THE ROLE OF ANTIMICROBIAL PEPTIDES IN THE INNATE IMMUNITY OF INSECTS AND THEIR MODE OF ACTION

by

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A Thesis
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of
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Bachelor of Science
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Spring Semester 2016
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DEDICATION

This is dedicated to my late grandmother Mrs. Ranjana Verma.

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I would like to thank my parents for their love and support. My sister Garima and my mother Nandini always motivated me to pursue my dreams. My grandfather has taught me the value of patience, something that has come in very handy during my difficult times. Most importantly I would like to thank Dr. van Hoek, my P.I, for showing incredible faith and trust in me and also for guiding me throughout my graduate career. I would also like to thank Dr. Kajal Gupta and Stephanie Barksdale for investing their time in teaching me valuable protocols and helping me write my manuscripts. Lastly I would like to thank my lab mates and friends who have always encouraged me and always cheered me up.

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3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium ..... MTT
3, 3'-dipropylthiadicarbocyanine iodide ..... $DiSC_3$ (5)
American Type Culture Collection ..... ATCC
Antimicrobial Peptide Database 2 ..... APD2
Cationic Antimicrobial Peptide ..... CAMP
Circular Dichroism ..... CD
Colony Forming Units ..... CFU
Confidence interval CI
Ethidium Bromide ..... EtBr
Half Maximum Concentration ..... EC_{50}
Lipopolysaccharide ..... LPS
Messenger Ribonucleic Acid ..... mRNA
Minimum Inhibitory Concentration ..... MIC
Molecular Weight ..... MW
Naja atra cathelicidin ..... NA-CATH
Optical Density ..... OD
Outer Membrane ..... OM
Phosphate Buffered Saline ..... PBS
Reverse Phase High-Performance Liquid Chromatography ..... RP-HPLC
Sheep Myeloid Antimicrobial Protein-29 ..... SMAP-29
Sodium Chloride ..... NaCl
Sodium Dodecyl Sulfate ..... SDS

ABSTRACT

THE ROLE OF ANTIMICROBIAL PEPTIDES IN THE INNATE IMMUNITY OF INSECTS AND THEIR MODE OF ACTION

Akanksha Kaushal, M.S.

George Mason University, 2016

Thesis Director: Dr. Monique L. van Hoek

Antimicrobial peptides (AMPs) are components of both vertebrate and invertebrate innate immune systems that are expressed in response to exposure to bacterial antigens. Naturally occurring AMPs from evolutionarily ancient species have been extensively studied and are being developed as potential therapeutics against antibiotic resistant microorganisms. In this thesis, a putative *Cimex lectularius* (bedbug, CL) defensin is characterized for its effectiveness against human skin flora including Gram-negative and Gram-positive bacteria. The bedbug defensin (CL-defensin), belonging to family of insect defensins, is predicted to have a characteristic N-terminal loop, an α-helix, and an antiparallel β-sheet, which was supported by circular dichroism spectroscopy. The defensin was shown to be antimicrobial against Gram-positive bacteria commonly found on human skin (*Micrococcus luteus, Corynebacterium renale, Staphylococcus aureus and Staphylococcus epidermidis*); however, it was ineffective against common skin

Gram-negative bacteria (*Pseudomonas aeruginosa* and *Acinetobactor baumannii*) under low-salt conditions. CL-defensin was also effective against *Micrococcus luteus* and *Corynebacterium renale* in high-salt (MIC) conditions. Our studies indicate that CL-defensin functions by depolarization and pore-formation in the bacterial cytoplasmic membrane. In addition to bedbugs, this thesis also focuses on characterizing antimicrobial activity of mosquito *Aedes albopitctus'* antimicrobial peptides against *Francisella. Francisella tularensis* is the cause of the zoonotic disease tularemia. In Sweden and Scandinavia, epidemiological studies have implicated mosquitoes as a vector. Prior research has demonstrated the presence of *Francisella* DNA in infected mosquitoes but has not shown transmission of tularemia from a mosquito to a mammalian host. We hypothesized that antimicrobial peptides may play a role in mosquito host-defense to *Francisella*. We established that *Francisella sp.* are susceptible to two Cecropin antimicrobial peptides derived from the mosquito *Aedes albopictus*. We also demonstrated induced gene expression of these peptides by *Francisella* infection C6/36 mosquito cell line. We demonstrated that mosquito antimicrobial peptides are active against *Francisella* by disrupting the cellular membrane of the bacteria. Thus, antimicrobial peptides may play a role in the inability of mosquitoes to establish an effective natural transmission of tularemia.

CHAPTER 1: INTRODUCTION

Innate Immunity in insects

Insects are one of largest and oldest classes belonging to phylum *Arthropoda*. Their evolutionary success is a testament to an effective immune response to a range of pathogens. Invertebrates do not possess an adaptive immune system and must depend entirely on innate immune defenses to protect themselves from pathogens. The innate immune system of insects involves recognition and activation of pattern recognition receptors (PRRs) that bind the conserved domains on pathogen- associated molecular patterns (PAMPs). This interaction results in activation of several signaling pathways that eventually produces molecules such as antimicrobial peptides (AMPs) that eliminates the pathogen (Tsakas & Marmaras, 2010). Insects are model organisms to study innate immunity defenses because their innate immune system is very similar to that of vertebrates. Insect immune systems have both humoral and cellular components. The humoral component is the one that produces AMPs (Tsakas & Marmaras, 2010). In insects, major sites of secretion of antimicrobial peptides are fat bodies, hemocytes and epithelial layers of gut and after production of AMPs they are released in the hemolymph (Govind, 2008; Tsakas & Marmaras, 2010).

Signaling pathways in insects

The organism *Drosophila melanogaster* is a powerful model organism to study immunity in insects and has helped us to understand the signaling pathways that are

involved in stimulating the innate immunity. *Drosophila* has two prime immune signaling pathways , Toll pathway and Imd pathway, that results in activation of NF-κB transcription factors (Myllymaki, Valanne, & Ramet, 2014; Silverman & Maniatis, 2001). Insects have as many as 13 genes for peptidoglycan recognition protein (PGRP) that recognizes and bind to peptidoglycan (PGN). Binding of PGN to receptors PGRP-LC, PGRP-LE, PGRP-LA activates the Imd signaling pathway whereas binding of PGN to receptors PGRP-SD and PGRP-SA activates the Toll signaling pathway (Myllymaki et al., 2014).

In the Imd signaling pathway, binding of PGN to these receptors results in recruitment of a signaling complex Imd, the adaptor protein dFadd, and apical initiator caspase Dredd which activated (polyubiquinated) cleaves amino terminal part of Imd (Meinander et al., 2012). DIAP2 then comes and binds to the cleaved Imd thereby ubiquinating (K63 linked) (Meinander et al., 2012) it and in-process activating Tab2/Tak1 complex which phosphorylates and activates IKK (Relish kinase complex) complex which in turn activates Relish proteins at N-terminus. The now activated "N-terminus" of Relish then translocates to the nucleus and activates the transcription of AMPs such as cecropin and diptericin (Myllymaki et al., 2014).

The toll pathway is another pathway that is activated in response to bacterial and fungal infections. As many as nine genes encoding for Toll-related receptors have been discovered in *Drosophila* (Valanne, Wang, & Rämet, 2011). The first step of the activation of Toll signaling pathway is cleavage of Toll receptor ligand Spatzle (Spz) by series of serine proteases Gastrulation defective (GD), Snake (SNK) and Easter (E). The

cleaved and processed SPZ dimer binds to the Toll receptor and activates it. The adaptor proteins MYD88, Tube (TUB) and Serine/threonine kinase Pelle (PLL) then interact with Toll receptor to make a signaling complex. PLL is autophosphorylated and recruits DL or DIF/CACT dimer to the signaling complex. CACT is complexed to either Dorsal (DL) or Dorsal-related immunity factor DIF. CACT and the DL/DIF dimer is phosphorylated and the phosphorylated CACT is degraded. Free phosphorylated DL/DIF dimer translocates to the nucleus to activate transcription of AMP genes (Myllymäki, Valanne, & Rämet, 2014)

Other pathways such as JAK/STAT pathways are essential for opsonization and phagocytosis of Gram-negative bacteria and thus play a very important role in innate immunity of insects.

Antimicrobial peptides in insects

AMPs are one of the most significant components of innate immunity, our first line of defense, and are expressed in response to bacterial, viral or fungal infections. Insects produce a very wide repertoire of antimicrobial peptides some of which such as defensins, cecropins and attacins are predominantly found in most of insect orders whereas others such as prolixicin (Ursic-Bedoya, Buchhop, Joy, Durvasula, & Lowenberger, 2011) and royalisin (Bílikova, Huang, Lin, Šimuth, & Peng, 2015) are specific to certain insect orders. Antimicrobial peptides have gained significant attention recently as a potential alternative or adjunctive therapy for antibiotics (Dean, Bishop, & van Hoek, 2011a). Previously conducted studies have identified antimicrobial peptides in humans (De Smet & Contreras, 2005), plants (Nawrot et al., 2014) and insects (Ravi,

Jeyashree, & Devi, 2011). Our group has been studying antimicrobial peptides from various sources including snakes, humans and alligators (Amer, Bishop, & van Hoek, 2010; Bishop et al., 2015; de Latour, Amer, Papanstasiou, Bishop, & van Hoek, 2010). Invertebrate insect peptides have been previously demonstrated to exhibit activity against Gram-positive (Ceřovský & Bém, 2014) and Gram-negative bacteria (Moore, Beazley, Bibby, & Devine, 1996). These peptides are typically small cationic proteins that are less than 5 kDa (Philippe Bulet & Stöcklin, 2005; Ganz, Selsted, & Lehrer, 2009) and target the cytoplasmic membranes of pathogenic microorganisms by recognizing acidic phospholipids on the surface of bacterial membrane (Kavanagh & Reeves, n.d.)

Insect Defensins

Insect defensins are usually 29-43 residues long peptides that are rich in cysteines (Philippe Bulet & Stöcklin, 2005; White, Wimley, & Selsted, 1995). They were first discovered in flesh fly *Sarcophaga peregrina* (Matsuyama & Natori, 1988) and from larvae of the black blowfly *Phormia* terranovae (Lambert et al., 1989). Antibacterial insect defensins such as Lucifensin have been widely documented and studied owing to their medical importance (Ceřovský & Bém, 2014). Antibacterial defensins are particularly active against Gram-positive bacteria; however, these peptides kill bacteria only in buffer of low of ionic strength. Gram-negative bacteria, yeast and filamentous fungi are not very susceptible to the antibacterial defensins (Philippe Bulet & Stöcklin, 2005). Most insect defensins are have not yet demonstrated any significant hemolytic activity. Antibacterial defensins have shown disruption of the permeability of the

cytoplasmic membrane as shown in *Micrococcus luteus* (Cociancich, Ghazi, Hetru, Hoffmann, & Letellier, 1993).

Much like the mammalian defensins, these peptides have six cysteine residues and three disulfide bonds that stabilize the overall structure, a carboxy-terminal β-sheet that runs anti-parallel and an amino-terminal loop as shown in **Figure 1**. Two of the three disulfide bridges are between the α- helix to one strand of the β-sheet and the third one is between N-terminal loop and second strand of β-sheet and stabilizes the interaction between N-terminal loop and β-sheet (Cornet et al., 1995; Maget-Dana, Bonmatin, Hetru, Ptak, & Maurizot, 1995). However, unlike mammalian defensin, insect defensins usually have an amphipathic α-helix domain (White et al., 1995). These defensins are found in fat bodies, hemolymph and thrombocytoids in the insects (White et al., 1995).

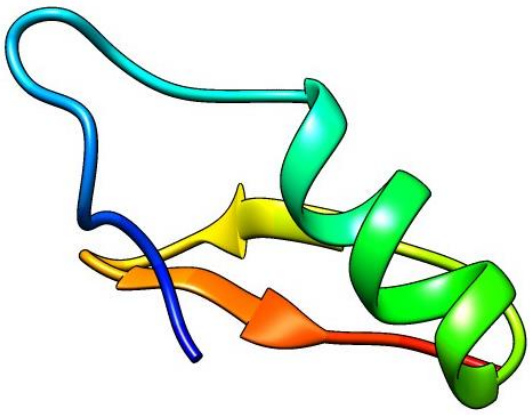


Figure 1: Three dimensional structure of Defensin D from *Aedes albopictus*

Insect Cecropins

Cecropins are 35-39 residue antimicrobial peptides that were first discovered in pupae of the cecropia moth (Hultmark, Steiner, Rasmuson, & Boman, 1980). They are active against both Gram-negative and Gram-positive bacteria (P Bulet, 1999; Rahnamaeian et al., 2015). Cecropin-melittin hybrid peptides have demonstrated excellent antimicrobial activity against *Staphylococcus aureus* (Boman, Wade, Boman, Wåhlin, & Merrifield, 1989). Cecropins are characterized by two amphipathic helices, as seen in **Figure 2** regions from residues 5-21 and a hydrophobic C-terminal region (Moore et al., 1996). The mechanism of action of cecropins is thought to be pore formation in the lipid membrane of bacteria and consequent destruction of the lipid bilayer, killing the bacteria (Brogden, 2005; Ferre et al., 2009). Gram-negative bacteria have an outer membrane that appears to make them more sensitive to cecropins (Moore et al., 1996) than Gram-positive bacteria, although both classes of bacteria have reported to have sensitivity to different cecropins.

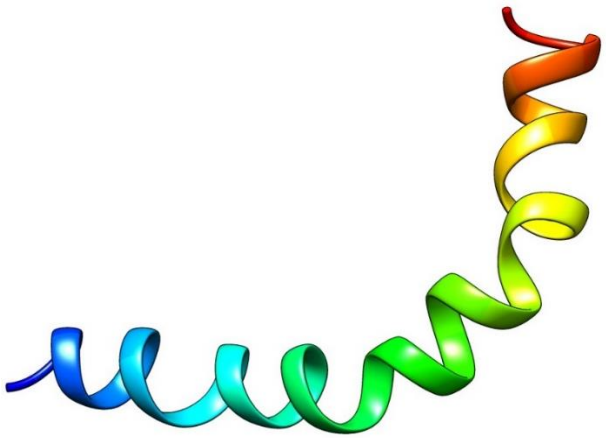


Figure 2: Three dimensional structure of Cecropin A1 from *Aedes albopictus*

Hypothesis and Aims

In this thesis we hypothesize that insect AMPs will be antimicrobial against bacterial pathogens and that these AMPs will function by disrupting the cytoplasmic membrane of the pathogen, either by creating small transient ion channels or multimeric pores. We also hypothesize that gene expression for *Aedes albopictus* AMPs will be also upregulated in response to bacterial infection. Finally we hypothesize that treatment of infected waxworms with mosquito AMPs will prolong the survival of waxworms as opposed to the untreated waxworms.

To predict the secondary structure of insect antimicrobial peptides the amino acid sequence of the putative peptides will be subjected to Swiss-model protein homology and subsequently to molecular modeling visualizing software Chimera. The peptide samples will be subjected to CD spectroscopy to determine secondary structure of the peptides. The selected bacterial strains will be treated with selected peptides serially diluted to determine the lowest concentration at which lytic activity against bacteria is observed under both high (MIC conditions) and low ionic (10mM phosphate buffer) concentrations. To determine the mode of action of the peptides, the bacteria will be treated with peptides and co-treated with ethidium bromide to determine the ability of peptide to form pores. The bacteria will also be treated with peptides and co-treated with $DiSC_3(5)$ to determine the depolarization induced in the membrane of bacteria by the peptides. For inducible peptides we will obtain the mRNA of the C6/36 cell line infected with *F. novicida* to measure the gene expression of the induced peptides. We will test if mosquito peptides can prolong the survival of infected waxworms. Finally we will assess cytotoxicity of peptides using tetrazolium dye MTT (3-(4, 5- Dimethylthiazol-2-yl)-2, 5-

diphenyltetrazoliumbromide). These studies will establish if these peptides can potentially be developed for therapeutics.

CHAPTER 2: CHARACTERIZATION OF *CIMEX LECTULARIUS* (BEDBUG) DEFENSIN PEPTIDE AND ITS ANTIMICROBIAL ACTIVITY

Introduction

Bedbug infestations are a global nuisance and after being nearly eliminated in 1950's through the use of carbamates and organophosphates, they have made a comeback following the ban on these insecticides and emerging resistance to the pesticides (Barbarin, Barbu, Gebhardtsbauer, & Rajotte, 2014). The CDC considers bedbugs a significant public health risk (Doggett, Dwyer, Peñas, & Russell, 2012). Bedbugs are obligate feeders on human blood and due to this close interaction, they carry some of the bacteria found on human skin. In previous work, the identified bacteria on bedbugs were found to be mostly Gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus*, the same bacteria commonly found on human skin (Cockburn et al., 2013; Reinhardt, Naylor, & Siva-Jothy, 2005). Gram-negative organisms were rarely found on bedbugs, but included *Walachia*, *Klebsiella* and *Stenotrophomonas maltophilia* (Cockburn et al., 2013; Reinhardt et al., 2005). The reasons for this disproportionate absence of Gram-negative bacteria are not known. In this study, we assessed the effectiveness of a bedbug defensin belonging to the Defensin-4 family of insect defensins against skin flora ("Life science projects in Japan," n.d.; Moriyama et al., 2012). Commonly found on human skin, these bacterial species (harmless otherwise) can cause infections in immunocompromised individuals. To

predict the secondary structure of CL-defensin we performed CD and bioinformatics analysis. To determine the mode of action of CL-defensin peptide, ethidium bromide (EtBr) uptake assay and cytoplasmic membrane depolarization assay were performed.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains tested in this study are listed in **Table 1** and were obtained from American Type Culture Collection (Manassas, VA). All microorganisms were cultured in nutrient broth (Difco Laboratories, Detroit, MI, USA) at 37^{o}C in shaking incubator at 200 rpm for 24 hours and were stored in -80^{o}C in 20% glycerol. The bacteria were plated on Mueller Hinton Agar for enumeration.

Table 1: Bacterial strains, MIC and EC50 values of CL-defensin

Bacteria	**ATCC strain no.**	**MIC µg/ml CL-Def**	**EC_{50} µg/ml CL-Def**	**95% CI µg/ml CL-Def**	**EC_{50} µM CL-Def**	**MIC µg/ml NA-CATH**	**EC_{50} µg/ml NA-CATH**	**95% CI µg/ml NA-CATH**	**EC_{50} µM NA-CATH**
C. renale	19412	41.66[a]	2.26	(1.51-3.56)	0.48	10.4[a]	9.57	(5.84 - 15.7)	2.29
M. luteus	4698	2.60[a]	7.21	(6.26-8.23)	1.53	10.4[a]	2.51	(2.23 - 2.83)	0.60
S. aureus	25293	-	2.17	(1.72-2.73)	0.46	83.33[a]	0.29	(0.27 - 0.32)	0.069
S. epidermidis	14990	41.66[b]	2.45	(1.87-3.29)	0.52	10.4[a]	0.52	(0.44 - 0.61)	0.12
A. baumannii	9955	-	>100	-	>21.31	10.4[a]	1.81	(1.41-2.33)	0.43
P. aeruginosa	9027	-	>100	-	>21.31	10.4[a]	0.63	(0.46 - 0.86)	0.15

a: Represents bactericidal concentration
b: Represents bacteriostatic concentration

Peptide and Bioinformatics analysis

The sequence of the CL-defensin was obtained from Life Science database archive ("Life science projects in Japan,"; http://togodb.biosciencedbc.jp/togodb/show/clest_cluster/Cl_contig0261#ja) and manufactured by China Peptides (Shanghai, China). The peptide was provided at 85-95% purity. The MW of the peptide was confirmed by using ESI-mass spectrometry to be 4692.45 g/mol and the purity was confirmed through RP-HPLC. Physiochemical and structural properties of CL-defensin were calculated using the Antimicrobial Peptide Database (APD2) (G. Wang, Li, & Wang, 2009; Z. Wang, 2004) and are listed in **Table 2**. Swiss model (http://swissmodel.expasy.org/interactive) (Guex & Peitsch, 1997) was used for protein modeling and for generating a Protein Data Bank (pdb) file encoding the predicted structure of the protein. A model of the peptide was generated using Chimera software, an extensible molecular modeling system (http://www.cgl.ucsf.edu/chimera/) (Pettersen et al., 2004).

Table 2: Sequence and Characteristics of CL-defensin from *Cimex lectularius*

Sequence	Molecular weight (g/mol)	Net charge	Hydrophobicity
ATCDLFSFQSKWVTPNHAACAAHCTA RGNRGGRCKKAVCHCRK	4692.45	+7	44%

Minimum Inhibitory Concentration

The MIC for CL-defensin against the microorganisms was determined in triplicate in cation-adjusted Mueller-Hinton broth (CA-MHC). Conditions were in accordance with

the current recommendation of the CLSI broth microdilution protocol ("Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that Grow Aerobically; Approved Standard- Ninth edition," n.d.). Briefly, a range of concentrations of the peptides was prepared by serial dilution and added to an equal volume of exponentially grown bacterial culture (50 µL) diluted to 1 x 10^5 CFU. Microtiter plates were incubated for 18-24 h (48 hrs for *M. luteus*) at 37°C. The MIC was defined as the concentration at which no microbial growth was observed spectrophotometrically. Wells containing only growth media was used as the positive control and growth media containing microbial cells without peptide was used as negative control.

Antimicrobial EC_{50} assay

The antimicrobial EC_{50} activity of CL-defensin against skin flora was determined as described previously in low-salt buffer (Amer et al., 2010). In a 96 well plate, 1 x 10^5 CFU of bacteria were incubated with various concentrations of CL-defensin (serially diluted 1:5) in 10 mM sodium phosphate buffer (pH 7.3). After a 3 hour incubation each well was further diluted 10^{-1}, 10^{-2} and 10^{-3} fold in 10 mM sodium phosphate buffer and each dilution was plated in triplicate on Mueller Hinton agar and incubated for 24 hours (48 hours for *M. luteus*). NA-CATH (4175.22 g/mol), a snake cathelicidin, was used as a positive control in the EC_{50} assays (Blower, Barksdale, & van Hoek, 2015; Dean et al., 2011a). The concentration of peptide required to kill 50% of bacterial population (EC_{50}) was determined by graphing percentage of surviving colonies, after the 24 - 48 hour incubation, as a function of log of peptide concentration and analyzing data using GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA) using the equation 1,

where Y corresponds to percentage of surviving bacteria at the given peptide concentration and X corresponds to the concentration of peptide in logarithmic function. The terms “Top” corresponds to upper boundary constrained to <100% and “Bottom” corresponds to lower boundary constrained to >0%. The “no peptide” treatment is graphed at 10^{-2} µg/ml

Equation 1: Percentage of surviving bacteria at the given CL-defensin concentration

$$Y = Bottom + (Top-Bottom)/ (1+10^{[(logEC50-X) \times Hill\ slope)]})$$

CD spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco J-1500 spectropolarimeter. 100 µg/ml of peptide was used in each experiment. Samples were allowed to equilibrate for 3 min prior to data collection at 25 °C in a 1 mm path length cuvette. Spectra were collected from 190 to 240 nm at 0.2-nm intervals, with a data integration time of 4 s and a 1 nm bandwidth. Data presented is an average of four spectra. Peptides were analyzed in 10 mM sodium phosphate and 60 mM sodium dodecyl sulfate (SDS) in phosphate buffer.

Bacterial cytoplasmic depolarization assay

Membrane depolarization assay was studied using $DiSC_3(5)$ as previously reported (Rodriguez, Papanastasiou, Juba, & Bishop, 2014; Wu & Hancock, 1999). Enumerated frozen bacteria were pelleted and washed twice in phosphate buffer (pH 7.2) and then resuspended to 4 X 10^7 CFU/mL in phosphate buffer containing 50 µg/mL

$DiSC_3(5)$. 100 µL of this suspension was added to wells of a black 96 well plate. The plate was incubated in a TECAN infinite F200 spectrofluorometer and monitored until fluorescence leveled off. Various concentrations of peptide in phosphate buffer (pH 7.2) were added to each well. Bacteria without peptide was used as a negative control and Triton X-100 was used as positive control. The plate was immediately returned to the spectrofluorometer. Readings were taken every 2 min for 10 min (excitation = 622 nm; emission = 670 nm).

Bacterial cytoplasmic membrane permeation

Bacterial cytoplasmic membrane permeation was monitored by using the ethidium bromide uptake assay. The ethidium bromide uptake assay was performed as previously detailed with some modifications (Li et al., 2012). Respective bacteria were grown until log phase in nutrient broth in a shaking incubator (37^{o}C). Bacteria were centrifuged (4^{o}C at 6500g), washed with PBS, and then adjusted to an OD 600 nm of ~ 0.1 in 10 mM Phosphate Buffer. 180 µL of bacteria was added to 10µL ethidium bromide (10 µM final concentration) and 50 µg/mL peptide in various concentrations. The plate was read in a TECAN infinite F200 spectroflurometer every 2 min for 30 min at 37^{o}C: (excitation = 540 nm, emission = 590 nm). ΔRFU was calculated by the equation 2.

Equation 2: Measurement of EtBr uptake following treatment with CL-defensin

RFU (desired concentration)-RFU (no peptide control).

Statistical Analysis

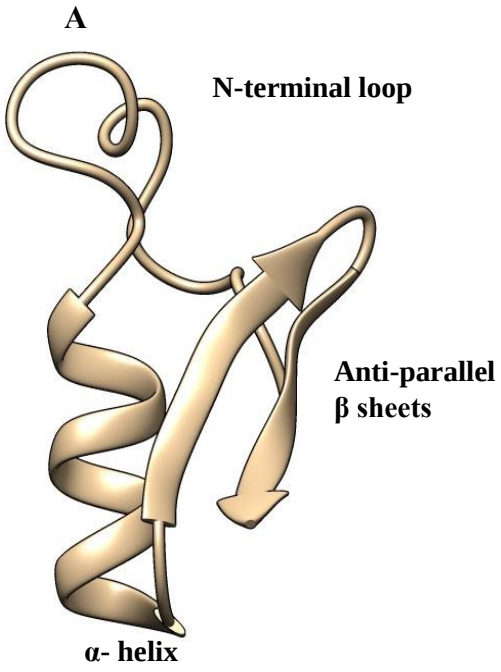
EC_{50}, $DiSC_3(5)$, MIC and EtBr uptake assays were performed in triplicates (n= 3). Standard deviations of the mean of each set are represented by error bars on each graph. Where the error bars cannot be seen, the error is very small. P-values for the depolarization assay are calculated using the Two-way ANOVA test. Confidence Interval (CI) (95%) for each EC_{50} determination is presented in **Table 1**, and represents significance of $p<0.05$.

Results

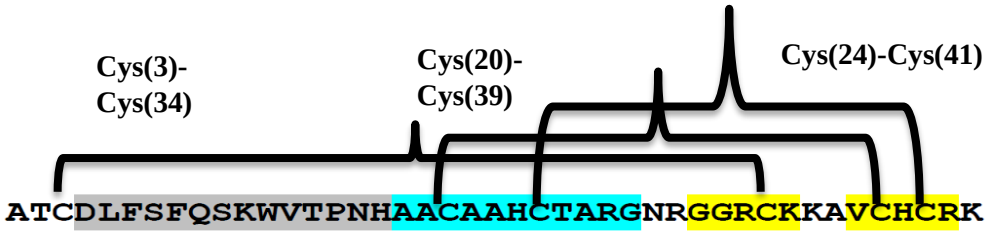
Three dimensional structure of CL-defensin

The amino acid sequence of CL-defensin was subjected to Swiss-model for protein homology modeling (Guex & Peitsch, 1997). Homologues for the CL-defensin sequence were found and sapecin was selected as a template for protein homology modeling since it shared 71% sequence homology with CL-defensin (PDB 1l4v.1.A). The pdb file was then converted to Chimera format (http://www.cgl.ucsf.edu/chimera/) to visualize the structure of CL-defensin. As seen in **Figure 3A,** CL-defensin is predicted to contain an N terminal loop, an α- helix and antiparallel β-sheet. The two antiparallel strands are connected by a turn consisting of two amino acids. The structure of CL-defensin is conventional with six cysteine residues at positions 3, 20, 24, 34, 39 and 41 and three predicted disulfide bridges (24-41, 20-39, 3-34) (**Figure 3B).** These results are in accordance with other studies conducted in defining the structure of insect defensin (Cornet et al., 1995). The secondary structure of antimicrobial peptide CL-defensin was further analyzed using circular dichroism (CD) spectroscopy, with a membrane-mimicking environment which was prepared by adding 60 mM of SDS in 10 mM of

sodium phosphate buffer (**Figure 3C**). The CL-defensin spectrum showed signals representing a peptide containing β-hairpin secondary structure and 14% percentage of α-helical contribution evidenced by an intense negative peak at ~ 205 nm and slight negative peak at 222nm (**Figure 3C**). The CD spectra confirmed the structure of CL-defensin predicted from 3-dimensional model of CL-defensin depicted in **Figure 3A**.



B



Gray= N terminal loop, Blue= Alpha – Helix, Yellow= Anti-parallel beta sheets

C

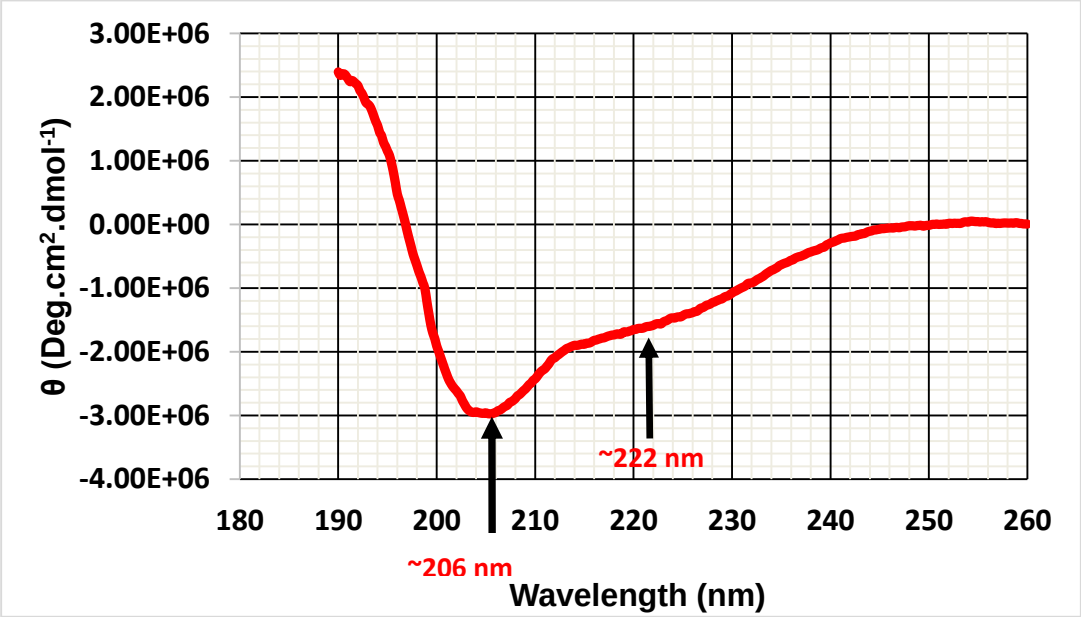
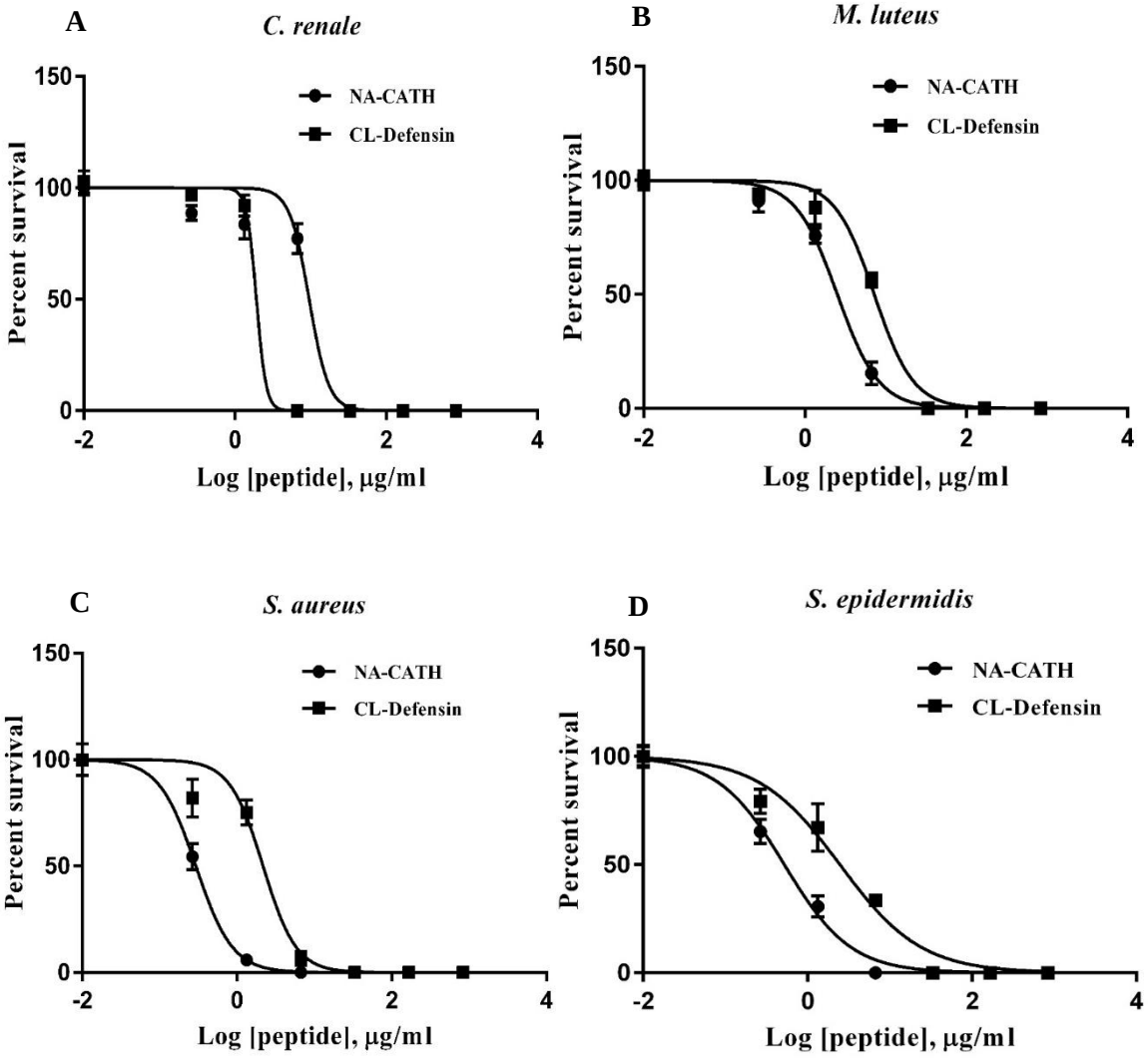


Figure 3: Structure of CL-defensin. A) Molecular visualization of CL-defensin B) intramolecular disulfide bridges in CL-defensin C) CD of CL-defensin. A) This figure represents a three dimensional view of the proposed structure of defensin as modeled by Chimera. In the figure the N-terminal loop, α-helix and antiparallel β-sheets are seen. B) The predicted N- terminal loop is highlighted in gray, α-helix in blue and antiparallel β- pleated sheets in yellow. The disulfide bonding between the six cysteine residues is also shown C) Circular dichroism of CL-defensin. The Circular Dichroism spectra (190 – 240 nm) of CL-defensin measured at a peptide concentration of 100 µg/mL dissolved in 60 mM of SDS in 10 mM of sodium phosphate buffer

Antimicrobial activity spectrum of CL-defensin

In this study, the antimicrobial effectiveness of CL-defensin was examined against bacteria commonly found on skin. The EC_{50} values of the peptide against these microorganisms are listed in **Table 1** along with the 95% confidence interval and their respective µM values. As seen in **Figure 4,** CL-defensin had significant antimicrobial activity against all Gram-positive bacteria tested **(Figure 4 A-D)** in low-salt buffer. CL-defensin had a bactericidal effect on both *C. renale* and *M. luteus* at concentrations of 41.66 µg/ml and 2.60 µg/ml respectively and a bacteriostatic effect on *S. epidermidis* at a concentration of 41.66 µg/ml in MIC (high-salt) conditions as shown in **Table 1**. However, the MIC values for other tested bacteria were above 300 µg/ml. These results suggest that CL-defensin has some antimicrobial activity at high salt concentrations such as those found in MIC conditions.


A
C. renale
B
M. luteus
C
S. aureus
D
S. epidermidis
NA-CATH
CL-Defensin
Percent survival
Log [peptide], µg/ml
150
100
50
0
-2
0
2
4

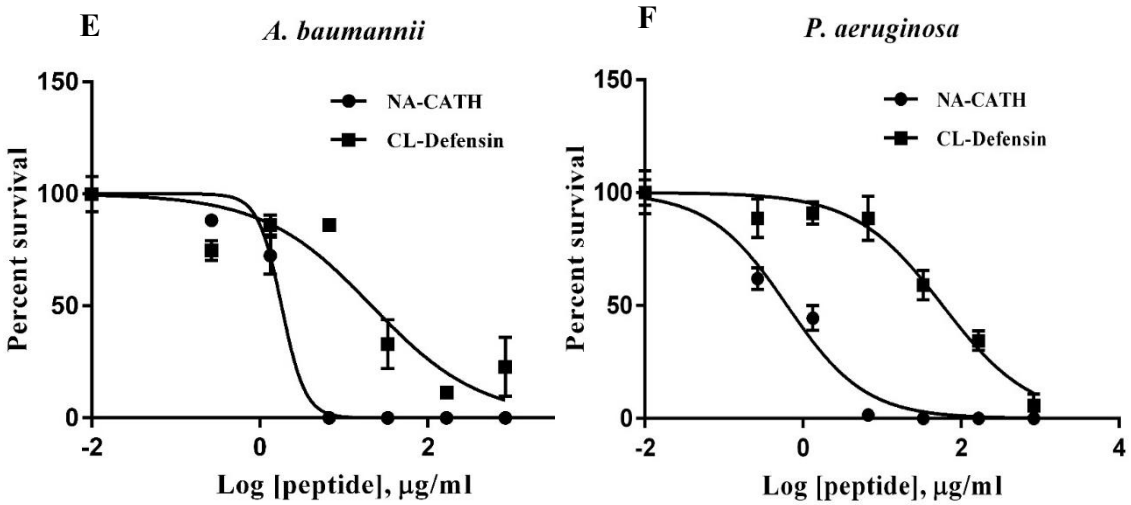
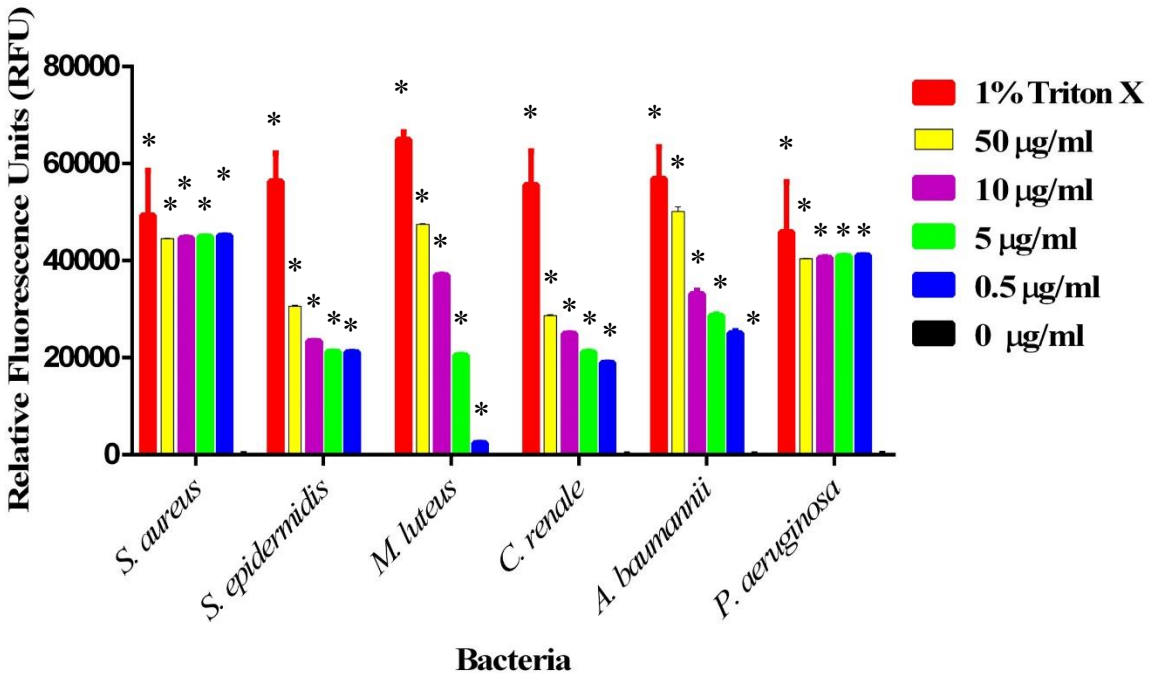


Figure 4: CL-defensin antimicrobial activity against the tested bacteria. Percent survival was calculated after an incubation period of 3 hours in 10 mM phosphate buffer and subsequent plating. (A) C. renale EC50 = 2.26 µg/ml (B) M. luteus EC50 = 7.21 µg/ml. (C) S. aureus EC50 = 2.17 µg/ml. (D) S. epidermidis EC50 = 2.45 µg/ml. (E) A. baumannii EC50>100 µg/ml. (F) P. aeruginosa EC50>100 µg/ml.

Proposed Mode of Action

To determine the mode of action of CL-defensin against selected microorganisms, we performed a cytoplasmic depolarization assay utilizing the $DiSC_3(5)$ dye, belonging to a family of membrane sensitive fluorescent dyes. Under normal conditions, the dye binds to the bacterial surface, and loses fluorescence. Depolarization releases the dye into the media, therefore increasing the fluorescent signal. Depolarization due to small ruptures in the membrane of cytoplasm leads to leakage of ions owing to proton flux (Epand, Pollard, Wright, Savage, & Epand, 2010) therefore disrupting the overall homeostasis of the bacteria. **Figure 5A** indicates that a significant increase in fluorescence was observed when all microorganisms were treated with various concentration of peptide. CL-defensin dissipates membrane potential in all microorganisms in as little as 0.5 µg/ml. To further investigate the mode of action of CL-defensin, we conducted an EtBr uptake assay to assess membrane permeabilization capability of CL-defensin. At high concentration of peptide, membrane permeabilization occurs that result in formation of large pores in the bacterial membrane. When we performed the ethidium bromide uptake assay we found that *C. renale* was most sensitive to membrane permeabilization by CL-defensin at 50 µg/ml as seen in **Figure 5B**. The peptide induces pore formation in other Gram-positive bacteria as well and also causes a small amount of membrane permeabilization in *A. baumannii*. However, *P. aeruginosa* was resistant to pore formation as evidenced by negative ΔRFU. Overall, we conclude that CL-defensin depolarizes membrane of both Gram-positive and Gram-negative bacteria. However, the peptide does not induce pore

formation in *P. aeruginosa* membrane and induces minimal pore formation in *A. baumannii* cytoplasmic membrane.



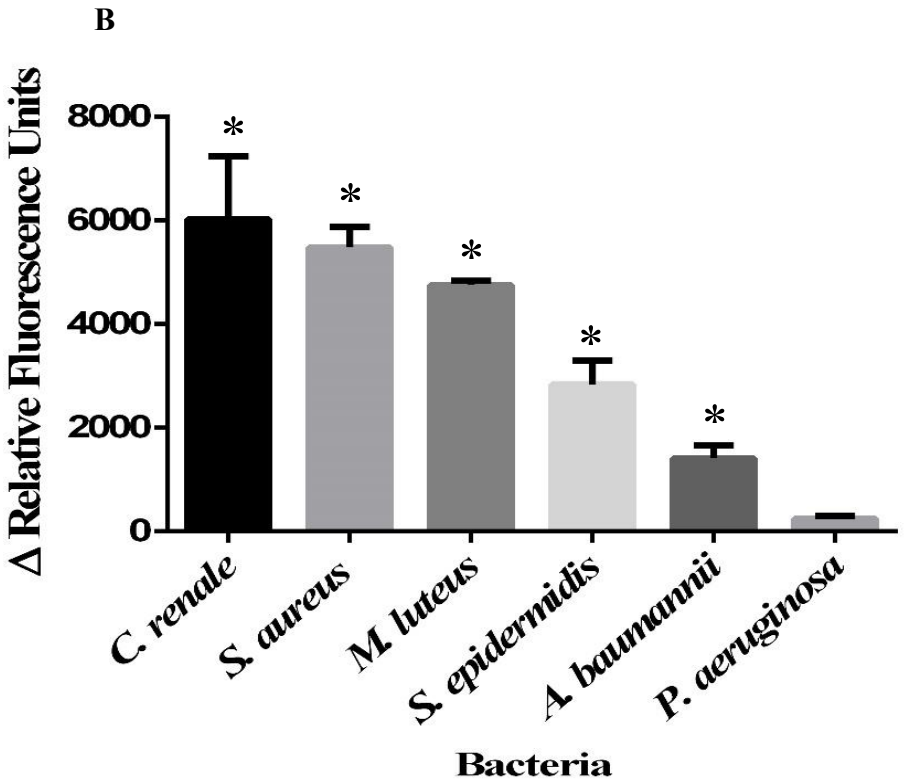


Figure 5: A) Depolarization and B) Membrane pore formation by CL-defensin of bacterial cytoplasmic membrane. A). All bacterial strains were incubated with a fluorescent dye DiSC3(5). Depolarization of membrane was observed at the peptide concentrations of 50 µg/ml, 5 µg/ml, 10 µg/µl and 0.5 µg/µl. Mean ± SD is shown. Error bars indicate the standard deviation from the mean. The asterisk (*) indicates p-value less than 0.0001. B) Kinetics of pore formation and binding of ethidium bromide to DNA with 50 µg/ml of peptide. Pore formation is indicated by increase in fluorescence that is a result of DNA binding by ethidium bromide and consequent formation of pores in the membrane of bacteria. Permeabilization of the membrane after 30 minutes was compared against no peptide control. Experiments were performed in triplicate (n=3). Mean ± SD is shown. The asterisk (*) indicates p-value less than 0.05

Discussion

In this study, we have identified and tested a defensin-type antimicrobial peptide from the bedbug, *C. lectularius*, called CL-defensin. The aim of the study was to discover the range of activity of this antimicrobial peptide against bacteria that it would normally encounter on its human host. We have demonstrated the antibacterial activity of this peptide under low-salt conditions against a panel of microorganisms that are commonly part of human skin flora. We have also demonstrated antibacterial activity of the peptide under MIC conditions against *M. luteus* (<1 μM) and against *C. renale* and *S. epidermidis* (under 10 μM). We found that CL-defensin has antimicrobial activity primarily against the panel of Gram-positive bacteria that are commonly found on human skin. The CL-defensin was ineffective against the two human pathogenic Gram-negative bacteria tested in this study, *P. aeruginosa* and *A. baumannii*. There may still be more antimicrobial peptides produced by bedbugs that would be effective against Gram-negative bacteria.

Antimicrobial insect defensins are more effective against microorganisms in conditions of low ionic strength. High-salt conditions usually negatively impact the activity of these peptides due to the initial binding of salt cations to microbial membranes thus decreasing the availability of favorable binding sites to the peptide (Lehrer, Lichtenstein, & Ganz, 1993; Walkenhorst, 2016). CL-defensin killed all the tested Gram-positive bacteria at 2 μM or less in low ionic conditions (10 mM phosphate buffer), yet was not lytic under high ionic conditions against *S. aureus* and Gram-negative microorganisms, *P. aeruginosa* and *A. baumannii.* In this study, however, we found that CL-defensin was bactericidal against *M. luteus* and *C. renale* and bacteriostatic against *S. epidermidis* even in high ionic (MIC) conditions. The *in vivo* physiological conditions

consists of high ionic strength (137 mM NaCl) (Lamberty et al., 1999); thus, antimicrobial activity of peptides against bacteria in such conditions is imperative for future therapeutic development. Other insect defensins that have shown a similar activity against *M. luteus* include defensin from a hemipteran insect *Pyrrhocoris apeterus* (MIC < 2 µM) (Cociancich et al., 1994) and termicin from *Pseudacanthotermes spiniger* (MIC 0.8-1.5 µM) (Lamberty et al., 2001).

It has been previously established that insect defensins function by disrupting the polarization of bacterial membrane and creating an ion imbalance across the membrane that eventually kills the targeted bacteria (Cociancich et al., 1993). In this study we have proved a substantial depolarization of both Gram-positive and Gram-negative bacteria in 10 minutes (**Figure 5A:** Data shown for 10 minutes). The relatively fast depletion of cytoplasmic membrane potential in the bacteria indicates that membrane polarization is most likely the primary mode-of-action of CL-defensin in Gram-positive bacteria. The level of depolarization at the concentration of 50 µg/ml was almost equivalent to that of Triton X-100. Another proposed mode for defensins is the formation of pores in the cytoplasmic membrane. These multimeric pores disrupt the intracellular stability of the cell by abnormal influx and efflux of cell contents eventually leading to cell death (Lehmann et al., 2002). We discovered that CL-defensin disrupts Gram-positive membranes by pore formation as depicted in **Figure 5B.** In low ionic condition, we observed some inhibition (~ 70-80%) of growth of Gram-negative bacteria *A. baumannii* and *P. aeruginosa* **(Figure 4E and 4F)** and the membrane depolarization and pore formation results obtained in this study are consistent with the results of EC_{50}

experiments. One of the major components of the Gram-negative bacteria is the presence of an outer membrane (OM). The function of this outer membrane is to restrict the entry of hydrophobic compounds (Epand et al., 2010). As seen from the sequence, CL-defensin is 44% hydrophobic and therefore the peptide may not traverse the OM sufficiently in order to access the cytoplasmic membrane leading to lysis. In future studies, additional compounds such Polymyxin B or EDTA could be utilized to increase the permeability of the outer membrane before treatment with peptide (Daugelavicius, Bakiene, & Bamford, 2000; Miki & Hardt, 2013). In conclusion, we have established that CL-defensin primarily kills Gram-positive bacteria and is not very effective against the Gram-negative bacteria tested in our study. We have also demonstrated that the bacterial cytoplasmic membrane is a target of CL-defensin. The discovered peptide can be considered as a template for new synthetic peptides that can function at high salt concentration.

CHAPTER 3: ANTIMICROBIAL ACTIVITY OF MOSQUITO CECROPIN PEPTIDES AGAINST *FRANCISELLA*

Introduction

Francisella tularensis, a Gram-negative coccobacillus, is a facultative intracellular pathogen, and is the causative agent of tularemia. Tularemia is a zoonotic disease that affects humans and wildlife. Two subspecies of *F. tularensis*, subsp. *tularensis* (Type A) and subsp. *holarctica* (Type B) are of significance since they cause human infections (Vonkavaara et al., 2013). The mode of transmission varies from handling of infected animals, vector borne transmission, airborne transmission or oral route (Fortier, Slayter, Ziemba, Meltzer, & Nacy, 1991; Petersen, Mead, & Schriefer). *Francisella novicida*, an environmental isolate, and *Francisella* Live Vaccine Strain (LVS), a live attenuated strain derived from the Holarctic strain of tularemia, are used as laboratory model strains to study infections of *F. tularensis* subsp. *tularensis*. *F. novicida* does not cause lethal tularemia in healthy humans but causes a tularemia-like disease in animals such as rabbits and mice (Ellis, Oyston, Green, & Titball, 2002). *F. tularensis* was designed as a biological weapon in the 20th century by United States and the former Soviet Union (Dennis et al., 2001) and is listed among Tier 1 agents declared by Department of Health and Human Services. Although *F. tularensis* is transmissible by aerosol, no human-human transmission has been demonstrated to date (Dennis et al., 2001).

Vector borne transmission is one of the most frequent types of transmission clinically observed in the northern hemisphere, with arthropods being a major vector in causing ulceroglandular and glandular manifestations of tularemia (Pérez-Castrillón, Bachiller-Luque, Martín-Luquero, Mena-Martín, & Herreros, 2001; Petersen et al.). The most common vector of *Francisella* in United States is the tick (Read, Vogl, Hueffer, Gallagher, & Happ, 2008), but in several European countries, such as Scandinavia and Sweden, epidemiological studies and case studies have implicated mosquitoes in tularemia outbreaks (Eliasson et al., 2002). Many research studies have demonstrated the presence of *Francisella* genes in all stages of mosquitoes infected with *Francisella* species (Bäckman, Näslund, Forsman, & Thelaus, 2015; Lundström et al., 2011; Mahajan, Gravgaard, Turnbull, Jacobs, & McNealy, 2011; Read et al., 2008; Thelaus et al., 2014). However, a natural transmission of tularemia from a mosquito to a susceptible mammalian host has not been demonstrated in the laboratory (Thelaus et al., 2014), although anecdotal reports in the literature suggest it is possible (Hanke et al., 2009). In the same study Thelaus et al established that 69±27% of infected *Aedes aegyptii* larvae tested positive for *F. tularensis* subsp. *holarctica,* but only 25±5% of adult mosquitos transstadially retained the *Francisella* DNA. These studies suggest that while mosquitoes may be vectors of *Francisella tularensis,* either *Francisella* loses its virulence during passage through the mosquito or is unable to survive the mosquito host-defense response long enough to establish a bite-mediated transmission.

One of the insect innate immune mechanisms involve the production of antimicrobial peptides in response to bacterial infections (Philippe Bulet & Stöcklin,

2005). These host-defense antimicrobial peptides are important components of the innate immune system that are found in many multicellular organisms and prokaryotes (van Hoek, 2014). They differ in their size and amino acid composition as well as the microbes they target. The most common group of insect peptides are insect defensins and the second most common group of peptides are insect cecropins (P Bulet, 1999). At least three very similar cecropins have been identified in the genome of Asian tiger mosquito *Aedes (A.) albopictus*, Cecropin A1, B and C (D Sun, Eccleston, & Fallon, 1998; Dongxu Sun, Eccleston, & Fallon, 1999), and highly similar peptides have been identified in the genomes of other mosquitoes such as *Culex (C.) pipiens*.

In the current study we investigated the activity of the two classes of insect antimicrobial peptides, defensins and cecropins, from the *A. albopictus* mosquito, a vector of many significant pathogens such as dengue, yellow fever and chikungunya. We then tested these peptides against *F. tularensis* subspecies *novicida*, as a model for the fully virulent *F. tularensis*. The peptides tested in this study are Defensin D, Cecropin A1 and Cecropin B. The antimicrobial activity of synthesized peptides was measured in low salt buffer against *F. novicida*, and we found that mosquito cecropins were particularly antimicrobial against *F. novicida* with EC_{50} values below 8 µM. We assessed membrane permeabilization and cytoplasmic depolarization of the peptides using the ethidium bromide uptake assay and $DiSC_3(5)$ release assay respectively. We concluded that mosquito cecropins act by disrupting membrane potential and eventually forming pores in the *Francisella* membrane.

In order to further study the mosquito host-response to being infected by *Francisella* bacteria, we established the C6/36 cell line model, a cultured mosquito cell line from *A. albopictus*. We determined that these cells can be infected by *F. novicida*. We then determined whether the gene expression of Defensin D and Cecropin peptides was altered in response to *Francisella* infection of the mosquito cells. We demonstrated a significantly elevated expression of Cecropin A1 and Cecropin B as well as Defensin D by C6/36 cell line in response to infection by *F. novicida.*

Materials and Methods

Bacterial strains

The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: *Francisella tularensis* spp. *novicida* Utah 112 NR-13) and *Francisella tularensis subsp. holarctica,* Strain 15 (Gaisky Live Vaccine Strain), NR-14. Bacteria were grown in Tryptic Soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with 1% cysteine at 37^{o}C in the shaking incubator at 200 rpm for 48 hours. Serial dilution and subsequent plating was performed in order to determine the final concentration of bacteria.

Peptides

The sequences of *A. albopictus* Defensin D (Uniprot O77217), Cecropin B (Uniprot Q963A9/Q9Y0Y0) and Cecropin A1 (Uniprot P81417) were custom synthesized by Peptide 2.0 (Chantilly, VA). **Table 3** lists the peptides, their sequences, net charge and mass, determined by Antimicrobial Peptide Database (G. Wang et al.,

2009; Z. Wang, 2004). Peptides were produced at 90-95% purity, and the sequence confirmed by HPLC and mass spectrometry.

Table 3: Sequences of mosquito antimicrobial peptides tested in this study

Peptide	Sequence	Hydro-phobicity	Molecular weight (g/mol)	Net charge
Cecropin A1	GGLKKLGKKLEGVGKRVFKASEKALPV**AV** G**I**KA**L**GK	44.44%	3676.57	+8
Cecropin B	GGLKKLGKKLEGVGKRVFKASEKALPV**LT** G**Y**KA**I**G	40%	3642.47	+7
Defensin D	ATCDLLSGFGVGDSACAAHCIARGNRGGY CNSKKVCVCPI	35%	4018.70	+2
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLR NLVPRTES	35%	4493.33	+6

Minimum Inhibitory Concentration

MIC assays were performed as previously described (Ahmad, Hunter, Qin, Mann, & van Hoek, 2010) with slight modification. The bacteria were cultured overnight in cation-adjusted Muller- Hinton broth (CA-MHC) supplied with 2% isovitalex. Conditions were in accordance with the current recommendation of the CLSI ("Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that Grow Aerobically; Approved Standard- Ninth edition," n.d.). The bacterial culture was then diluted 1:50 to achieve ~ 2 x 10^6 cfu/ml. A range of concentrations of the peptides was prepared by serial dilution and added to an equal volume of bacteria. Microtiter plates were incubated for 18

h at 37°C. The MIC was defined as the concentration at which no microbial growth was observed spectrophotometrically via readings of optical density (OD) (TECAN, Switzerland) at 600 nm. Wells containing only media and growth media containing *F. novicida* without peptide were used as controls.

Antimicrobial EC_{50} assay

96 well plates (Falcon, Corning NY) were prepared with various dilution of peptide in 10mM phosphate buffer (pH 7.2) supplemented with 0.1% cysteine (Han, Bishop, & van Hoek, 2008); the highest concentration of peptide was 833.33 µg/ml and subsequent 1:5 serial dilutions were performed. The wells were then inoculated with 1 x 10^5 colony forming units (CFU) of *F. novicida*. After a 3 hour incubation each well was further diluted 10^{-1}, 10^{-2} and 10^{-3} fold and each dilution was plated in triplicate. The plates were then incubated for 24 hours for *F. novicida* and colonies were counted after the incubation. The concentration of peptide required to kill 50% of bacterial population was determined by graphing percentage of surviving colonies, after the 24 hour incubation, as a function of log of peptide concentration. The data was analyzed through GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA) using the equation:

$$\mathbf{Y = Bottom + (Top-Bottom)/ (1+10^{[(logEC50-X) \times Hill\ slope)]})}$$

where Y corresponds to percentage of surviving bacteria at the given peptide concentration and X corresponds to the concentration of peptide in logarithmic function. The terms "Top" corresponds to upper boundary constrained to 100% and "Bottom" corresponds to lower boundary constrained to 0%. The "no peptide" treatment is graphed at 10^{-2} µg/ml. The antimicrobial activity of the mosquito peptides was compared to lytic

activity of LL-37, a human peptide that is extremely effective against *F. novicida* (Amer et al., 2010).

Bacterial cytoplasmic depolarization assay

Membrane depolarization assay was studied using $DiSC_3(5)$ as previously described (Rodriguez et al., 2014; Wu & Hancock, 1999). Enumerated bacterial stocks were centrifuged (6500 g for 10 min at 4ºC) pelleted and washed twice in 10 mM phosphate buffer (pH 7.2) and then diluted to 4 X 10^7 CFU/mL in 10mM phosphate buffer containing 50 µg/mL $DiSC_3(5)$. 100 µL of this suspension was added to wells of a black 96 well plate (Ultracruz Polypropylene Microplate, Santacruz, CA). The plate was incubated in a TECAN Microplate Multimode reader. A change in the fluorescence was continuously monitored until a steady reduction in fluorescence is reached (evidenced by a flat line) in the fluorescence graph indicating maximum uptake of the dye by the intact membrane. The experimental wells are treated with various concentrations of *A. albopictus* peptides (50µg/mL, 10 µg/mL and 5 µg/mL) diluted in 10mM phosphate buffer (pH 7.2). Bacteria without peptide was used as a negative control and LL-37 and 1% Triton X-100 was used as positive control. The plate was immediately returned to the spectrofluorometer. Readings were taken every 2 min for 20 min (excitation = 622 nm; emission = 670 nm).

Bacterial cytoplasmic permeation assay

Pore formation in *F. novicida* cytoplasmic membrane was assessed using the ethidium bromide uptake assay. The membrane permeability assay was performed as previously described with some modifications (Li et al., 2012). Briefly, *F. novicida* was

grown until log phase in tryptic soy broth (Difco laboratories, Detroit, MI, USA) containing 0.1% cysteine (37ºC). Bacteria were centrifuged (6500 g for 10 min at 4ºC) and washed with PBS, and then diluted to an OD_{600} of ~ 0.1 in10 mM phosphate buffer. In a 96 well black plate (Ultracruz Polypropylene Microplate, Santacruz, CA), 180 µL bacterial culture was then mixed with 10µM ethidium bromide and incubated with 50 µg/ml of either Cecropin A1, Cecropin B and Defensin D. LL-37 was used as positive control. The plate was then immediately read in a TECAN infinite F200 spectroflourometer every 2 min for 20 min at 37ºC: (excitation = 535 nm, emission = 590 nm). The fluorescence ΔRFU (Relative Fluorescence units) was calculated by the formula: RFU (50 µg/ml of tested peptide)-RFU (no peptide control).

MTT cell proliferation assay

The toxicity of the peptides was assessed using tetrazolium dye MTT. Active cells are able to reduce the yellow color of the MTT dye to water insoluble dark-blue formazan precipitate that can be dissolved using DMSO as the vehicle solvent. The result is a purple color solution that can be analyzed spectrophotometrically. The concentration of live cells is directly proportional to the purple color of the solution. The larger the number of live cells, the darker the solution. A549, human lung carcinoma cell line (ATCC) cultured in DMEM supplemented with 10% FBS, were used in the experiment. 10^4 cells were seeded in 96 well plates (Falcon, Corning NY) for 24 hours. After 24 hour incubation the wells were treated with 120 µg/ml of peptide. Wells with A549 cells without peptide was used as negative control and wells with cells treated with 1% Triton X-100 was used as positive control. The plate was again incubated for 24 hours. On the

third day both experimental and control wells were treated with 10 µg of MTT dye. After a 4 hour incubation all but 25 µl of media was aspirated out. 50 µl of 0.1mM DMSO was then added to dissolve the dark blue crystallized formazan. After a brief incubation with DMSO, the absorbance of the plate was recorded (540 nm). Percent survival was calculated as:

[(Absorbance of experimental wells) - (Absorbance of media only)/ (Absorbance of A549 cells only) - (Absorbance of media only)] x 100

In-vivo survival assay of *Galleria mellonella*

Galleria mellonella (wax moth caterpillars) were obtained from Vanderhorst Wholesale (Saint Marys, OH, USA). Survival assay of wax moth caterpillar was conducted as previously described (Dean, Bishop, & van Hoek, 2011b). Briefly, ten caterpillars of equal size/weight were randomly assigned to each group and placed into labeled petri dishes. A 1-ml tuberculin syringe was used to inject 10 µl of 3×10^6 CFU/ml of *F. novicida* into each caterpillar's hemocoel. After a 60 minute incubation to allow the infection can occur, the caterpillars were then injected with 10 µl of either water, 10 µg of Cecropin A1, Cecropin B or Defensin D in the dorsal side of the caterpillar. The insects were then observed daily for their survival status.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Mosquito C6/36 cell infection assay:

Aedes albopictus cell line C6/36 (ATCC) was grown in Eagles modified essential medium (ATCC) supplemented with 10% fetal bovine serum at 28^{o}C. For the infection assay, cells were plated into a 24 well dish, and wells were infected with 50 MOI of *F.*

novicida. After 2 hours the cells were washed with buffer. Gentamicin protection is performed 2 hours after the initial infection by incubating with a high-pulse of gentamicin (20µg/ml). RNA of the infected cell line was extracted using RNeasy mini kit from Qiagen.

Quantitative RT-PCR

C6/36 cells were infected with *F. novicida* as described earlier. Total RNA was extracted using RNeasy Mini Kit (Qiagen). The RNA was eluted with 50 µl of nuclease free water. qRTPCR was performed using the Bio-Rad CFX Connect Real-Time PCR Detection System and SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) with oligonucleotides listed in **Table 4**. 18S rRNA was used as a control. Relative transcript quantification was accomplished using the CFX Manager Software (Bio-Rad), by first normalizing transcript abundance [based on the threshold cycle value (Ct)] to 18S rRNA followed by determining transcript abundance ratios. Melting curve analyses were employed to verify specific single product amplification. Amplification products were verified by electrophoresis on a 2% agarose gel, visualized by EtBr staining (data not shown).

Table 4: Primers used in this study for qRT-PCR

Peptide	Primer sequence
Cecropin A1	Forward Primer: GAGTCGGCAAACGAGTCTTC Reverse Primer: TTGAACCCGGACCATAAATC
Cecropin B	Forward Primer: GTTTGCGCTTGTTCTGCTTA Reverse Primer: TCCCACCCAGTTAGAACAGC
Defensin D	Forward Primer CGGACGAAGCTCAGTCTGTT Reverse Primer GACGCACACCTTCTTGGAGT
18S rRNA	Forward Primer: TCAAAATTAAGGGTAGTGGT Reverse Primer: GACTTCAACTGGCTTGAACT

Statistical Analysis

EC_{50}, MIC, $DiSC_3(5)$, EtBr uptake and MTT assays were performed in triplicates (n= 3). Standard deviations of the mean of each set are represented by error bars on each graph. Where the error bars cannot be seen, the error is very small. Statistically significant differences were assessed using Student's *t* tests for EtBr and MTT assay, 2-way ANOVA for depolarization $DiSC_3(5)$ assay and Kaplan Meier survival curve for *Galleria mellonella* survival. Confidence Interval (CI) (95%) for each EC_{50} determination is presented in **Table 5**, and represents significance of $p < 0.05$.

Results

Susceptibility of *F. novicida* to mosquito AMPs

We determined the antimicrobial activity of *A. albopictus* peptides against *F. novicida* and their activity was compared to LL-37, a human peptide with known antimicrobial activity against *F. novicida* (Amer et al., 2010). *F. novicida* was treated with various concentrations of Cecropin A1, Cecropin B and Defensin D in the presence of Buffer Q (pH 7.2) to determine their antimicrobial activity, measured as the

concentration of peptide that kills 50% of the bacteria, EC_{50}. The EC_{50} value for Cecropin B was determined to be 4.64 µg/ml **(Figure 6A**) and for Cecropin A1 it was determined to be 20.09 µg/ml **(Figure 6B)**. Defensin D was not antimicrobial against *F. novicida* (EC_{50} was more than 100 µg/ml as seen in **Figure 6C**). The EC_{50} value for LL-37 was 2.73 µg/ml. The EC_{50} values of these microorganisms are listed in **Table 5** along with the 95% confidence interval ($p<0.05$) and their respective µM values. None of the peptides were effective against *F. novicida* in MIC conditions (high salt conditions). The highest concentration of peptide tested was 300 µg/ml; however, even at the highest concentration, growth of *F. novicida* was not inhibited (data not shown). This result is consistent with that reported by Moule et al (Moule et al., 2010) who tested *Drosophila* Cecropin against *F. novicida* in a Kirby Bauer Disc Inhibition assay and found no zone of inhibition under high salt conditions.

Table 5: EC50 values of mosquito antimicrobial peptides against *F. novicida*.

Peptide	**EC_{50} (µg/ml)**	**EC_{50} (µg/ml) 95% CI**	**EC_{50} (µM)**	**EC_{50} (µM) 95% CI**
Cecropin A1	20.09	(14.85-27.17)	5.46	(4.04-7.39)
Cecropin B	4.64	(3.44-6.25)	1.27	(0.94-1.71)
Defensin D	>100	-.	>25	-
LL-37	2.73	(2.37-3.13)	0.53	(0.46-0.60)

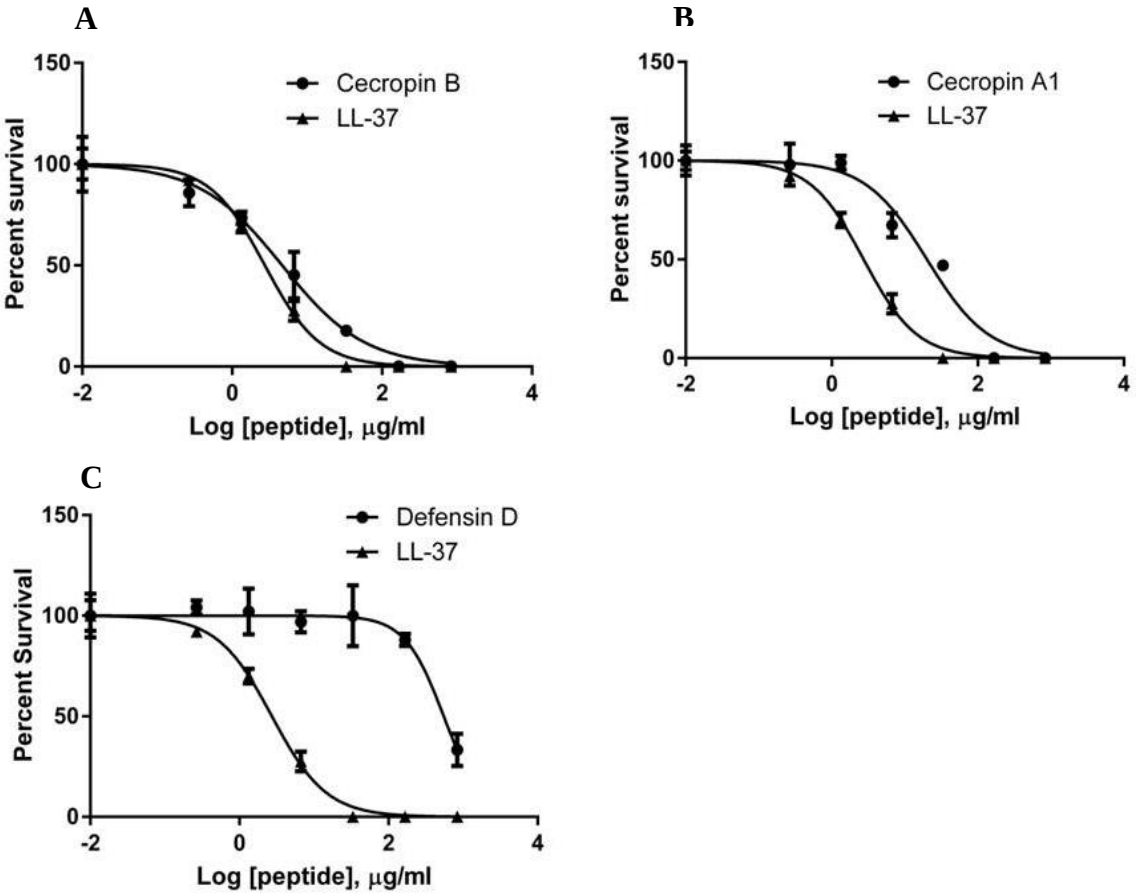


Figure 6: Antimicrobial activity of mosquito antimicrobial peptides against *F. novicida*. *F. novicida* was incubated for 3 h with a range of peptide concentrations in Buffer Q (pH 7.2), and percent (%) survival was calculated as the ratio of CFUs before and after incubation. Cecropin B was able to kill 50% of the *F. novicida* bacteria at 4.64 μg/ml **(Fig. 6A)** whereas Cecropin A1 was able to kill 50% of the *F. novicida* bacteria at 20.09 μg/ml **(Fig. 6B)**. Defensin D was not able to completely eliminate bacteria even at the highest concentration of peptide **(Fig 6C)**

Mosquito AMPs dissipate membrane potential in *F. novicida*

Antimicrobial peptides generally target the cellular membrane of bacteria in order to exert their antimicrobial effect. Therefore we assessed the bacterial membrane integrity in response to the tested peptides. One of the ways that cell membrane integrity is

compromised is due to formation of small, voltage dependent channels leading to leakage of potassium ions (Epand et al., 2010; Kagan, Selsted, Ganz, & Lehrer, 1990). To determine the effect of *A. albopictus* peptides on the cytoplasmic membrane of *F. novicida* we conducted a membrane potential displacement assay. We performed the cytoplasmic depolarization assay with *F. novicida* utilizing the $DiSC_3(5)$ dye. The undamaged cytoplasmic membrane takes up the $DiSC_3(5)$ dye which results in reduction of fluorescence of dye. The addition of depolarizing compounds results in the release of the dye in the surroundings and a sudden increase of fluorescence is observed. **Figure 7** indicates a concentration dependent increase in fluorescence when *F. novicida* was treated with a range of concentrations of different peptides. All the peptides tested dissipated the membrane potential in *F. novicida* at less than 5 µg/ml indicating that depolarization of cytoplasmic membrane is a mechanism employed by insect antimicrobial peptides to kill *F. novicida*. However, both Cecropin A1 and Cecropin B were significantly better at depolarizing the *F. novicida* cytoplasmic membrane than Defensin D, consistent with the EC_{50} results. 1% Triton X-100 and LL-37 were used as positive control in the depolarization study of the peptides (Fernandez-Lopez et al., 2001; Gupta, Singh, & van Hoek, 2015). As seen in **Figure 7** both Cecropin A1 and Cecropin B at 50 µg/ml reached a fluorescence level equivalent to that of 1% Triton X-100, indicating complete depolarization. Defensin D exhibited significantly less membrane depolarization when compared to Cecropin B and Cecropin A1. In conclusion, all three peptides demonstrated significant levels of depolarization (p values < 0.0001).

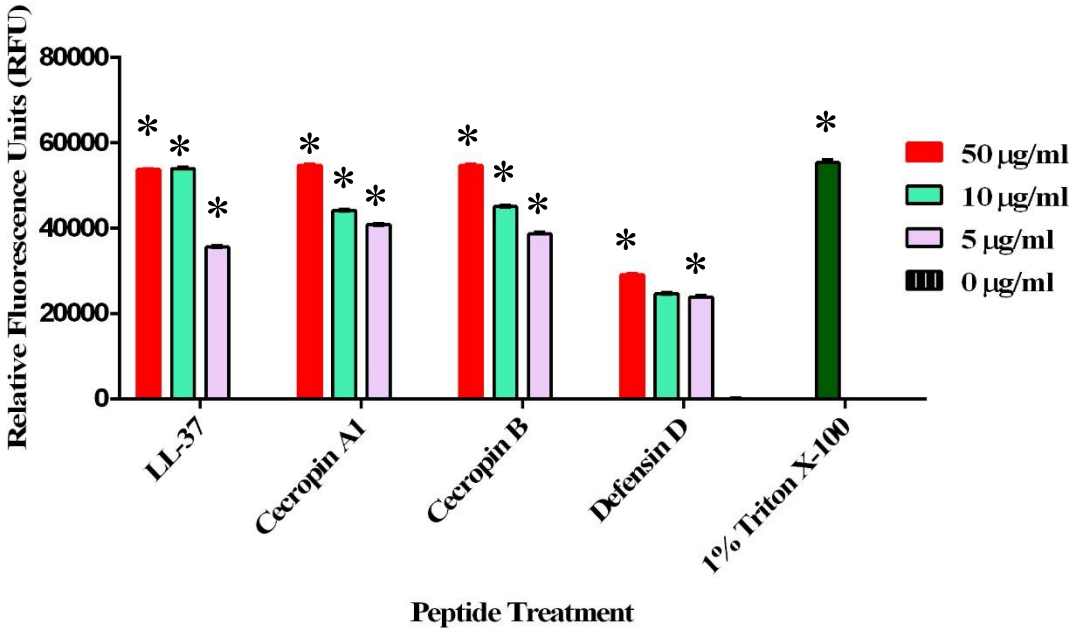


Figure 7: Release of $DiSC_3(5)$ dye by depolarized membrane in *F. novicida*. After the incubation of *F. novicida* with $DiSC_3(5)$, the dye has aggregated in the cellular membrane as seen by reduction in fluorescence in the untreated sample. With the addition of antimicrobial peptides, the fluorescence significantly increases as the cationic dye is released from the depolarized membrane into the media (seen in the 5 µg/ml, 10 µg/ml and 50 µg/ml). Both cecropins significantly depolarize the membrane with as little as 5 µg/ml. Defensin D also depolarizes the membrane; however it is less effective than Cecropin B and Cecropin A1. (*) represents p value <0.0001

Mosquito AMPs forms pores in cytoplasmic membrane of *F. novicida*

Previous studies have demonstrated that the concentration of peptides can have different consequences on the bacterial membrane (Yi, Chowdhury, Huang, & Yu, 2014).

At low peptide-to-lipid ratios, small transient pores and ion-permeable channels are formed in the membrane that result in depolarization of the membrane (Silvestro, Gupta, Weiser, & Axelsen, 1997; Silvestro, Weiser, & Axelsen, 2000). At high peptide-to-lipid ratios, large pores can be formed (Silvestro et al., 1997, 2000). Therefore, in order investigate the pore formation capability of the *A. albopictus* peptides, we conducted the EtBr uptake assay, in which the pore size must be large enough for EtBr to pass through and intercalate with the bacterial DNA. It was observed that *F. novicida* was most sensitive to membrane permeabilization by Cecropin B as seen in **Figure 8** evidenced by a significant RFU difference between the control and treated bacteria (p values <0.05). Cecropin A1 and LL-37 have almost the same effect evidenced by similar ΔRFU values. Defensin D demonstrates some pore formation relative to no-peptide treatment, but much less than the Cecropins and it is not as effective as the Cecropins in killing the bacteria. Overall, we conclude that the Cecropin peptides tested in this study permeabilize *F. novicida* by forming large pores in the membrane.

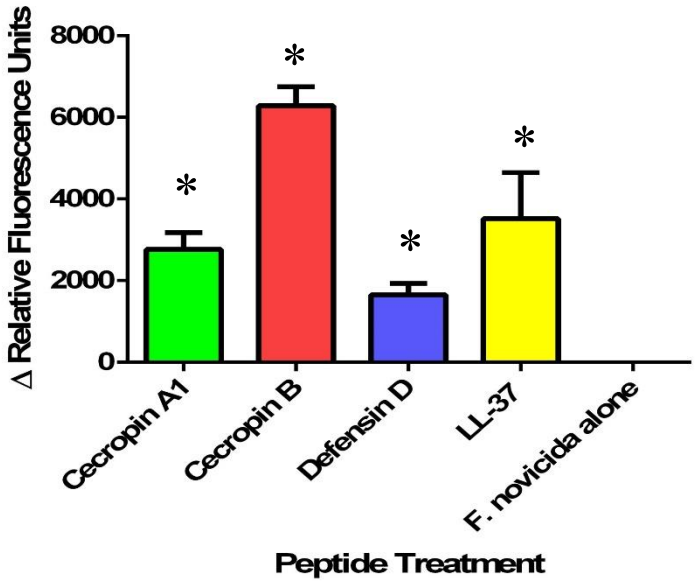


Figure 8: Pore formation as measured by EtBr in *F. novicida* membrane. EtBr accumulation by bacterial cell membrane treated with 50 µg/ml of peptide is shown in the figure. Pore formation is measured by the increase in fluorescence as ethidium bromide influx in the destroyed cell increases and EtBr binding to DNA occurs. Fluorescence was monitored every two minutes for 20 minutes. The fluorescence after 20 minutes was compared against no peptide control. Experiments were performed in triplicate (n=3). (*) represents p value < 0.05.

Cytotoxicity of mosquito peptides

One concern regarding the therapeutic use of antimicrobial peptide is that of cytotoxicity of these peptides. Many potent antimicrobial peptides, such as SMAP-29, have a broad range of antibacterial activity yet are significantly hemolytic and cytotoxic (Dawson & Liu, 2009). To understand the cytotoxic activity of peptides tested in this study, we analyzed the survival of A549 cells in response to treatment with peptides. Cell death was measured after 24 hours of addition of the peptide. We observed that Cecropin

A1 and LL-37 were slightly cytotoxic (p-value < 0.05) compared to cells-only control whereas cells treated with 1% Triton X-100 (used as positive control) were all lysed (p-value <<0.05). Cecropin B and Defensin D were not significantly cytotoxic to the A549 cell lines (**Figure 9**).

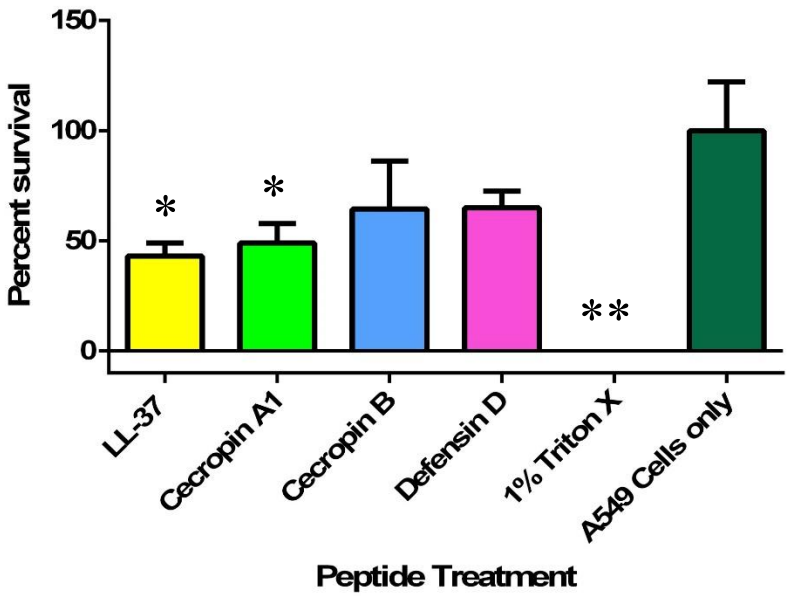
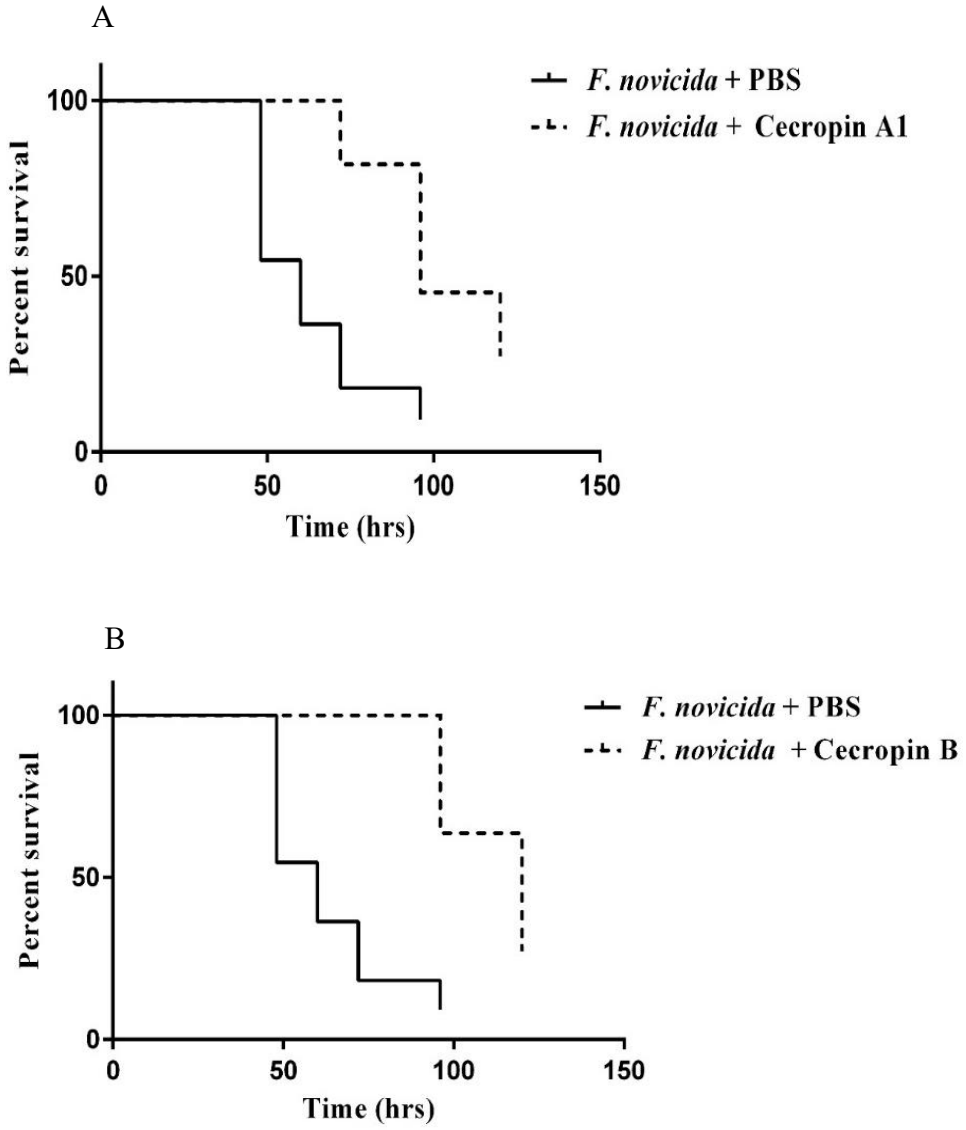


Figure 9: Cytotoxicity of mosquito AMPs. Percent survival is calculated as the ratio of A549 cells treated with peptide to A549 cells without treatment. P-values are represented on the figure. The graph shows the effect of the three mosquito peptides Cecropin A1, Cecropin B and Defensin D and control peptide LL-37 against A549 cells. 1% Triton X-100 was used as positive control. (*) represents a significant decrease in experimental absorbance relative to the control (A549 cells only) (p value <0.05) and (**) represents very significant decrease in the experimental absorbance relative to the control (p value <<0.05).

Galleria mellonella survival assay results

The larval stage of *Galleria (G.) mellonella*, wax moth caterpillar, has been used as an *in vivo* model to study infections caused by wide range of bacteria such as *Pseudomonas aeruginosa*, and the effect of treatment by different antimicrobial agents (Ahmad et al., 2010; Dean et al., 2011b; Dean & van Hoek, 2015; McKenney et al., 2012). *Francisella*-infected *G. mellonella* was previously used as an alternative infection model system for *in vivo* effect of peptides and antibiotics against *F. tularensis* LVS infections (Aperis et al., 2007). Our group has successfully established *G. mellonella* as an *in vivo* infection and treatment model for *F. novicida* infections (Ahmad et al., 2010; Dean & van Hoek, 2015; McKenney et al., 2012). The larvae do not have an adaptive immune system, but have resistance to microbial infections via cellular and humoral defenses (Aperis et al., 2007; Mahajan et al., 2011). The analysis of insect responses to pathogens can provide an accurate indication of the mammalian response to a pathogen. We used *G. mellonella* as an alternative to the mouse model of *Francisella* infection to test our hypothesis that treatment with antimicrobial peptide Cecropin A, and Cecropin B can prolong the survival of *Francisella* infected caterpillars *in vivo*. *G. mellonella* were infected with 3×10^4 CFU bacteria/larva of *F. novicida* and then treated with a single dose of 10 μg in 10 μl of Cecropin A, Cecropin B and Defensin D. Control groups (no infection) consisted of no injections, or injections of PBS (to measure trauma related to injections). *Francisella*-infected *G. mellonella* did not survive past 100 hours post-infection. Control groups survived for more than 120 hours. Infected groups were treated with a single dose of 10 μg of antimicrobial peptides. Cecropin treated *G. mellonella* had statistically significant prolonged survival times when compared to infected groups (p-

value < 0.005) (**Figure 10A and 10B**), however the Defensin D treated *G. mellonella* did not have increased survival compared to untreated (p=0.275) (**Figure 10C).**



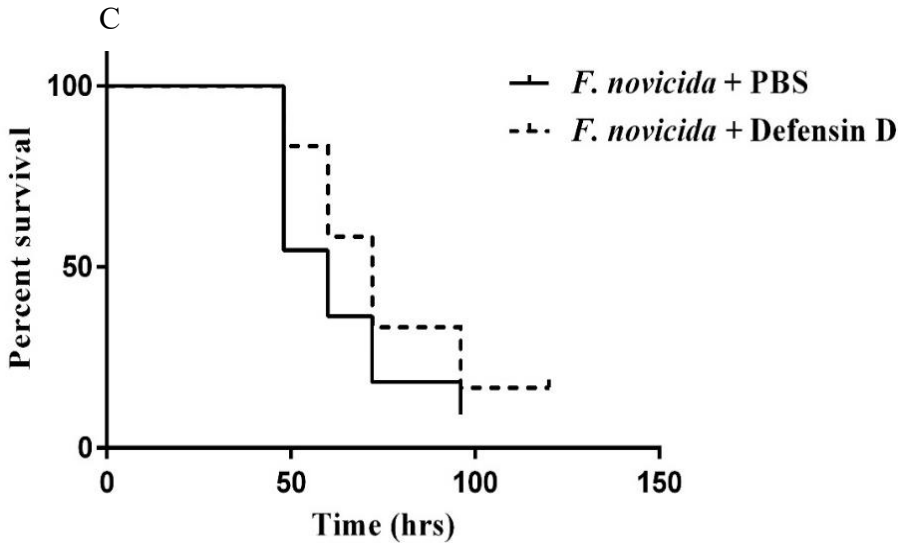


Figure 10: *Galleria mellonella* survival curve assay results. Antimicrobial peptide Cecropin A1 and Cecropin B prolonged the survival of *G. mellonella* infected with 3×10^6 CFU *Francisella* (10A and 10B). Defensin D treated *F. novicida* infected group did not show prolonged survival (10C). Non-infected control groups consisted of no injection, PBS injection or 10 μg in 10 μl antimicrobial peptide injection. The infected control group received *F. novicida* injection, then PBS. A single dose of 10 μg of antibiotic, or 10 μg in 10 μl antimicrobial peptide Cecropin A1, Cecropin B and Defensin D was given one hour after bacterial infection.

Induction of mRNA expression of AMPs by *F. novicida* infected C6/36 cells

Following *F. novicida* infection of A549 cells, the mRNA expression levels for Cecropin A1, Cecropin B and Defensin D were found to have increased by 7.34-, 2.19- and 2.10-fold respectively, relative to the levels of the corresponding mRNA in uninfected control cells (**Figure 11**). The observed increase in Cecropin A1 mRNA was comparable to that of results reported by Lowenberger et. al (Lowenberger et al., 1999). They reported strong expression of *A. aegyptii* Cecropin in immune activated mosquitoes.

Similar results were also observed by Magalhaes et. al (Magalhaes et al., 2008) where they also reported 4-fold change in transcript level of *A. aegyptii* Defensin following 60% and 100% infection with parasitemia. Comparable results have also been obtained for Drosophila Cecropins induced by *Francisella* (Moule et al., 2010).

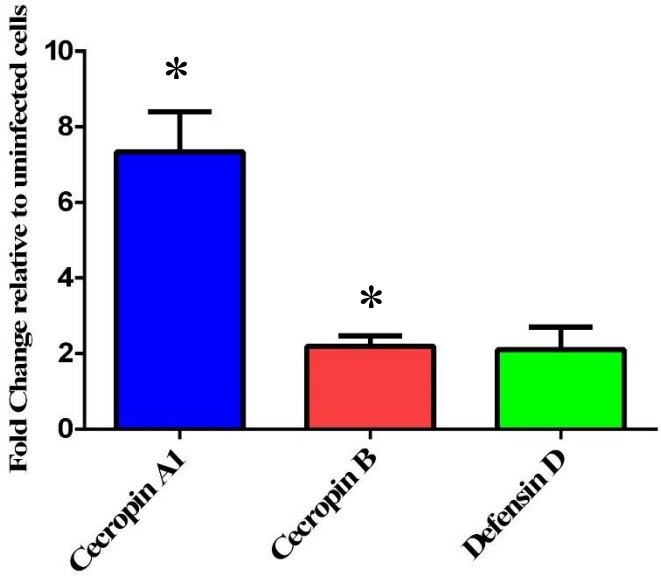


Figure 11: Expression of mosquito AMPS in *F. novicida* infected C6/36 cells. Induction of Cecropin A1, Cecropin B and Defensin D gene expression in C6/36 cells following *F. novicida* infection. C6/36 cells were left uninfected (control) or were infected with *F. novicida* at MOIs of 1:50 for 18 hours. The expression of mRNA induction was determined as described above. Total RNA was extracted, and RT-PCR was conducted to quantify Cecropin A1, Cecropin B and Defensin D mRNA levels using the delta-delta CT method. As a negative control, RNA was omitted from reverse transcription and PCR amplification (no RNA). The 18S rRNA gene was used as a housekeeping gene. Data shown are representative of three separate experiments. In the analysis, results are represented as the mean ± SD from at least three independent experiments. *, $P < 0.05$.

Discussion

Aedes albopictus, the Asian tiger mosquito, is a prolific human-feeding mosquito, with a range that includes Northern Europe, which is the main area of Type B tularemia cases. In this study, we have tested two classes of insect antimicrobial peptides from *A. albopictus*, cecropins and defensins, against Gram-negative *F. novicida* and also determined the mechanism of action of these peptides. We have established that at least two *A. albopictus* antimicrobial peptides were able to exert significant direct antimicrobial activity against *F. novicida in vitro*. Both Cecropin A1 and Cecropin B had EC_{50} values less than 10 µM. Defensin D did not have any significant direct antimicrobial activity towards *F. novicida*. We observed that all three peptides demonstrated depolarization and pore formation although Cecropin A1 and B were significantly more active than Defensin D. We then demonstrated that treatment with Cecropin B and Cecropin A1 demonstrated prolonged waxworm survival. *F. novicida* infected C6/36 cells show an elevated expression of all three peptide genes compared to the unstimulated cells. Finally, we demonstrated that Cecropin B and Defensin D were not cytotoxic against human A549 cells.

We observed an interesting slight but significant difference between the activity of Cecropin A1 and Cecropin B, especially the EC_{50} values. As seen in **Table 3** Cecropin A1 and Cecropin B have minor differences between the amino acid sequences, charge and molecular weight. *A. albopictus* Cecropin A1 has 44.44% hydrophobic amino acids whereas Cecropin B has 40% hydrophobic amino acids. A previous study investigating the effects of hydrophobicity on the antimicrobial activity of peptides has established that

increasing peptide hydrophobicity increases antimicrobial activity of peptide, and an increase in hydrophobicity beyond a certain maximum results in peptide self-association which restricts the peptide access to the bacterial cytoplasmic membrane (Chen et al., 2007; Datta et al., 2001). In another study, it was established that most Cecropin peptides have similar antimicrobial activity against common Gram-negative bacteria such as *Escherichia coli* and *Serratia marscescens*. However, Cecropin B had better antimicrobial activity than Cecropin A1 with a slightly better range of activity against less susceptible microorganisms (Hultmark, Engström, Bennich, Kapur, & Boman, 1982). *Francisella* is a polymyxin B, and it is known to form biofilms (Durham-Colleran, Verhoeven, & van Hoek, 2010; van Hoek, 2013). We observed Cecropin B was slightly more antimicrobial than Cecropin A1 towards *F. novicida*. The marginally lower antimicrobial potential of Cecropin A1 may be attributed to the higher hydrophobicity of this peptide.

Cecropin peptides derived from insects are generally more effective against Gram-negative bacteria and insect defensin peptides are generally active against Gram-positive bacteria. However, lytic activity of most peptides is observed to be compromised *in vitro* under high ionic conditions which mimic the physiological salt levels (Dorschner et al., 2006; Lamberty et al., 1999). Therefore it is uncertain as to how these peptides defend the host *in vivo,* given their lack of antimicrobial activity in the conventional MIC conditions (high salt). Consistent with these previous results, we found that *A. albopictus* peptides are not antimicrobial in high salt (MIC) conditions *in vitro*. However, the

peptides were well able to kill *F. novicida* in low ionic conditions (10 mM phosphate buffer).

Since peptides as antimicrobials are being explored as an alternative to antibiotics, we assessed the ability of the mosquito peptides to prevent or treat *in vivo* infection by *F. novicida* in the waxworm caterpillar model system. *G. mellonella* has been established as an invertebrate host model system to study *F. tularensis* infections (Aperis et al., 2007; Dean & van Hoek, 2015; McKenney et al., 2012). In this study we determined that the *F. novicida* infected waxworms showed prolonged survival when treated with Cecropin A1, Cecropin B and but not Defensin D as compared to the untreated, infected waxworms. Through this particular experiment we have demonstrated that while the MIC conditions are a gold standard for assessing *in vitro* capability of peptides to kill bacteria, the physiological environment *in vivo* may contain many factors that may prove to be helpful to peptide activity. In this case, the peptides were able to prolong *F. novicida* waxworm survival *in vivo* when injected in the hemocoel of the insect despite showing no effect in MIC assays.

The mechanism of action for most insect antimicrobial peptides is believed to be their interaction with the cytoplasmic membrane of bacteria (Nakajima et al., 2003). More specifically, the mode of action of the peptides depends on the peptide-to-lipid ratio and composition of the bacterial cellular membrane. At low peptide-to-lipid ratios and low peptidoglycan composition (such as Gram-negative organisms), a slow leakage of cellular contents is observed (Manzini et al., 2014). α-helical peptides, such as cecropins, attach to the lipid head group region which results in membrane thinning (Brogden,

2005). Peptides at low concentration orient in a parallel resulting in tiny transient pores in cellular membrane and gradual leakage of ions (Brogden, 2005). At high peptide-to-lipid ratios, the orientation changes from parallel to perpendicular forming large monomeric pores. Low and high peptide concentrations are relative to the type and amount of lipid composition of the microorganisms (Brogden, 2005).

The specific mode of action of mosquito peptides is not known. Therefore in this study we investigated both aspects of mosquito peptide interaction with *Francisella* bacterial membrane through fluorescence studies. First, we measured the dissipation of membrane potential by the use of cationic dye $DiSC_3(5)$. The dye is incorporated into intact membrane and that results in decrease in fluorescence of the dye. However when a membrane is depolarized, the dye is released into the media and consequently a dramatic increase in fluorescence is observed. The concentration range of peptide tested in depolarization study was 0-50 µg/ml. In this study, significant depolarization of *F. novicida* membrane was observed, even at lowest concentration (5 µg/ml), in the first few minutes of adding the peptides. At 50 µg/ml, the fluorescence is almost equivalent to 1% Triton X-100 (**Figure 7)**. Observing this we decided to evaluate the second aspect of peptide interaction: formation of large multimeric pores utilizing ethidium bromide uptake assay. At 50 µg/ml, we noted significant pore formation in *Francisella* bacteria treated with Cecropin B, and Cecropin A1 relative to negative control (untreated bacteria) **(Figure 8)**. The human cathelicidin peptide LL-37 was used as a positive control in the pore formation study.

Previously, we had demonstrated the induction of expression of antimicrobial peptide genes in mammalian host cells in response to *Francisella* bacterial infection (Amer et al., 2010; Han et al., 2008). We examined a cultured mosquito cell line, C6/36 cells (derived from the mosquito *Aedes albopictus*) to see if the mosquito antimicrobial peptide genes were induced in their expression following infection by *F. novicida.* This system will model the host-response of mosquito cells to potential infection by the *Francisella* bacteria. Previously, mammalian defensins, such as human beta defensin peptide-2 (hBD2) and human beta defensin peptide-3 (hBD3) have shown differential antimicrobial activity particularly against *Francisella* (Han et al., 2008) and are also highly expressed in response to *Francisella* infections (Han et al., 2008). The human cathelicidin peptide LL-37 is also induced in expression in response to *Francisella* infection of the human lung epithelial A549 cells (Amer et al., 2010). Insect immune response is similar to mammalian immune response and involves some of the same types of effector molecules and pathogen recognition receptors (Müller, Vogel, Alber, & Schaub, 2008). Therefore, we did an experiment to see if there was a similar induction of insect host-defense antimicrobial peptide genes in response to *F. novicida* infection. We first determined that infection of these cells by *Francisella* bacteria did not cause cell death by 18 hours. By qRT-PCR, we determined that Cecropin A1 had the significantly highest expression in the C6/36 cell line infected with *F. novicida* (7.3-fold) compared to the uninfected C6/36 cells. Cecropin B also had significantly higher expression in the infected cell line (2.2-fold). However, Defensin D only had a 2.1-fold increase in gene expression compared to the uninfected cells. Previous reports have also shown elevated

expression of these peptide genes in C6/36 cells lines when stimulated with LPS, although the expression of Defensin D was shown to be higher than Cecropin B in those studies (Mizutani et al., 2003; Moon, Walker, & Goodbourn, 2011). In conclusion, we have established that antimicrobial peptides from *Aedes albopictus* tested in our study were able to kill *F. novicida* in low ionic conditions *in vitro*. We have also demonstrated that the peptides target the cellular membrane of *F. novicida* and in low peptide-to-lipid ratios (5-10 µg/ml) dissipates membrane potential and in high peptide-to-lipid ratios (50 µg/ml) forms pores in cytoplasmic membrane. We have also showed prolonged survival of *F. novicida* infected *Galleria mellonella* wax worm larvae when treated with Cecropin B and Cecropin A1. Lastly, we have shown that *Aedes albopictus* C6/36 cells have significantly induced mRNA expression of Cecropin A1 (7.34-fold) and Cecropin B (2.19-fold) genes in response to *F. novicida* infection. While depolarization and pore formation seem to be the accepted mode of action for most insect peptides, the presence of intracellular targets are unknown, which could be the focus of future studies. Thus, the innate immune host-defense system of mosquitoes is able to produce antimicrobial peptides in response to *Francisella* infection, and those peptides have antimicrobial activity against *Francisella* bacteria *in vitro* and *in vivo* in the waxworm model.

CHAPTER 4: CONCLUSION

In conclusion, in this thesis we have established that defensin from *Cimex lectularius* tested in our study was able to all Gram-positive bacteria in low ionic conditions *in vitro* and *M. luteus* and *S. epidermidis* in MIC conditions. The Cecropins from the mosquito, *Aedes albopictus*, were antimicrobial against *F. novicida* under low ionic conditions as well. We have also established that all the peptides target the cellular membrane of the pathogen and in low peptide-to-lipid ratios (5-10 µg/ml) dissipate membrane potential and in high peptide-to-lipid ratios (50 µg/ml) form pores in cytoplasmic membrane of the pathogen. We have also showed prolonged survival of *F. novicida* infected *Galleria mellonella* wax worm larvae when treated with Cecropin B and Cecropin A1. Lastly, we have shown that *Aedes albopictus* C6/36 cells have significantly induced mRNA expression of Cecropin A1 (19-fold) and Cecropin B (9-fold) genes in response to *F. novicida* infection. While depolarization and pore formation are the accepted mode of action for most insect peptides, the presence of intracellular targets are unknown, which could be the focus of future studies.

Isolating Insect AMPs: Challenges

There still remain a lot of challenges that need to be tackled for further research in the field of insect AMPs. The first and the foremost challenge is effectively predicting and isolating AMPs from the insect. The original aim of the chapter two was to infect

bedbugs with Gram-positive and Gram-negative bacteria and subject the lysate to bio-prospector to obtain insect AMP sequences and test them for antimicrobial activity. However, it was not feasible due to the inability of this technique to identify known cathelicidins or defensins in other samples so instead we decided to custom synthesize a putative defensin sequence discovered in one of the databases and test that for antimicrobial activity. Effective prediction of potential AMPs remains our number one priority. Another challenge is to identify all the potential antimicrobial peptides expressed by the insects in- response to infection. However traditional methods only allows the identification of peptides expressed in over- abundance. In addition, ex-vivo analysis of expression of peptide genes in response to bacterial infection is only possible for those insects whose cell lines have been established. Although genomic comparison between insects belonging to similar orders can allow for prediction of more AMPs, there are only limited number of organisms whose genome is fully mapped. *Cimex lectularius* genome for example is only partially available and therefore it is a continuing challenge to identify novel insect AMPs expressed after pathogenic infection.

Insect AMPs: Applications in nature

Insect AMPs are being investigated as a potential alternative to antibiotics; however, over-expression of recombinant insect AMPs such as gallerimycin and sarcotoxin lA in tobacco plants have also shown to confer resistance to certain fungi such as *Rhizoctonia solani* and *Pseudomonas syringae* (Mitsuhara et al., 2000). Induced expression of another proline-rich peptide called Metchnikowin in barley also increases its overall resistance to pathogens (Rahnamaeian et al., 2009). Transgenic rice, *Oryza*

sativa, expressing Cecropin A from silk moth showed enhanced resistance to fungi *Magnaporthe grisea* (Coca et al., 2006). These studies suggest that broad-spectrum pathogen resistance could be achieved using insect AMPs.

Insect AMPs have a broad spectrum antimicrobial activity which is not limited to prokaryotes or viruses. Some AMPs are also functional against parasites Some peptides, such as, Defensin A, are now being engineered in arthropod vectors of clinical significance such as *Aedes aegypti* to confer resistance to bacterial pathogens thereby reducing indirect transmission of diseases (Yi et al., 2014).

Insect AMPs: Future directions

Most insect AMPs isolated function under the conditions of low ionic strength. Future research should focus developing synthetic AMPs that are functional at the physiological ionic concentration. The natural insect AMPs that are discovered can be utilized as a template for development of synthetics AMPs. In addition to that, future studies can also focus on testing small fragments of strongly antimicrobial AMPs. Certain AMP sequences are very long and although these AMPs have a range of antimicrobial activity, it may not be very cost effective to develop them for therapeutics. Testing individual small fragments of the sequence may reveal parts of the AMP sequence that is more antimicrobial than the other fragments thereby reducing the cost of development (de Latour et al., 2010; Dean et al., 2011a, 2011b) .

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