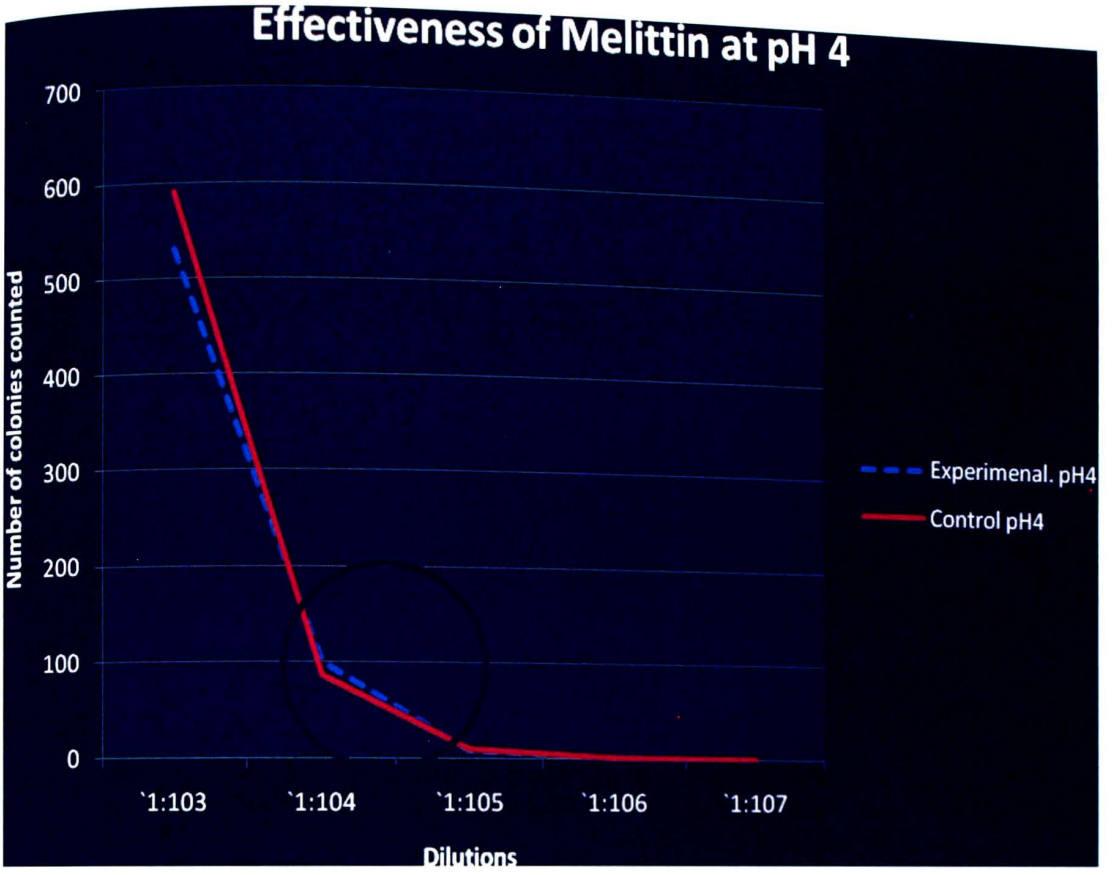

Figure 10. The ability of melittin to clear *S. aureus* from solution after exposure to pH 4 was statistically relevant. However the graphed results show unpredictable killing between dilutions $1:10^4$ and $1:10^5$. The control exposed melittin to molecular water before adding it to *S. aureus* solution. The experiment exposed melittin to a pH 4 solution before adding it to a S. aureus solution. The results were then compared to determine if pH 4 had an effect on the killing ability of melittin. Standard deviation at for the control at $1:10^3$ is 590 ± 231.37 at control $1:10^4$ it is 85.75 ± 46.91, at control $1:10^5$ it is 9.5 ± 11.73, at control $1:10^6$ it is 1 ± 1.14. Standard deviation for experimental at $1:10^3$ is 529.75 ± 122.01 at experimental $1:10^4$ it is 100.5 ± 24.28, at experimental $1:10^5$ it is 8.75 ± 2.5, at experimental $1:10^6$ it is 0.75 ± 0.96.

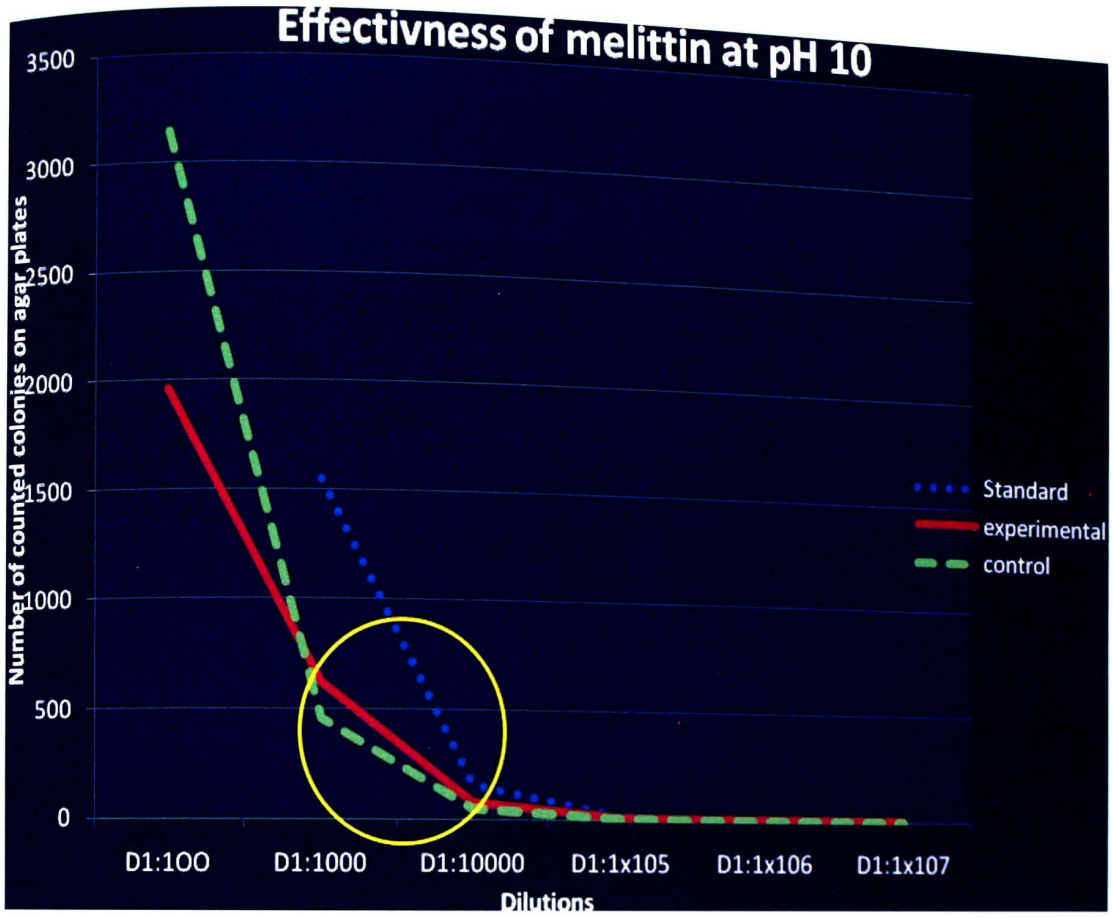

Figure 11. Effectiveness of pH 10 was shown here indicated that there was some fluctuation with results. The standard group contained nutrient broth, *S. aureus* at, and 10ul of molecular water for consistent volume. The control tubes contained molecular water, *S. aureus*, and melittin. The experimental tubes contained pH solution *S. aureus*, and melittin. The standard gives the expected countable colonies of *S. aureus* and a comparison for the experimental and control groups. The graph indicates that pH 10 had some effects on the killing ability of melittin on *S. aureus*. Standard deviation for control 1:1000 is 459.25 $\pm$ 29.39, at control 1:10000 it is 49.75 $\pm$ 13.05, at control 1:1x10^5 it is 5.25$\pm$ 3.86, at control 1:1x10^6 it is 0.5 $\pm$ 0.60, at control 1:1x10^7 it is 0.25 $\pm$ 0.5. Standard deviation for experimental 1:1000 is 624 $\pm$ 64.91, at experimental 1:10000 it is 79.5 $\pm$ 12.07, at experimental 1:1x10^5 it is 11.75$\pm$ 3.5, at experimental 1:1x10^6 it is 3.25 $\pm$ 4.57, at experimental 1:1x10^7 it is 3.75 $\pm$ 4.11.

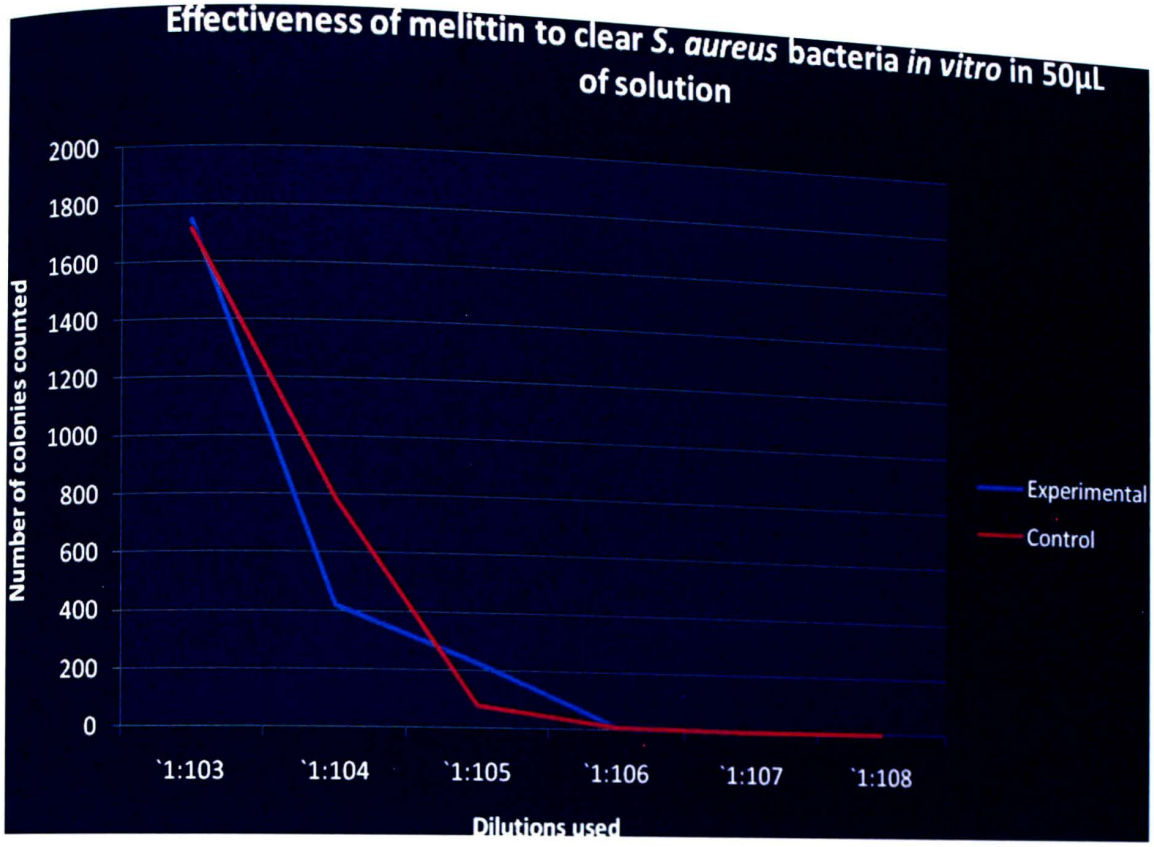

Figure 12. Occurrence of melittin and *S. aureus* interacting increased in a volume of 50µL. The result were not shown to be statistically relevant, however, there was unpredictability of killing effect on S. aureus by melittin. The control group contained 50µL of NB and 20µL of S. aureus solution with a wavelength of 400nm with an ABS of 0.503. The experimental group contained 50µL of NB, approximately $4x10^5$ *S. aureus* organisms and 100 pg of melittin. The tubes were serial diluted down to the $1:10^8$ and plated on nutrient agar. The colonies were then counted and compared. Standard deviation at of control at $1:1x10^3$ is 1712 $\pm$ 457.71, at control $1:1x10^4$ it is 786 $\pm$ 226.07, at control $1:1x10^5$ it is 76 $\pm$ 18.53, at control $1:1x10^6$ it is 7 $\pm$ 3.61, at control $1:1x10^7$ it is 1 $\pm$ 0.45. Standard deviation for experimental at $1:1x10^3$ is 1744 $\pm$ 333.71, at experimental $1:1x10^4$ it is 419 $\pm$ 57.49, at experimental $1:1x10^5$ it is 227 $\pm$ 21.85, at experimental $1:1x10^6$ it is 7.75 $\pm$ 6.24.

Figure 13. Occurrence of melittin and *S. aureus* interacting increased in a volume of 100µL. The result were not shown to be statistically relevant, however, there was unpredictability of killing effect on S. aureus by melittin. The control group contained 100µL of NB and approximately $4x10^5$ *S. aureus* organisms. The experimental group contained 100µL of NB, approximately $4x10^5$ *S. aureus* organisms and 100 pg of melittin. The tubes were serial diluted down to the $1:10^8$ and plated on nutrient agar. The colonies were then counted and compared. Standard deviation at of control at $1:1x10^3$ is 2430 $\pm$ 319.61, at control $1:1x10^4$ it is 340 $\pm$ 106.82, at control $1:1x10^5$ it is 39 $\pm$ 27.83, at control $1:1x10^6$ it is 11 $\pm$ 5.35. Standard deviation at of experimental at $1:1x10^3$ is 1992 $\pm$ 483.22, at experimental $1:1x10^4$ it is 455.25 $\pm$ 89.17, at experimental $1:1x10^5$ it is 60 $\pm$ 19.42, at experimental $1:1x10^6$ it is 12 $\pm$ 5.79.

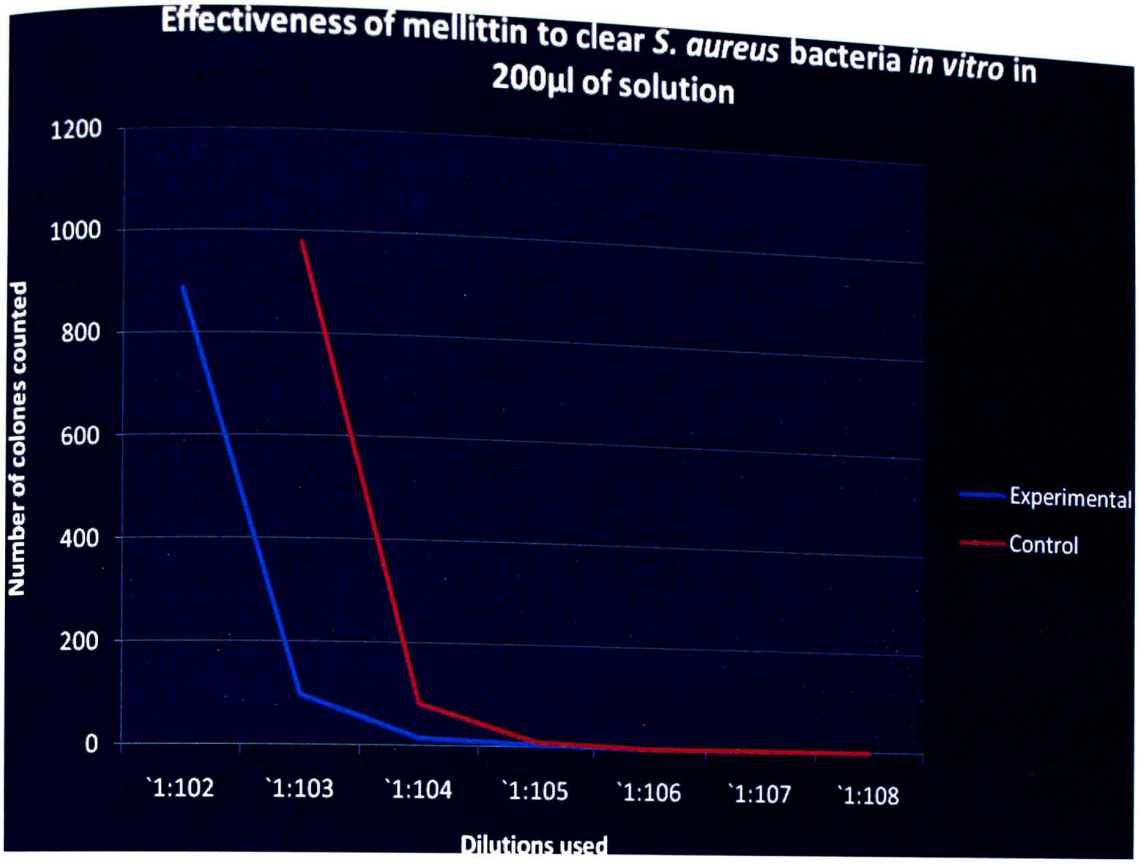

Figure 14. Occurrence of melittin and *S. aureus* interacting increased in a volume of 200µL. The result were not shown to be statistically relevant. This volume was shown to have strong predictability of the killing effect of melittin on *S. aureus*. The control group contained 200µL of NB and approximately $4x10^5$ *S. aureus* organisms. The experimental group contained 200µL of NB, approximately $4x10^5$ *S. aureus* organisms, and 100 pg of melittin. The tubes were serial diluted down to the $1:10^8$ and plated on nutrient agar. The colonies were then counted and compared. Standard deviation at of control at $1:1x10^3$ is 977.33 $\pm$ 74.33, at control $1:1x10^4$ it is 81 $\pm$ 9.54, at control $1:1x10^5$ it is 9.67 $\pm$ 8.62. Standard deviation of experimental at $1:1x10^2$ is 885.33 $\pm$ 516.63, experimental at $1:1x10^3$ is 97 $\pm$ 39.23, at experimental $1:1x10^4$ it is 14 $\pm$ 7.

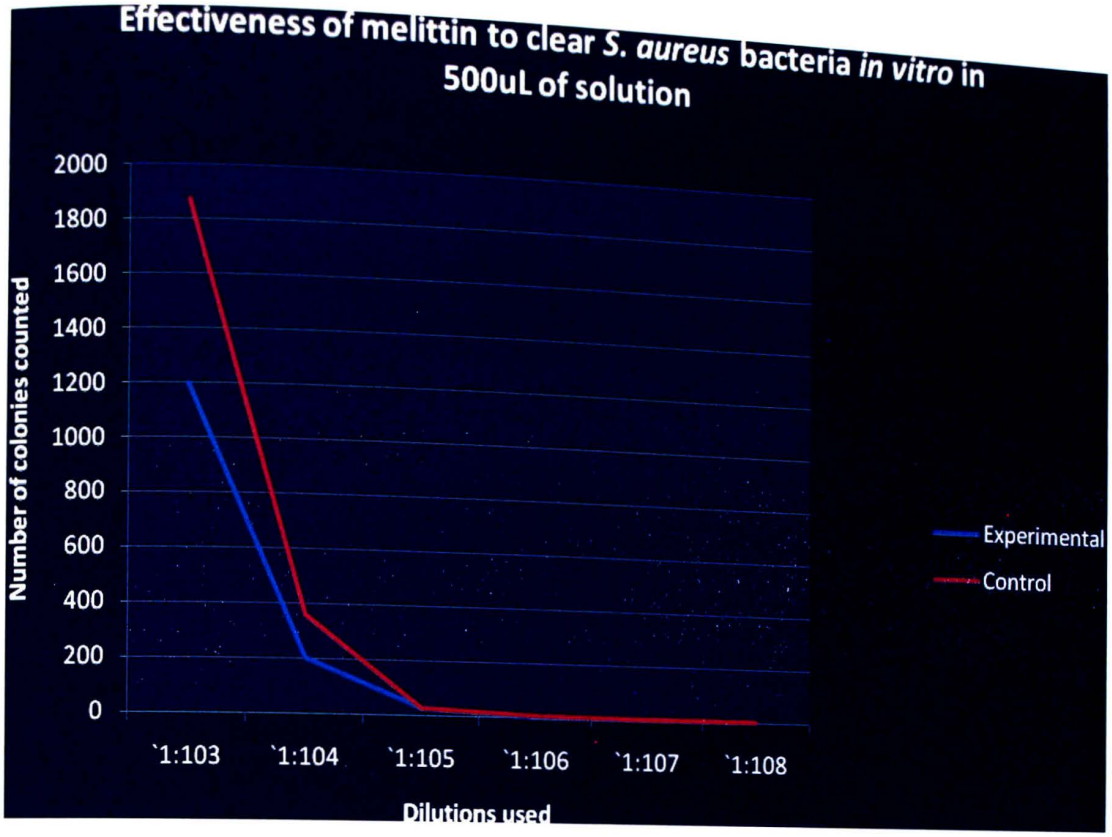

Figure 15. Occurrence of melittin and *S. aureus* interacting increased in a volume of 500µL. The results were not shown to be statistically relevant. Longer incubation might have increased the occurrence of S. aureus in melittin. The control group contained 500µL of NB and approximately $4x10^5$ *S. aureus* organisms. The experimental group contained 500µL of NB, approximately $4x10^5$ *S. aureus* organisms, and 100 pg melittin. The tubes were serial diluted down to the $1:10^8$ and plated on nutrient agar. The colonies were then counted and compared. Standard deviation at of control at $1:1x10^3$ is 1870 ± 583.38, at control $1:1x10^4$ it is 358 ± 111.07, at control $1:1x10^5$ it is 24.25 ± 12.89, at control $1:1x10^6$ it is 3 ± 1.83, at control $1:1x10^7$ it is 0.75 ± 1.5. Standard deviation of experimental at $1:1x10^3$ is 1198 ± 209.35, experimental at experimental $1:1x10^4$ it is 203 ± 50.12 at control $1:1x10^5$ it is 23.5 ± 11.15, at control $1:1x10^6$ it is 2.5 ± 2.38, at control $1:1x10^7$ it is 0.25 ± 0.5.

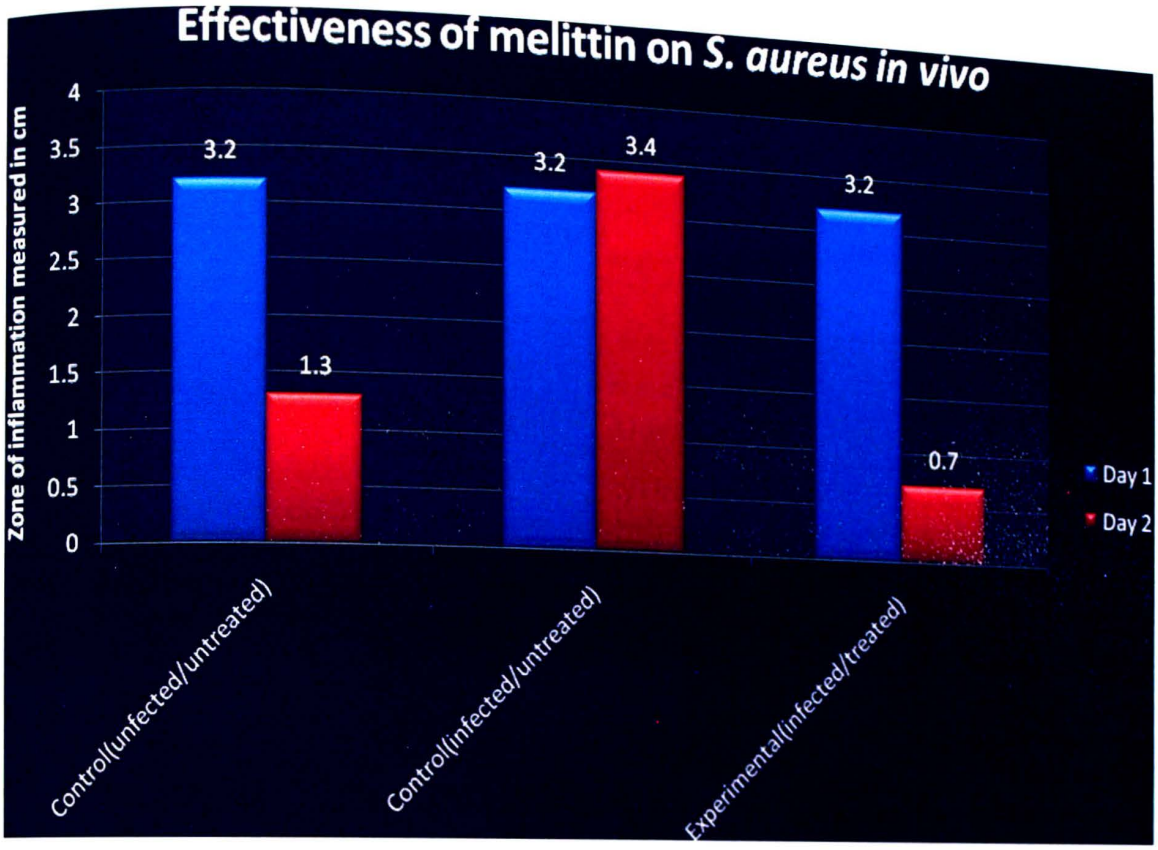

Figure 16. Effectiveness of melittin on clearing *S. aureus* infection *in vivo*. Results indicated that there was a significant difference between day 1 and day 2 in the control group (uninfected/untreated) and the experimental group. Control (uninfected/untreated) group punctured a 2mm hole in the mice model's ear but did not infect or treat the mice. Control (infected/untreated) group punctured a 2mm hole in the mice model's ear, infected the mice with S. aureus but did not treat with melittin. The experimental group punctured a 2mm hole in the mice model's ear infected the mice with S. aureus and also treated them with melittin. Standard deviation for the control mice (uninfected/untreated) day one was 3.2 $\pm$ 0.16, standard deviation for the control mice (uninfected/untreated) day two was 1.3 $\pm$ 0.4. Standard deviation for the experimental mice (infected/untreated) day one was 3.2 $\pm$ 0.16, standard deviation for the experimental mice (infected/untreated) day two was 3.4 $\pm$ 0.2. Standard deviation for the experimental mice (infected/treated) day one was 3.2 $\pm$ 0.16, standard deviation for the experimental mice (infected/treated) day two was 0.7 $\pm$ 0.2.

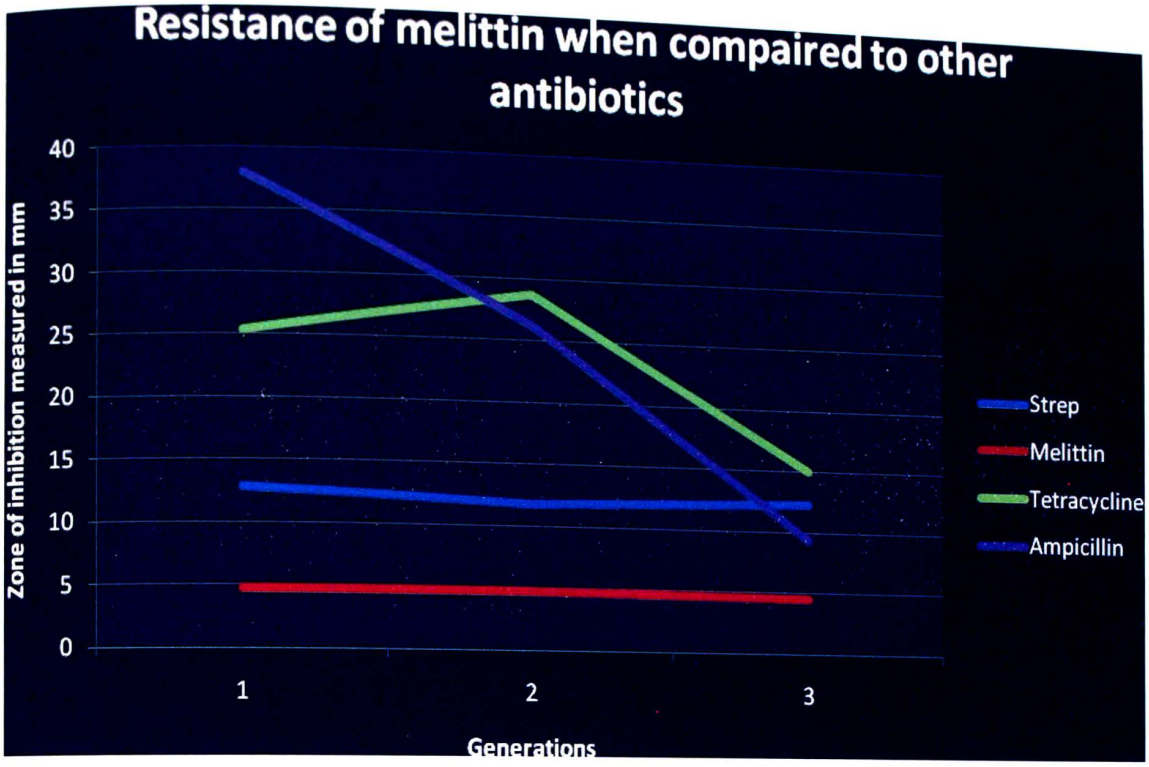

Figure 17. *S. aureus* was shown to develop a resistance to ampicillin and tetracycline was shown to develop after only 2 generations. *S. aureus* developed a resistance to Streptomycin after 4 generation. *S. aureus* was unable to build up a resistance to melittin over several generations.

Discussion

The goal of this study was to determine if melittin was effective at clearing *S. aureus* infection both *in vitro* and *in vivo*. This results support the hypothesis in that melittin was able to clear *S. aureus* infections *in vitro* and *in vivo*. Melittin cleared 90% of *S. aureus* bacteria *in vitro* in just 15 minutes. This indicates that melittin is a possibility for use as an alternative to current antibiotic treatments. An important finding relating to melittin use as an alternative drug therapy was finding that S. aureus was unable to build up a resistance to melittin. Although these findings support that melittin can be used to treat *S. aureus* infections and potentially MRSA, there are certain conditions that have to be followed to ensure the effectiveness of melittin.

To examine the possibility that melittin had a killing effects on *S. aureus* after being plated, melittin was centrifuged out of solution prior to plating. When comparing the two experimental groups (one plated with melittin and one without) to the control the results showed that melittin did not have a noticeable killing effect on *S. aureus* after being plated with or without melittin. This indicated that *S. aureus* was being killed in solution.

Shelf-life was an important aspect of antimicrobial treatments. An experiment was designed to show how long melittin can be left at room temperature before starting to lose the ability to kill *S. aureus* in solution. This experiment indicated that melittin had a relatively short shelf-life. When left at room temperature for an extended period of time melittin the effectiveness it had

on clearing *S. aureus* dropped. The experiment showed that when left out melittin only retained its ability to kill *S. aureus* for two days before the colony count stared to rise. This does not indicate however that melittin cannot be used as a novel drug treatment; it simply showed that melittin has to be kept at a temperature between 4 and 20°C. An experiment was not completed to find the most effective temperature for melittin to work under, this simply was too show a positive or negative result.

To determine if melittin was able to be denatured it was exposed to different pH concentrations. If melittin is easily denatured when exposed to a non neutral pH, especially an acidic pH the condition in the stomach would not be favorable. This test was to indicate if melittin might only be able to be used as a venous or topical therapy. It would be expected at melittin would work more effectively at a pH 7. The statistical evidence so no difference between pH 4, pH 7, and pH 10. The graphical results however showed to be inconsistent between the control and experimental groups in pH 4 and pH 10. At pH 4 and pH 10 the colony counts between the control and experimental groups throughout the dilution series were unpredictable. This may indicated that the 15 minute incubation period may not have been enough to completely denature all of the melittin. Melittin was shown to work the best under the condition of pH 7. While this study focused on topical application of melittin, this experiment was designed to the optimal pH for melittin activity.

Volume was varied to show the effects that constant amount of melittin had on a constant amount of *S. aureus*. This experiment indicated that the

effective volume, (that would produce predictable results) was 200 µL. This experiment also produced very interesting results in the remaining volumes. In the 50 µL and 100 µL experiment the results were inconsistent throughout the dilutions. This may indicate that the volume in these tubes were too low, increasing the possibility of melittin and *S. aureus* interacting. The results could also indicate the melittin will congregate on the first *S. aureus* bacteria that it comes in contact with instead of spreading out in solution when the volume is low. The 500 µL volume indicated that, that specific volume might have been too high. The larger volume decreased the incidence rate of melittin and *S. aureus* encountering. Increasing the incubation time could have given the melittin efficient time to encounter the *S. aureus* bacteria been.

Production of a melittin gel to use topically on mouse model C3H/HeJ to indicated melittin was able to clear *S. aureus* infection *in vivo*. Three groups were used in this experiment to determine melittin's killing ability. Control 1 (uninfected&untreated), this group was to show if the puncture itself in the ear caused any lasting inflammatory response. Control 2 (infected&untreated), indicated the inflammation causes by exposure to *S. aureus* bacteria and when left untreated would persist in infection. Experimental group (infected&treated) was used to show the ability of melittin to clear *S. aureus* infection on the ear of the mice. The control 2 and experimental groups were compared to show that melittin had a significant killing ability on *S. aureus* in vivo.

Considering the mounting problem of microbial resistances to various antibiotics, it was important to examine the susceptibility of *S. aureus* to melittin

over generations of mutational time. Multiple generations of *S. aureus* were observed and showed no resistance to melittin which might best be explained by the mode of action that melittin employs. Since melittin does not inhibit cell formation or protein synthesis like some antibiotics but rather, forms a hole directly into the cell membrane, *S. aureus* was unable to build up a resistance to this mechanism, probably because it simply lacks the basic genetic tools begin a resistance. Since melittin was able to keep its killing ability over several generations it makes melittin a possible treatment for MRSA infections.

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Vita

Christina Louise Russell graduated from Parkview High School in Lilburn Georgia in 2000. She then began work on her undergraduate degree at Georgia Perimeter College while working full time. She obtained her Associates degree in Pre Medicine in 2005. She then transferred to Austin Peay State University in Clarksville Tennessee to finish her bachelor's degree in biology with a minor in chemistry. During her studies she volunteered at local hospitals in the emergency and surgical departments. Also while finishing her undergraduate degree, she attended EMT-IV school and received her certification in May 2008. After which she took many opportunities to volunteer and work as an EMT. During this time Christina also began work on her Master's in biology. She was awarded a competitive academic scholarship by Austin Peay to complete her Master degree working in the anatomy and physiology department. After graduating with a Master in biology Christina hopes to go on and further her medical career, eventually attending medical school and earning her M.D.