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# The Effects of Honeybee (Apis mellifera) Antimicrobial Peptides on Paenibacillus larvae

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THE EFFECTS OF HONEYBEE (*APIS MELLIFERA*) ANTIMICROBIAL
PEPTIDES ON *PAENIBACILLUS LARVAE*


By

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ABSTRACT

**The Effects of Honeybee (*Apis mellifera*) Antimicrobial
Peptides on *Paenibacillus larvae***

By

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American Foulbrood Disease (AFB) is the most detrimental bacterial disease that affects honeybee larvae (*Apis mellifera*) worldwide. The etiological agent of AFB is the Gram-positive, spore-forming bacterial pathogen, *Paenibacillus larvae*. Treatment with antibiotics, specifically oxytetracycline, has led to the development of antibiotic resistance in *P. larvae*. Therefore, there is a pressing need for an alternative treatment method. The overall goal of this project was to test naturally occurring, active antimicrobial peptides (AMPs), which are produced in the adult honeybee, against vegetative *P. larvae*. These active AMPs could potentially be used as a prophylactic treatment to prevent *P. larvae* infection of honeybee larvae. The use of AMPs is a superior approach to preventing/treating AFB than common practices, such as antibiotics, due to the unlikeliness of developing AMP-resistant bacteria and the prevention of burning and destroying hives. My research first focuses on adapting and creating two *in vitro* methods in order to test the antimicrobial activity of honeybee AMPs against vegetative *P. larvae*. Once methods were established, a total of five AMPs were tested against *P. larvae* either individually or in certain combinations. I found that four of the five AMPs were capable of inhibiting *P. larvae* growth; however, only two AMPs met my threshold requirements and were deemed suitable candidates for AFB prophylaxis. Furthermore, based on statistical significance, none of the AMP combinations tested inhibited the growth of *P. larvae* more than the single active AMP. Overall, my research

demonstrates that honeybee AMPs do inhibit the growth of *P. larvae in vitro* and have the

potential to be used in use in treatments and /or prophylaxis strategies for AFB.

# ACKNOWLEDGEMENTS

DEDICATION

To my family,

for always believing in me and

supporting me in any venture I dare take.

Thank you and I love you.

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CHAPTER 1

INTRODUCTION

1.1. The Importance of Honeybees in Agriculture

Approximately one-third of crops produced in the United States are dependent on pollination performed by European honeybees (*Apis mellifera*) (Kaplan, 2012). Some crops, such as almonds, are entirely dependent on honeybees for pollination. While other crops, such as alfalfa, apples, avocados, citrus fruits, cotton, cucumbers, pumpkins, strawberries, and soybeans rely on honeybee pollinators in most cases (indicated in Table 1). For a more extensive list, refer to Morse and Calderone, 2000. It has been estimated that honeybees generate upwards of 215 billion dollars in worldwide agricultural yields (Arbia & Babbay, 2011). Thus, any disorder affecting the honeybees, including those highlighted below, will have devastating effects on agriculture, the agricultural economy, but also upon our ability to feed the ever-increasing human population (Aizen, Garibaldi, Cunningham, & Klein, 2008).
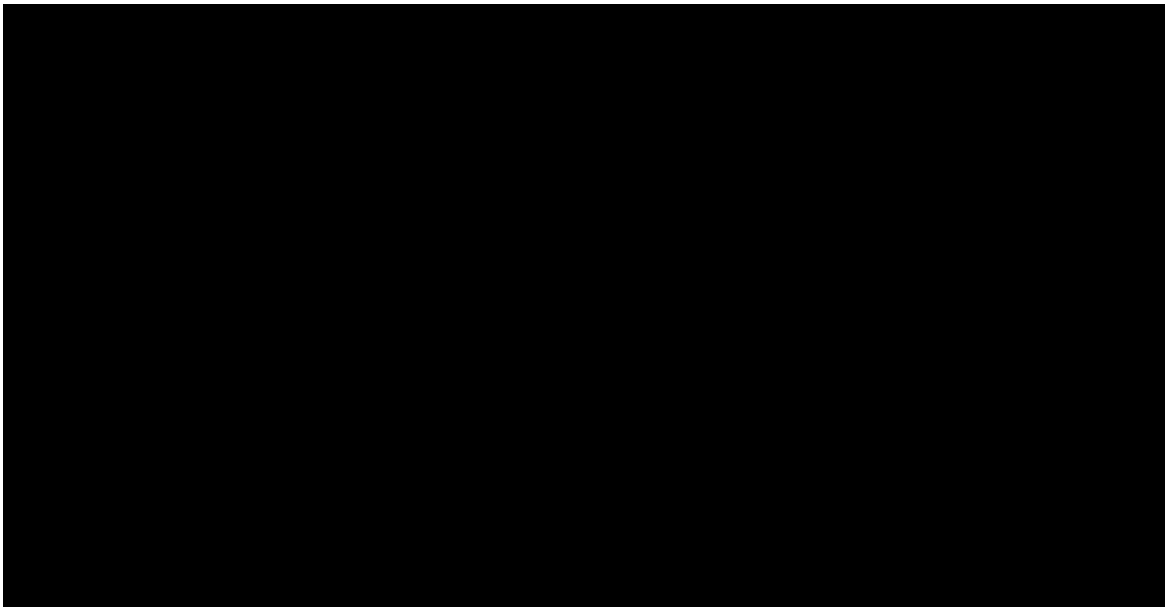
Declines in the honeybee population are expected annually, especially during the cold winter months; however, the expected loss is between twelve and fourteen percent. Having said that, most of the population is replenished during the summer months during the queen's active, egg-laying season when she can lay over 2,000 eggs a day. Despite these annual events, there has been a dramatic drop in the honeybee population since the 1940s. In fact, losses within beekeeper's populations has been so large that in the last 70 years roughly 5 million hives are down to about 2.5 million (Johnson, 2010). Consequently, it is now commonplace for honeybee colonies to be shipped between various crops depending upon the seasonal demand, sometimes travelling thousands of miles (Walsh, 2013). Many factors have been attributed to the loss in honeybees, including, but not limited to: Colony Collapse Disorder, the ectoparasitic mite *Varroa destructor* and associated Disfigured Wing Virus, and American Foulbrood Disease (Arbia & Babbay, 2011). Since my research focuses on alternative treatment strategies for American Foulbrood Disease, only this disease will be described in more detail below.

1.2. American Foulbrood Disease

American Foulbrood Disease (AFB) is caused by the bacterial pathogen, *Paenibacillus larvae*, which is found globally and can bring devastation to beekeepers with infected hives (Neuendorf, Hedtke, Tangen, & Genersch, 2004). A colony, or hive, with a rampant infection will sustain a significant loss to the brood, which are defined as the larvae within the colony. Without the new brood to replace the older workers, the whole colony is in jeopardy and is likely to die. Yet, some colonies are strong enough to recover from the losses and rebound from infection. Furthermore, there are some colonies of honeybees, such as those in South America and Africanized honeybees, which can resist infection from the pathogen. This phenomenon has been characterized by the observation that these

resistant colonies produce more propolis (a waxy substance produced by worker bees and used as structural support in the hive) than do their docile European honeybee counterparts (Danka & Villa, 1994).

AFB only affects honeybee larvae, while adult honeybees are completely resistant to infection (Hitchcock & Stoner, 1979; Wilson, 1971). As the larvae mature, they become more resistant to infection and require a higher infectious dose of *P. larvae* spores, the infectious agent, in order to display signs of disease (Bamrick, 1967; Brodsgaard, Ritter, Hansen, & Wolfgang, 1998).  The pathogen primarily infects the first, second, and third instar larvae; there are conflicting reports as to whether fourth instar larvae are susceptible to infection (Figure 1). Once the larvae have pupated and matured into adult honeybees, they will be completely resistant to infection (Bamrick, 1967; Genersch, 2010; Rinderer & Rothenbuhler, 1969). The infectious dose is low, since ingestion of ten or fewer spores can lead to the death of a larva during the susceptible stages (Chan et al., 2011; Genersch, 2010).
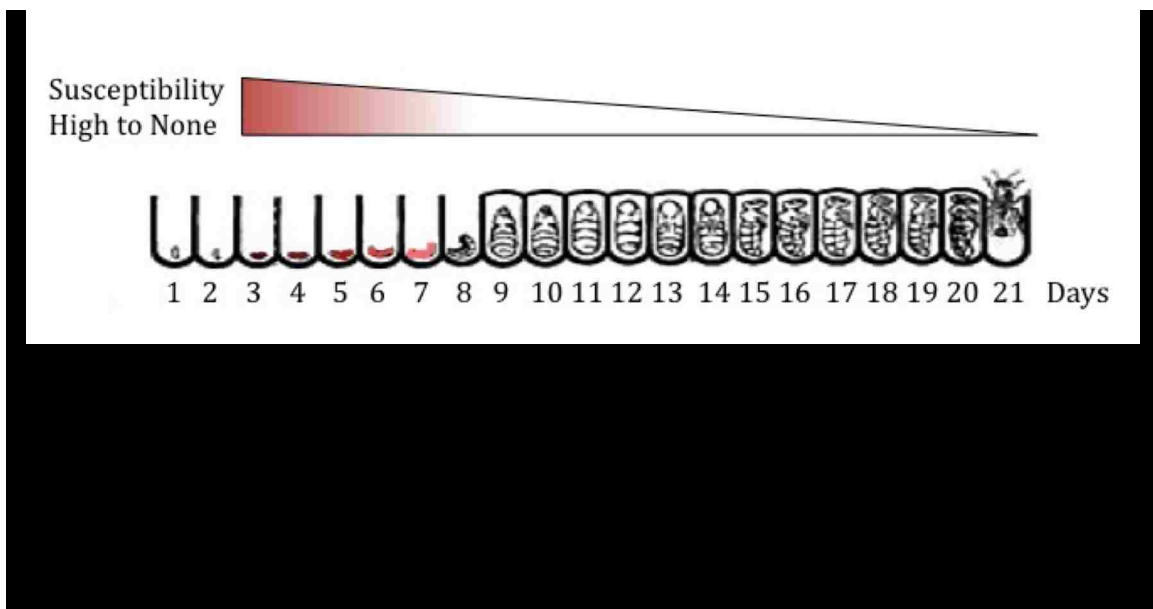
Once ingested, the infectious spores enter the foregut of the larva. Within 24h of ingestion, chemical signals within the foregut of the larva are then received by the spores causing them to germinate (Jay D Evans, 2004). The resulting vegetative cells then migrate to the midgut where they survive off the food the larva ingests. During this period, the vegetative *P. larvae* cells are multiplying and dividing as if they were commensal organisms. Once an abundant threshold level of vegetative cells accumulates within the larval midgut, proteases are secreted, eventually degrading the underdeveloped peritrophic matrix of (PM) of the larva (Garcia-Gonzalez & Genersch, 2013; Genersch, 2010; Wang & Granados, 2000; Yue, Nordhoff, Wieler, & Genersch, 2008). Two of the most characterized enzymes are enolase and an unnamed metalloprotease. Enolase is commonly found as a cytosolic enzyme involved in the glycolytic pathway, but it has been found to elicit a strong immunogenic and toxic response in honeybee larvae (Antúnez, Anido, Arredondo, Evans, & Zunino, 2011; Antúnez, Anido, Evans, & Zunino, 2010). An unnamed, secreted metalloprotease has also been found to elicit a strong immunogenic and toxic response in honeybee larvae (Antúnez et al., 2010; Antúnez, Arredondo, Anido, & Zunino, 2011). Both of these enzymes have been found within the cytosol of the vegetative cells and surrounding the spores of *P. larvae* and have been implicated in the degradation of the PM of the honeybee larva midgut (Antúnez, Anido, et al., 2011; Antúnez et al., 2010; Antúnez, Arredondo, et al., 2011). The PM is composed of a colloidal chitin, which is a major component and serves as a protective barrier for the epithelium beneath. Once the PM has been degraded, the vegetative *P. larvae* cells penetrate the underlying epithelium by the use of a paracellular route. The *P. larvae* cells eventually enter the hemocoel leading to septicemia and, ultimately, the death of the larva. Finally, the larva is turned into a brown, colloidal substance, called a "ropy mass." This ropy mass is formed by the secreted proteases of *P. larvae* (Antúnez, Anido, Schlapp, Evans, & Zunino, 2009; Genersch, 2010; Yue et al., 2008).The resulting ropy mass of dead

brood produces a foul odor, hence the name "American Foulbrood". The ropy mass dries out, forming a scale that consists of over 2.5 billion *P. larvae* spores. Spores are formed throughout the entire process, but the majority of spores are formed during the conversion of the larva into the ropy mass. These spores will be distributed throughout the colony and will infect more brood if the scale is not removed or if the cell containing the scale is not capped off with wax (Genersch, 2010).

Spores are introduced into colonies by three common routes, although others may be possible. The first mode of transmission is by nurse bees feeding contaminated food to the larvae. Nurse bees ingest royal or worker jelly and then visit cells containing larvae and feed each larva a portion of food.  Royal and worker jellies are food sources that contain different amounts of hormones and nutrients depending on whether that larva is destined to become a queen or a worker bee. By feeding contaminated food to the larvae the nurse bee has effectively sentenced to death the larvae she was attempting to rear (Alippi, 1999; Fries & Camazine, 2001; Genersch, 2010). The second common route of infection occurs during robbing where a strong colony pirates and loots a smaller, weaker colony for its resources. The transfer of resources, such as pollen (bee bread) or nectar products (honey or royal jelly), may lead to the transfer of spores and hence the disease (Lindström, Korpela, & Fries, 2008). The third mode of transmission occurs by human interactions with their honeybee colonies. This includes any tools or clothing the beekeeper may use to handle the colony. In a manner similar to robbing, there is a transfer of spores from one colony to another. Generally, frames are removed from a strong colony and placed in a weaker colony to assist with food stores. If those frames contain spores, the weaker colony may not recover if the pathogen infects its larvae (Alippi, 1999; Genersch, 2010).

Diagnosis of an infected hive can be accomplished either in the field or laboratory, with the quickest detection method being in the field. As mentioned previously, AFB is

characterized by an unpleasant odor, which is commonly compared to the smell of sweaty gym socks. If the beekeeper smells this, they may remove a frame containing brood from the colony and observe whether ropy masses are present. This is accomplished by sticking a toothpick or other sterile instrument into a cell of the beehive. The ropy mass will adhere to the toothpick and will travel up the side of the wall of the cell. Once it reaches the top, the ropy mass will stretch 1 to 2 cm from the wall before breaking (Poncea-Andronescu & Curcă, 2009).

Molecular techniques can also be used for the detection of *P. larvae*. For example, cultivation of the spore-containing brood scales can be used as an inoculum to grow vegetative cells either in liquid or solid media. Those cells can then be used for various molecular techniques, such as polymerase chain reaction (PCR), biochemical profiling, or phage sensitivity tests, in order to identify the pathogen. Other techniques that can be utilized are microscopy and immunological techniques, such as immunofluorescence and enzyme-linked immunosorbent assay (ELISA) (Alippi, 1999; de Graaf et al., 2006; Poncea-Andronescu & Curcă, 2009). While all the laboratory techniques listed take more time to diagnose the presence of AFB in comparison to the standard field technique, they are more sensitive and accurate in detecting and identifying the pathogen.

However the pathogen is detected, most beekeepers are reluctant to report the presence of the pathogen (some states require reporting incidences of AFB). This reluctance is due to the destructive nature of eradicating the pathogen through burning of the hive. Beekeepers do not want to lose entire hives and associated tools, as they are sources of revenue and a significant investment. Therefore, the prevalence of the disease may be underestimated due to underreporting.

1.3. The Organism *Paenibacillus larvae*

The bacterial pathogen *P. larvae* is a gram-positive, endospore-forming bacillus responsible for the honeybee brood disease AFB. The oval-shaped spores are the infectious agent that can remain viable for many years, even more than 70 years from a dormant sample (Alippi, 1999; Genersch, 2010); therefore, *P. larvae* spores are incredibly resilient. Spores can withstand the test of time, UV radiation, and even chemical challenge. The only known host of *P. larvae* is the honeybee larvae. Extensive characterization of this organism began over a century ago and has continued ever since (Genersch, 2010).

G. F. White characterized the pathogen in 1906 when he attempted and failed to isolate another spore-forming, honeybee pathogen, *P. alvei* (White, 1906). White originally named the organism *Bacillus larvae*, but it was later renamed *Paenibacillus larvae* based on characteristic 16S rRNA signatures among group 3 bacilli (Ash, Priest, & Collins, 1993). *P. larvae* was once classified into two subspecies, *P. l. larvae* and *P. l. pulvifaciens*, due to the different colors of the colonies produced. The former produces grayish-white colonies that are smooth and shiny, while the latter produces reddish-orange colonies. Some other differences between the two subspecies were also discovered based on different enterobacterial repetitive intergenic consensus (ERICs) that are based on 16S rRNA gene sequences. ERICs are palindromic sequences that occur in enteric bacteria, as well as vibrios, and only within intergenic regions of transcribed regions (Wilson & Sharp, 2006). *P. larvae* has ERICs that are classified using the numbers I-IV. With an increasing ERIC number classification, the *P. larvae* strain is considered to be less virulent. *P. l. larvae* was classified as belonging to ERICs I and II, while *P. l. pulvifaciens* belongs to ERICs III and IV (Genersch, 2010). However, after a genetic comparison was conducted, the two organisms were not significantly different enough to classify them under the subspecies level, so the subspecies nomenclature was dropped (Heyndrickx et al., 1996).

1.4. Treatment Methods of AFB

Current methods to treat hives infected with *P. larvae* include the use of antibiotics, specifically oxytetracycline (OTC) and chloramphenicol (Arbia & Babbay, 2011; Genersch, 2010; Miyagi, Peng, & Chuang, 2000), burning the hive and all associated tools (Alippi, 1999), or the use of tylosin tartrate (Elzen et al., 2002; Peng et al., 1996). The first method of using OTC is very controversial, primarily due to the overuse of the antibiotic which has led to the emergence of OTC-resistant strains of *P. larvae* (Miyagi et al., 2000). To compound the issue, residual antibiotics may infiltrate honeybee products, such as honey and propolis (which is also eaten), which are sold commercially. Consequently, the use of antibiotics has been banned in many countries, particularly those belonging to the European Union, because of the potential ramifications it could have on the long-term health of consumers (Dharmananda, 2002).

Beekeepers have previously attempted fumigating or washing infected hives and tools with chemical detergents in hopes of salvaging their tools. This method proved to be ineffective when healthy colonies were becoming infected with residual spores from the tainted tools (Arbia & Babbay, 2011). The current universally accepted method of treatment is destruction of the hive and associated tools by fire. This process is extremely destructive and results in multi-million dollar losses to the beekeeping community (Alippi, 1999). As mentioned previously, spores are incredibly resilient structures and only the extreme heat produced by burning the hive can destroy them.

Finally, the newest treatment against American Foulbrood is the use of tylosin tartrate. This compound is generally fed to pigs as an antimicrobial compound. The powder is sprinkled on and within the hive to prevent *P. larvae* growth, which has been reported to be effective (Elzen et al., 2002; Miyagi et al., 2000; Peng et al., 1996); however, the length of

time this antibiotic will remain effective and the long-term effects of its use are unknown

(Peng et al., 1996).

1.5. Honeybee Immunity

The immune system of the honeybee helps prevent disease at the individual level as well as at the colony level.  Honeybee immunity is subcategorized into two main branches: social immunity and innate immunity (described in Figure 2). Both branches work in tandem to prevent disease within the entire colony, between individuals, and within individuals (Evans & Spivak, 2010), which is explained in more detail below.
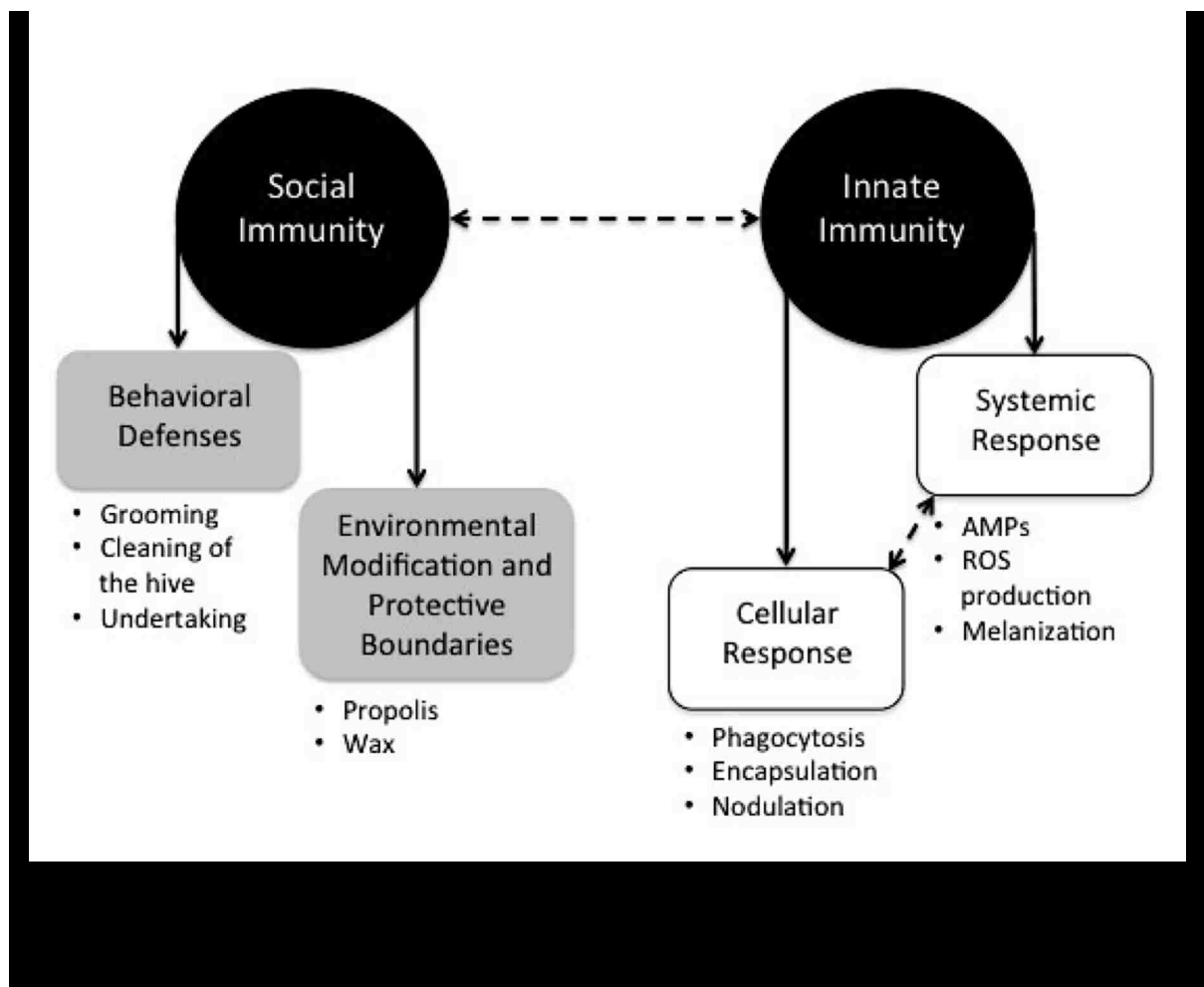
### 1.5.1. Social Immunity

Social immunity is a broad form of immunity that can be generalized into two main categories: behavioral defenses and modification of the environment. Behavioral defense describes the hygienic behavior of the honeybee which includes, but is not limited to, grooming themselves or each other (auto- and allo-grooming, respectively), extensive cleaning of the hive of debris and carcasses that could be potentially infectious, and undertaking, or the process of cleaning up after the dead (Evans & Spivak, 2010). All these behaviors work to protect the bees within the colony; however, if the bees take more than 48 h to respond to the carcass or debris within the hive, the pathogen can spread throughout the colony (Evans & Spivak, 2010).

The other half of social immunity involves modification of the environment, specifically the components within and immediately surrounding the hive. Typically, honeybees in the wild live in a hollowed out portion of a tree where they remove the outer layer of decaying wood to expose the hardwood. This cavity is then layered with propolis, a combination of wax and collected plant resins, to protect the wood from fungal decay and reduce exposure to the external environment. These behaviors are also observed in beekeepers' hives (Evans & Spivak, 2010; Simone, Evans, & Spivak, 2009). As mentioned previously, some forms of propolis can decrease the instances of AFB (Danka & Villa, 1994). These two levels of social immunity, hygienic behavior and protective boundaries, such as propolis, provide a necessary first line of defense against many of the honeybee pathogens, but alone this is not sufficient. The innate immune response within each individual honeybee, handles the rest.

1.5.2. Innate Immunity

The innate immune response of the honeybee is broken down into two categories: cellular immunity and systemic immunity. Both systems work in tandem to provide a robust immune response to microbial infection. These systems have been characterized extensively in *Drosophila melanogaster*, as well as other insects, such as cecropin moths and *Anopheles* mosquitoes (Evans & Spivak, 2010; Lavine & Strand, 2002; Lemaitre & Hoffmann, 2007); therefore, many of the same principles can be applied to the honeybee, *A. mellifera*. However, some differences do exist which may stem from the fact that the honeybee is a social insect (Evans et al., 2006). After a genome-wide analysis, it was reported that honeybees have roughly two thirds fewer immune genes than solitary insects, such as *D. melanogaster* and *Anopheles* (mosquito) (Evans et al., 2006).

The first category of the innate immune response, cellular immunity, is composed of many specialized cell types, collectively known as hemocytes, which are found both free-floating and stationary in the hemolymph (Hoffmann & Reichhart, 1997). The main hemocytes that have been studied include plasmatocytes, granulocytes, lamellocytes, and crystal cells. Each has a specialized function and are all derived from the precursory stage prohemocyte (Hoffmann & Reichhart, 1997; Lavine & Strand, 2002; Lemaitre & Hoffmann, 2007). These various cell types perform a variety of functions such as phagocytosis (plasmatocytes), nodule formation (granulocyte and lamellocytes), and encapsulation (lamellocyte). Nodule formation and encapsulation involve various hemocytes that aggregate around the invading microorganism and remove large numbers of invading cells within two hours of infection, but do not trigger the expression of antimicrobial genes (Hoffmann, 1995; Hoffmann & Reichhart, 1997; Vilmos & Kurucz, 1998). Unlike the previously mentioned hemocytes, crystal cells do not adhere to their target cells like plasmatocytes, granulocytes, and lamellocytes and do not directly kill pathogens. Instead,

crystal cells contain phenoloxidase (PO) precursors that participate in melanization (a process that falls under the category of systemic immunity). Consequently, crystal cells can be considered the cellular  bridge to systemic immunity (Hoffmann & Reichhart, 1997).

Systemic immunity is comprised of three different processes: melanization, the production of reactive oxygen species (ROS), and antimicrobial peptides (AMPs). Melanization occurs at the wound site in order to prevent invading microorganisms from spreading further. Crystal cells migrate to the wound site and readily release their contents, mainly prophenoloxidase (proPO). Once the crystal cell has released its contents, proPO is enzymatically converted into PO, which is oxidized to quinones. The quinones polymerize into melanin which deposits around invading microorganisms (Hoffmann & Reichhart, 1997; Schmid, Brockmann, Pirk, Stanley, & Tautz, 2008; Tang, 2009).  Melanization is generally associated with the encapsulation process which contributes to the killing of the invading pathogen (Vilmos & Kurucz, 1998).

ROS production provides a source of protection against gut invading microbes (Lemaitre & Hoffmann, 2007).  Upon infection, there is an immediate formation of ROS in the gut of *Drosophila* through NADPH-dependent oxidase enzyme and dual oxidase enzymatic activity (Ha, Oh, Bae, & Lee, 2005). ROS are oxygen-containing molecules, such as superoxide radicals, hydrogen peroxide, and nitric oxide and are extremely reactive. Acids can also be formed to defend against microbial invasion from these precursor molecules (Dowling & Simmons, 2009; Fang, 2004). These ROS are typically derived from oxidation-reduction reactions occurring naturally within the body of the organism and used as signaling molecules (Dowling & Simmons, 2009). However, when a microbial infection is detected, production of these ROS is ramped up through the enzymatic pathways listed above.  ROS can damage bacterial cells by creating double strand breaks in DNA, protein residue modifications within the microbial cell and/or on the microbial cell surface. Many

mechanisms have been elucidated due to the different environments in which ROS are found (Fang, 2004; Fang, 2011). In order to protect the host DNA, RNA, and proteins from the deleterious effects of the ROS, following the ROS response the host produces enzymes and non-enzymatic mechanisms to reduce associated damage and to clear up any remaining ROS (Dowling & Simmons, 2009; Fang, 2004).

### 1.5.2.1. Antimicrobial Peptides

Antimicrobial peptides (AMPs) are a component of the innate immune system. These peptides have broad-spectrum antimicrobial properties against bacteria, as well as fungi and viruses (Izadpanah & Gallo, 2005). AMPs are ubiquitous throughout nature and are present in plants (Castro & Fontes, 2005), invertebrate and vertebrate animals (Izadpanah & Gallo, 2005). Generally, in honeybees, these peptides are synthesized in the fat body or specialized glands in an inactive form, called the pro-peptide, and are subsequently proteolytically cleaved upon infection. Once the pro-peptide sequence is cleaved, the AMP becomes active and able to function as an antimicrobial. In humans, AMPs are part of the innate immune response, the first line of defense, before the acquired immune system generates a specific response (Xu, Shi, & Chen, 2009). However, honeybees lack an acquired immune system, so they depend on the function of AMPs and other components of their innate immune response. AMPs are generally positively charged, allowing them to be attracted to the negatively charged, bacterial cell envelope. Once attached to the bacterial cell envelope, the peptide can either enter the cytoplasm where they render the pathogen non-functional through a variety of mechanisms (different classes of AMPs have different mechanisms), or kill the pathogen directly through the process of pore formation, which leads to cell lysis. Although there are others, the specific honeybee AMPs that are the focus of this research include: apidaecins, abaecins, hymenoptaecins and

defensins (a brief summary of characteristics can be seen in Table 2). A more detailed

description of the honeybee AMPs used in this study can be found in Chapter 3.
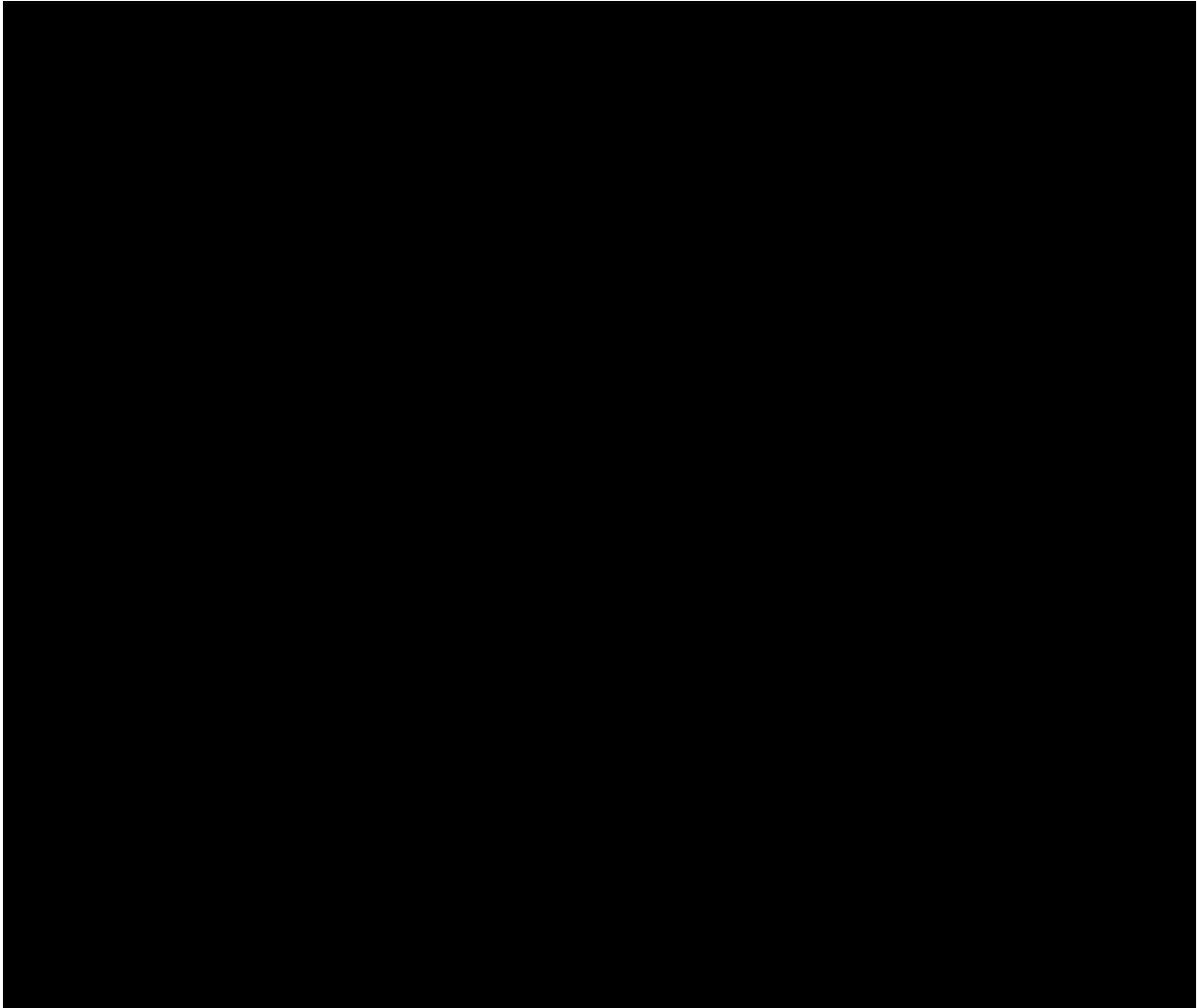
1.6. Overall Goal of the Project & Rationale

Treatment of AFB Disease with antibiotics is not ideal due to the development of antibiotic resistance in *P. larvae*. Additionally, the prolonged consumption and use of antibiotic-containing honeybee products could potentially cause detrimental effects in humans (Arbia & Babbay, 2011; Chan et al., 2011). As described previously, antibiotics have been banned in European countries for these reasons. Therefore, there is a pressing need to develop an alternative treatment strategy that can circumvent these issues.

In 2004, Nathan suggested the concept of alternative methods for treatment of infectious disease, specifically human disease. One potential alternative treatment method is the use of AMPs (Nathan, 2004), which is the focus of my research. AMPs are an attractive alternative treatment option for several reasons. First, AMPs are naturally occurring peptides, generally between fifteen and forty-five amino acids in length, with broad-spectrum antimicrobial specificity (Boman, 2003). Second, they are a safer option than antibiotics due to the fact that they only attack bacteria, fungi, and viruses (Parisien, Allain, Zhang, Mandeville, & Lan, 2008). This eliminates the potential of harming the organism, while eradicating infection. Third, AMPs employ a wider variety of mechanisms used to eliminate microbes in comparison to conventional antibiotics (Sang & Blecha, 2008). Fourth, far less compound needs to be used. For example, the minimal inhibitory concentration (MIC) for some AMPs ranges from 1-8 μg/ml (Hancock & Chapple, 1999), in comparison to some antibiotics that are used at an effective concentration of 25 μg/ml and even higher, like chloramphenicol. Finally, bacteria are less prone to developing resistance to AMPs, even those that are multi-drug resistant, such as methicillin-resistant *Staphylococcus areus* (Boman, 2003; Hancock & Chapple, 1999; Parisien et al., 2008; Sang & Blecha, 2008). For example, it has been shown that after 20 passages of different organisms with AMP concentrations close to the MIC that no resistant mutants were recovered

(Hancock & Chapple, 1999). Thus, AMPs are promising candidates as an alternative treatment method.

Importantly, the concept of alternative treatment method for AFB has never been proposed, and yet there is a dire need for one due to the declining population of honeybees. Currently, a USDA grant awarded to the School of Life Sciences and the Chemistry Department at UNLV is focused on developing alternative treatment methods using a either germinant inhibitors, lysogenic phage, AMPs or a combination thereof. By adapting a series of treatments to prevent infection and treat infection (if it manifests), these alternative treatment strategies can be utilized to prevent and treat AFB from occurring within a colony and save the beekeeping industry millions of dollars.

The overall goal of this project is to use AMPs found in the adult honeybee, which are resistant to infection by *P. larvae*, in order to identify a potential alternative treatment to prevent *P. larvae* infection of honeybee larvae. Like human infants that do not have a fully developed immune system, it is possible that the larvae are susceptible to infection with *P. larvae* because they do not possess the active AMPs, which are present in the adults and form an important part of an insect's immune response. Therefore my research involves testing active AMPs, which are found in the adult honeybee, to determine whether these naturally occurring compounds are active against *P. larvae* and see if they could be used as a remedy for this disease. The use of AMPs is a superior approach to preventing/treating AFB than common practices, such as antibiotics (Sang & Blecha, 2008), due to the unlikeliness of developing AMP-resistant bacteria and the prevention of burning and destroying hives.

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CHAPTER 2

PROTOCOLS TO TEST THE ACTIVITY OF ANTIMICROBIAL PEPTIDES AGAINST THE HONEY

BEE PATHOGEN *PAENIBACILLUS LARVAE*


2.1. Forward

This chapter is based on a manuscript that has been accepted for publication in The Journal of Microbiological Methods authored by Jasmin C. Khilnani and Helen J. Wing. This work is based on the collaborative research headed by Michelle Elekonich that was conducted in both the School of Life Sciences and Chemistry Department at UNLV. All work focusing on the activity of antibacterial peptides in honeybees is my own under the advisement of my mentor, Helen J. Wing.  This manuscript has been reformatted for this thesis.


2.2. Keywords

*Paenibacillus larvae*, American Foulbrood disease, Antimicrobial compounds, honey bee

2.3. Abstract

*Paenibacillus larvae* is the causal agent of the honey bee disease American foulbrood. Two enhanced protocols that allow the activity of antimicrobial peptides to be tested against *P. larvae* are presented. Proof of principle experiments demonstrate that the honey bee antimicrobial peptide Defensin 1 is active in both assays.

2.4. Introduction

American Foulbrood disease (AFB) is a contributing factor in the decline of honey bee

(*Apis mellifera*) populations (Evans and Schwarz, 2011). It is the most destructive and

widespread of all the bee brood diseases (Shimanuki, 1983). Infectious spores of *P. larvae*

infect honey bee larvae within 72 hours of eclosion (hatching from the egg), but adults are

resistant to AFB. Despite its name, AFB is found worldwide, wherever honey bees are kept

(Matheson, 1993). While antibiotics can be used as effective prophylaxes, they are not

recommended for the treatment of colonies that are actively infected, because this can i)

select for resistant strains of *P. larvae* (Murray et al., 2007), ii) lead to traces of antibiotics in

honey, and iii) trigger vegetative *P. larvae* cells to sporulate, where they remain as

endospores until antibiotic levels decrease, at which time a new infection can occur

(Genersch, 2010; Lindstrom et al., 2008). Consequently, there is need to improve upon the

current strategies used to control this disease, and so alternative treatment strategies

and/or prophylaxes for AFB are currently being investigated (Alvarado et al., 2015;

Alvarado et al., 2013; Beims et al., 2015; Chan et al., 2009; Ghorbani-Nezami et al., 2015;

Gonzalez et al., 2015; Oliveira et al., 2015; Piana et al., 2015).

Antimicrobial peptides (AMPs) synthesized within the resistant adult honey bee

provide an attractive solution to this problem because they have the potential to be used to

fortify the natural immune system of the susceptible larvae. Furthermore, it has been

proposed that bacteria are unlikely to develop resistance to these AMPs, because these

natural compounds will not present a new selection pressure for these bacteria (Boman,

2003). Although methods to measure the activity of antimicrobials on *P. larvae* exist (de

Graaf et al., 2013), these protocols require relatively large quantities of the antimicrobial

and are not optimized for larger molecular weight (>500 Da) molecules like AMPs. Instead

they were developed to test the resistance/susceptibility of *P. larvae* to commercially available antibiotics (de Graaf et al., 2013).

Here, we present and compare two newly enhanced protocols that allow the antimicrobial activity of low abundance, high molecular weight compounds, like AMPs, to be tested against *P. larvae* in semi-solid and liquid media. The first protocol is a modified zone of inhibition assay, in which test compounds are applied directly to a well in a low percentage agar plate. The second protocol is an adaptation of a minimal inhibitory concentration (MIC) assay, in which the antimicrobial activity of compounds is tested in 96-well plate format. The advantage of both of these protocols over currently published methods is that they allow the antimicrobial activity of candidate compounds that are high in molecular weight, low in supply or otherwise prohibitively expensive to be tested.

2.5. Methods

2.5.1. Growth of Bacterial Strains

 In this study, our newly adapted methods were used to test the activity of two active honey bee AMPs against either *P. larvae* strain B-3554 (American Tissue Culture Collection number; NRRL B-3554) or *E. coli* MC4100. For each of our growth inhibition assays, cultures were grown from frozen glycerol stocks (20% v/v glycerol) in R2B medium (EMD Millipore) at 3x concentration (3x R2B). This medium was chosen for these studies because i) *P. larvae* grows well in this medium, ii) it is commercially available, iii) it has similar composition to MYPGP, a medium  used for this kind of assay previously (de Graaf et al., 2013), and iv) unlike other commonly used broths, such as Brain Heart Infusion (BHI) medium, it does not contain sodium chloride, which has been shown to inhibit the function of honey bee AMPs (Casteels et al., 1989; Casteels et al., 1990; Chi et al., 2003). Cultures were routinely grown for 16 h at 37°C with aeration in an orbital shaker set to 325 rpm

(MaxQ 4000 Thermo Scientific), sub-cultured (1:20) and grown for an additional16 h at

37°C with aeration. This method of growth was found to minimize *P. larvae* cell aggregation,

which became important for our 96-well plate assays.


   2.5.2. Zone of Inhibition Assay

   For our modified zone of inhibition assays, 500 μl of *P. larvae* and 250 μl of *E. coli*

overnight culture were harvested and pelleted by centrifugation. The supernatant was

discarded, cells were resuspended in 150 μl of fresh 3x R2B, and cell suspensions were

spread onto 3x R2A plates containing 1.5% (w/v) agar (reduced from the standard 2%).

These inocula ultimately allowed the growth of robust bacterial lawns. Once the cell

suspension had been absorbed, a hole was punched into the center of the agar using a

sterile, pre-cut pipet tip, and 2 μl of the AMPs at a concentration 4 mg ml$^{-1}$ (solubilized per

manufacturer's instructions) was added to the hole. Plates were sealed with Parafilm® to

prevent dehydration and incubated at 37°C. Zones of inhibition were measured after 48 h.

All experiments were done in triplicate.


   2.5.3. 96-well Plate Assay

   For our 96-well plate assays, overnight cultures of *P. larvae and E. coli* cultures were

diluted in 3x R2B medium to an OD$_{600}$ of about 0.2. The inner wells of a clear, flat-bottomed,

96-well plate (Greiner Bio-One) containing 5 μl of AMP (ranging from 200 to 0.2 mg ml$^{-1}$) or

distilled water were filled with 95 μl of inoculum. To minimize evaporation and other edge

effects, outermost wells of plates were filled with 100 μl of water. Plates were incubated for

24 h at 37°C in a Tecan m200 plate reader, which shook the plate in orbital mode at 336

rpm for 5 min every 15 min (amplitude of 1.5 mm). Five absorbance scans at 600 nm were

taken every 15 minutes and the average was reported. Growth curves were plotted to

determine whether the AMP affected cell growth by comparing the antimicrobial-treated samples to water-treated controls. On each plate five replicate wells were used per condition and all experiments were independently replicated three times.

2.6. Results

Using these approaches, two AMPs produced by adult honey bees, the bacteriostatic compound apidaecin (Anaspec; Fremont, CA; MW 2.1 KDa) and the bactericidal compound defensin 1 (Peptide 2.0, Chantilly, VA; MW 5.5 KDa), were tested against *P. larvae* and *E. coli* [*E. coli* was previously demonstrated to be sensitive to apidaecin in liquid assays (Casteels et al., 1989) while defensin 1 was previously shown to be active against *P. larvae* in a polyacrylamide gel overlay experiment (Bachanova et al., 2002)]. As expected, apidaecin exhibited detectable antibacterial activity against *E. coli* in both the zone of inhibition assay (8 mm in diameter; Figure 3A) and the 96-well plate assay over the 24 h incubation period (Figure 3E). In contrast, defensin 1 did not display antimicrobial activity against *E. coli* in either assay (Figure 3, C & G), which is consistent with previous findings that demonstrate this AMP is primarily active against Gram-positive bacteria (Ilyasov et al., 2013). When the antimicrobial activity of apidaecin was measured against *P. larvae*, no growth inhibition was detected in the zone of inhibition assays (Figure 3B) but surprisingly certain concentrations displayed modest but significant antibacterial activity against *P. larvae* in the 96-well plate assay over the 24 h incubation period (Figure 3F). In contrast, as predicted (Bachanova et al., 2002) defensin 1 displayed robust antimicrobial activity against *P. larvae* in both the zone of inhibition (6 mm in diameter; Figure 3D) and the 96-well plate assay (Figure 3H).
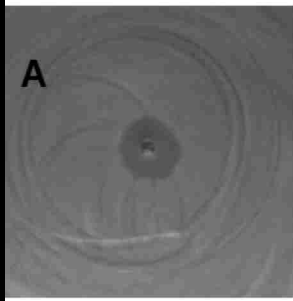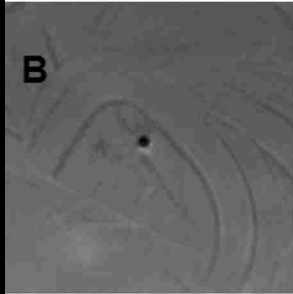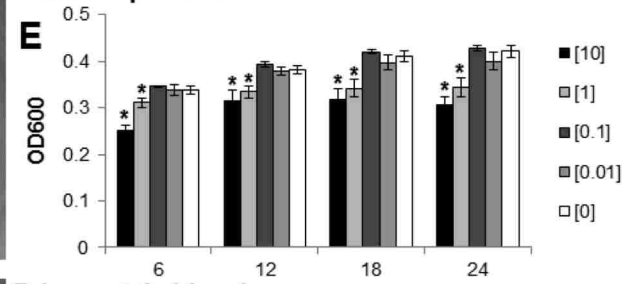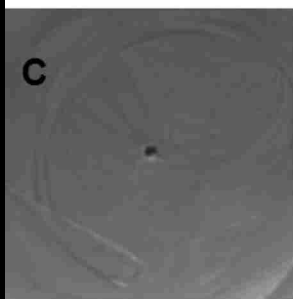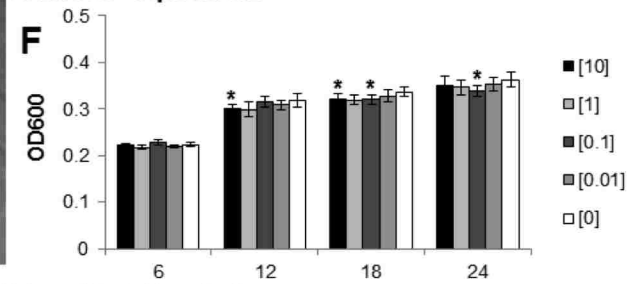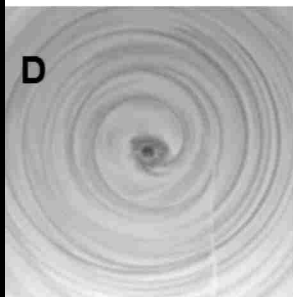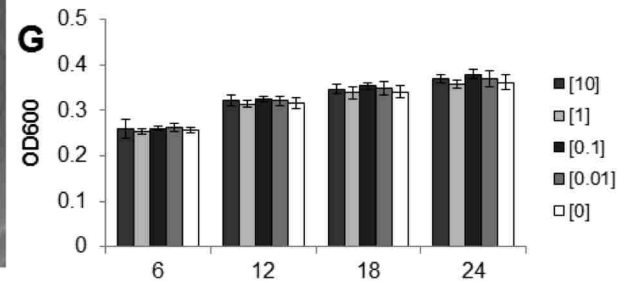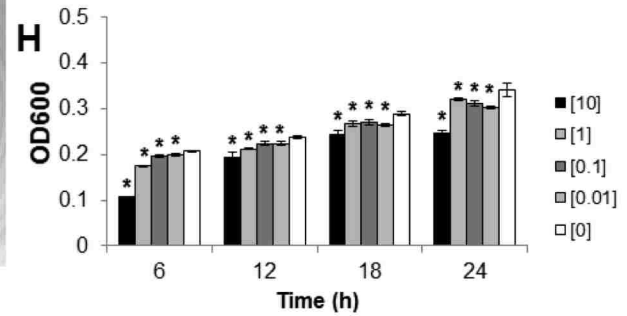
## 2.7. Conclusions

To conclude, this study demonstrates that two newly adapted protocols allow the antimicrobial activity of high molecular weight, low abundance compounds, like AMPs, to be tested against *P. larvae*. Each of the methods provide significant improvements over currently existing protocols [described in (de Graaf et al., 2013)], which were initially developed to test the resistance/susceptibility of *P. larvae* to commercially available antibiotics. In the zone of inhibition assay, by placing the AMP in a hole in the center of the agar plate, reducing the agar concentration and removing sodium chloride from the growth medium, the activity and diffusion of the AMPs through the agar was less impeded. As a result, for the first time apidaecin was found to be active against *E. coli* in semi-solid medium, which was not the case when we initially used the disc diffusion assay (de Graaf et al., 2013) to test the antimicrobial activity of this compound (data not shown). This demonstrates the newly adapted zone of inhibition assay is an improvement over the currently published disc diffusion assay (de Graaf et al., 2013). In the 96-well plate assay, by decreasing the volume of *P. larvae* cultures to 100 ml, much less test compound was required, making these assays much more suitable for testing the activity of low abundance compounds than the published MIC assays (de Graaf et al., 2013). For the compounds tested in this study, the 96-well plate assay proved to be more sensitive than the zone of inhibition assay, allowing even the modest antimicrobial activity of apidaecin to be detected against *P. larvae* (compare Figure 3F to 3B). Nevertheless, we strongly recommend the use of both protocols when initially characterizing the antimicrobial activity of compounds like AMPs because the activity of these compounds may vary in semi-solid and liquid media. In summary, the protocol adaptations and the recommendations presented in this work will prove useful to those evaluating high molecular weight (up to 5.5 KDa), low abundance antimicrobials for use in treatments and /or prophylaxis strategies for AFB.

## 2.8. Acknowledgements

## 2.9. References

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CHAPTER 3

CHARACTERIZATION OF HONEYBEE ANTIMICROBIAL PEPTIDES AGAINST THE HONEYBEE

PATHOGEN *PAENIBACILLUS LARVAE*

3.1. Introduction

*Paenibacillus larvae* is the most devastating bacterial pathogen of *Apis mellifera*

(honeybees) and is the causative agent of American Foulbrood Disease (AFB). In spite of its

name, AFB is found globally. Spores are the infectious unit of this Gram-positive pathogen

and are easily transmissible throughout the colony, either through normal honeybee

nursing activity or through human beekeeping practices. AFB occurs when spores infect the

first, second and third instar larvae within the hive. Once ingested by the larvae, the spores

germinate within the midgut and the resulting pathogenic vegetative cells lead to the onset

of AFB, which is characterized by the presence of a foul smelling, brown, glue-like substance

called a "ropy mass". If left untreated by the honeybees or the beekeeper, AFB leads to the

demise of the entire colony (Genersch, 2010).

Adult honeybees are resistant to infection with *P. larvae* spores (Crailsheim &

Riessberger-Gallé, 2001; Wilson, 1971). I hypothesize this is because adult honeybees have

a fully functional innate immune system that harbors active antimicrobial peptides (AMPs).

Generally, there is a basal-level of AMPs in the hemolymph of adults honeybees that is

synthesized in the adipocytes found in the fat body, epithelial cells, and various hemocytes

(Shen et al., 2010). Upon any microbial infection, the synthesis of AMPs is up-regulated

through either the Toll or Imd (Immune deficiency) pathways (Lemaitre & Hoffmann,

2007). The Imd pathway is responsible for the synthesis of AMPs that target Gram-negative

pathogens, while the Toll pathway is responsible for the synthesis of AMPs that target

Gram-positive and fungal pathogens (Evans & Spivak, 2010; Lemaitre & Hoffmann, 2007).

Interestingly, one category of AMPs, the defensins, is synthesized by the signaling of both

the Imd and Toll pathways (a summary of these pathways can be seen in Fig. 4) (Hoffmann, 1995; Hoffmann & Reichhart, 1997; Lemaitre & Hoffmann, 2007; Lemaitre, 2004). My study focuses on five out of the six characterized cationic AMPs synthesized by the honeybee: apidaecin, abaecin, hymenoptaecin, defensin 1, and defensin 2.
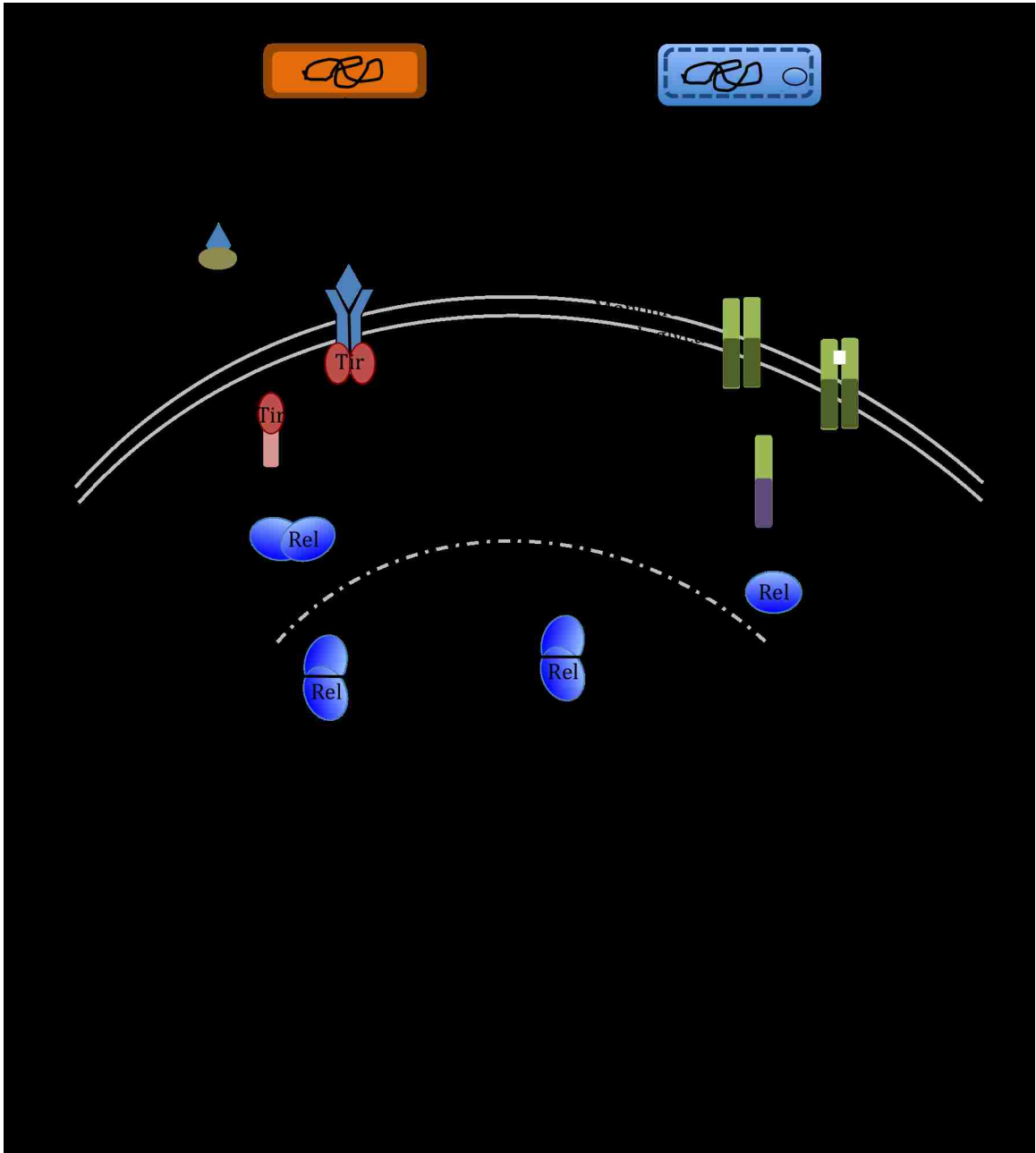
The first three honeybee AMPs to be characterized are all reported to be primarily active against Gram-negative bacteria. Apidaecin (Casteels, Ampe, & Jacobs, 1989) has been the most extensively studied out of the five. This proline-rich (33% proline) AMP is 18 amino acids long and has a linear structure (Casteels et al., 1989; Li, Ma, & Zhou, 2006). Apidaecin works in a bacteriostatic fashion by inhibiting the protein chaperone, DnaK (Li et al., 2006). Next, abaecin was characterized. It is made up of 34 amino acids, has a linear structure, is proline-rich (29% proline), and also works in a bacteriostatic manner. The exact mechanism of its inhibitory action has yet to be elucidated but it has been speculated that it may work on the bacterial membrane (Casteels et al., 1990; Shen et al., 2010). Hymenoptaecin, the third AMP to be characterized, is entirely unique to honeybees. It consists of 93 amino acids, is glycine-rich (19% glycine) but the exact structure has yet to be determined. Hymenoptaecin has been shown to form pores in the inner and outer membranes of gram-negative bacteria, it is bactericidal in nature, but the specific mechanism of action remains unclear (Casteels, Ampe, Jacobs, & Tempst, 1993) (see Table 3).

The final two honeybee AMPs this research focuses on, defensin 1 (or royalisin) and defensin 2, belong to the family of defensins that is present throughout all eukaryotes. Defensins are predominantly active against Gram-positive bacteria and fungi. The category of defensins are characterized to work in a bactericidal manner by forming pores in the cellular membrane (Ganz, 2003). Honeybee defensins 1 and 2 are cysteine-rich, 12% and 7% respectively, and each contains three disulfide bridges that link β-pleated sheets (Ilyasov, Gaifullina, Saltykova, Poskryakov, & Nikolaenko, 2013; Ilyasov, Gaifullina, Saltykova, Poskryakov, & Nikolenko, 2012). Both of these characteristics are hallmarks of the defensin family (Ganz, 2003). Defensin 1 is unique in that it is the only honeybee AMP produced in specialized cells of the hypopharyngeal, mandibular, and thoracic salivary

glands. Defensin 2 is then secreted into bee products, such as royal jelly and honey (Ilyasov, Gaifullina, Saltykova, Poskryakov, & Nikolaenko, 2013; Ilyasov, Gaifullina, Saltykova, Poskryakov, & Nikolenko, 2012). So far, defensin 1 is the only honeybee AMP to be tested against *P. larvae,* and it was found to display antimicrobial activity (Bachanová et al., 2002; Ilyasov et al., 2013; Ilyasov et al., 2012) (see Table 3). Defensin 2 is formed in the fat body and hemocytes, like the other AMPs, and circulates throughout the hemolymph (Ilyasov, Gaifullina, Saltykova, Poskryakov, & Nikolaenko, 2013; Ilyasov, Gaifullina, Saltykova, Poskryakov, & Nikolenko, 2012).

None of these five honeybee AMPs have been tested for their potential to be used as alternative treatments for AFB. Alternative treatment methods are necessary because current methods of *P. larvae* eradication are either insufficient or destructive (i.e. oxytetracycline resistance and burning of hives, respectively) (Arbia & Babbay, 2011). AMPs are an appealing form of alternative treatment of AFB for the following reasons: 1) they display antimicrobial activity at lower concentrations than conventional antibiotics (Hancock & Chapple, 1999), 2) they have more modes of action than conventional antibiotics (Sang & Blecha, 2008), 3) they have broad antimicrobial activity (Boman, 2003; Hancock & Chapple, 1999; Sang & Blecha, 2008), 4) they are safer than antibiotics in that they do not have side effects on host cells, and 5) bacterial resistance is less likely to develop against AMPs in comparison to antibiotics (Boman, 2003; Hancock & Chapple, 1999; Parisien, Allain, Zhang, Mandeville, & Lan, 2008; Sang & Blecha, 2008). For these reasons, I decided to pursue the use of naturally occurring AMPs found in the adult honeybee as an alternative treatment method for AFB.

The aim of my research was to test the five honeybee AMPs described above against vegetative *P. larvae* cells *in vitro* in order to find potential alternative treatment candidates. The activity of the AMPs was tested in two assays. A zone of inhibition assay, which tested them in a semi-solid medium, and a liquid culture assay conducted in microtiter (96-well) plate format. My goal was to find an AMP that killed vegetative *P. larvae* cells or inhibited its growth in both assays. Those AMPs could then be investigated further in future *in vivo* studies testing active *P. larvae* infection in honeybee larvae.
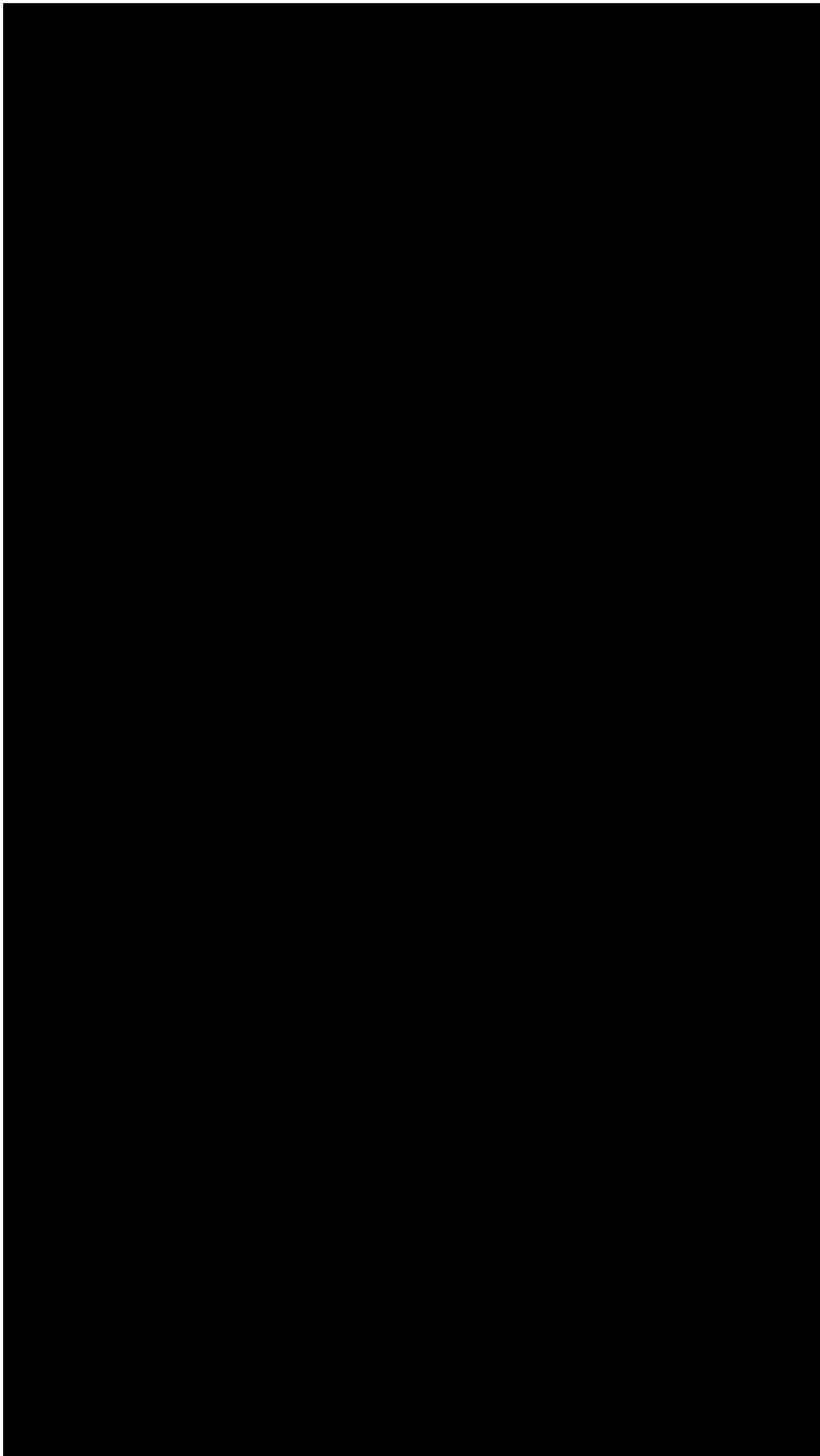
3.2. Methods

3.2.1. Source and Storage of AMPs

All honeybee AMPs were synthesized commercially since no difference in antimicrobial activity has been detected in relation to honeybee purified AMPs (Peter Casteels et al., 1989). All AMPs were received from the manufacturer as a lyophilized powder. Since no functional differences exist between the different apidaecin isoforms (IA, IB, and II) (Casteels et al., 1989), apidaecin IB was used for these studies because it is commercially available (Anaspec; Fremont, CA) and comprises 85-90% of the apidaecin content in the hemolymph of an infect honeybee. Any reference to apidaecin therefore means apidaecin IB. Abaecin was synthesized to a 95% purity level from GenScript (Piscataway, NJ). Hymenoptaecin and defensins 1 and 2 were synthesized to a 95% purity level from Peptide 2.0 (Chantilly, VA). Apidaecin, abaecin, hymenoptaecin, and defensin 1 were solubilized in sterile, distilled water according to manufacturer's instructions. Defensin 2 was solubilized in acetonitrile, sterile distilled water, and methanol (using a 1:2:1 ratio) according to manufacturer's instructions. All peptides were stored for up to 6 months at -20 °C.

3.2.2. Growth Conditions of Organisms

Refer to Chapter 2 for growth conditions for *Escherichia coli* (strain MC4100), and *P. larvae* (strain NRRL B-3554). *P. alvei* (strain 33A3) was subject to the same growth conditions as *E. coli*.

3.2.3. Zone of Inhibition Assay

The ZoI assay was developed from disk diffusion assays (de Graaf et al., 2013) in order to maximize detection of zones of clearance. Refer to Chapter 2 for a more detailed description of the procedure. All AMPs categorized as bacteriostatic (apidaecin and abaecin) were positively controlled with chloramphenicol, as this antibiotic works via a bacteriostatic mechanism. The three AMPs categorized as bactericidal (hymenoptaecin and defensins 1 and 2) were positively controlled with ampicillin, since it is classified as a bactericidal antibiotic. The AMPs, chloramphenicol and ampicillin were used at stock concentrations of 4, 25, and 100 mg/ml, respectively. Water served as the negative control in these assays, because it was the solvent for most of the AMPs tested. Each trial was replicated three times.

3.2.4. 96-well Plate Assay

The 96-well plate assay was developed *de novo*. For a more detailed description of the assay, refer to Chapter 2. Chloramphenicol served as the bacteriostatic control for apidaecin and abaecin, and ampicillin served as the bactericidal control for hymenoptaecin, and defensins 1 and 2. AMPs were dissolved in sterile distilled water to achieve final concentrations of 200, 100, 10, 1, 0.1, and 0.01 µg/ml once added to the wells. Water served as a negative control for all assays. Each test condition was replicated in five separate wells on the same plate and data from three independent plates were collected.

Each trial produced a 24 h growth curve with readings taken every 15 minutes for each organism in the presence of the AMP, the control antibiotic and water (representative data for apidaecin can be found in the Appendix). This generated 3880 data points per trial. To facilitate analysis and data presentation the $OD_{600}$ of the water-treated control (denoted as [0]) was normalized to 100% and each treatment was expressed relative to [0] at the 0, 6,

12, 18 and 24 h time points. Relative optical density readings for the three individual trials were then averaged for an overall percentage decrease ± standard error. Results with p-values less than 0.05 were considered statistically significant and denoted with an asterisk (*). However, since my goal was to find a honeybee AMP which caused a substantial decrease in the growth of *P. larvae*, only AMPs with overall percentage decreases greater than 5% were considered relevant in the context of these assays. Therefore, any AMP that met these requirements was considered an active compound.

### 3.2.5. Statistical Analysis

P-values were calculated using a student's *t*-test under the assumption of equal variances and compared to the [0] control for their respective time points. All statistics were performed using Microsoft Excel and the StatPlus®:mac LE.2009 program on representative data.

### 3.3. Results

AMPs used in this work were previously characterized as either primarily active against Gram-negative or Gram-positive organisms (Table 3) (Bachanová et al., 2002; Casteels et al., 1990; Casteels et al., 1989, 1993). Consequently, *E. coli* and *P. alvei* were used throughout this work as representative Gram-negative and Gram-positive organisms, respectively. In all experiments, control treatments behaved as anticipated: the bacteria treated with water (denoted as [0]) all grew and those treated with the antibiotics, chloramphenicol or ampicillin, displayed a reduction in $OD_{600}$ indicative of either growth inhibition or direct killing. Representative images of ZoI plates can be seen in the Appendix.

3.3.1. *In vitro* Testing of Apidaecin for Antimicrobial Activity

In the ZoI assay, the Gram-negative organism, *E. coli,* displayed a noticeable zone of clearance of at least 11.17 mm ± 0.31 (average diameter ± standard error) when challenged with apidaecin (Table 4). Although the diameter of the zone of clearance was smaller at 48 h than at 24 h, this was likely caused by the bacteria forming a full bacterial lawn between 24 h and 48 h. Similar decreases in the zone of inhibition were routinely seen at the 48 h time point for all AMPs with detectable antibacterial activity. As expected, the Gram-positive organism, *P. alvei,* displayed no zone of clearance when challenged with apidaecin. Similarly, when the test subject, *P. larvae,* was grown in the presence of apidaecin no zone of inhibition was detected, indicating *P. larvae* is not susceptible to apidaecin in this assay.



| | Average (mm) ± Standard Error | |
|---|---|---|
| Distilled Water | Cm | Apidaecin |

In the 96-well plate assay, *E. coli* (Figure 5a) challenged with apidaecin resulted in statistically significant changes in relative $OD_{600}$ when grown in the presence of 200, 100, and 10 µg/ml concentrations from 6 h of growth and onwards when compared to the [0] control. At 24 h, however, the 200, 100, 10, and 1 µg/ml concentrations resulted in statistically significant percentage decreases in $OD_{600}$ that ranged from 40 ± 2.8% to 12 ± 3.3% (average ± standard error). Although other statistically significant changes in relative

optical density were observed for *E. coli*, they did not reach the threshold requirements for substantial percentage decrease (p ≤ 0.05 and a percentage decrease greater than 5%, respectively). The data collected for *P. alvei*, (Fig. 5b) demonstrate that there was substantial activity of apidaecin at the 200 and 100 µg/ml concentrations between 12 h and 18 h. The percentage decrease was at its greatest during 18 h, 16 ± 2.5% and 6.0 ± 0.7%, respectively. This demonstrates that apidaecin is active against *P. alvei* at concentrations greater than or equal to 200 µg/ml (Casteels et al., 1989). At 24 h, however, only the 1 µg/ml concentration produced a substantial and statistically significant percentage decrease in optical density (6.5 ± 1.5%), which suggests that apidaecin at the 100 and 200 µg/ml concentrations may display growth phase dependent activity against *P. alvei*. Nevertheless, based on these results, I conclude that apidaecin is active against *P. alvei*.

       For the test subject *P. larvae*, only the 1 µg/ml concentration of apidaecin (Fig. 5c) resulted in a statistically significant percentage decrease in relative $OD_{600}$ at 6 h, but the decrease observed (3.0 ± 0.9%) did not meet the threshold requirements for susceptibility. There were no percentage decreases at 24 h when compared to the [0] control for any of the concentrations tested. Therefore, I conclude that *P. larvae* is not susceptible to apidaecin in the liquid culture assay. Overall, the results of the ZoI and 96-well plate assays are consistent with one another and demonstrate that *P. larvae* is not susceptible to apidaecin.
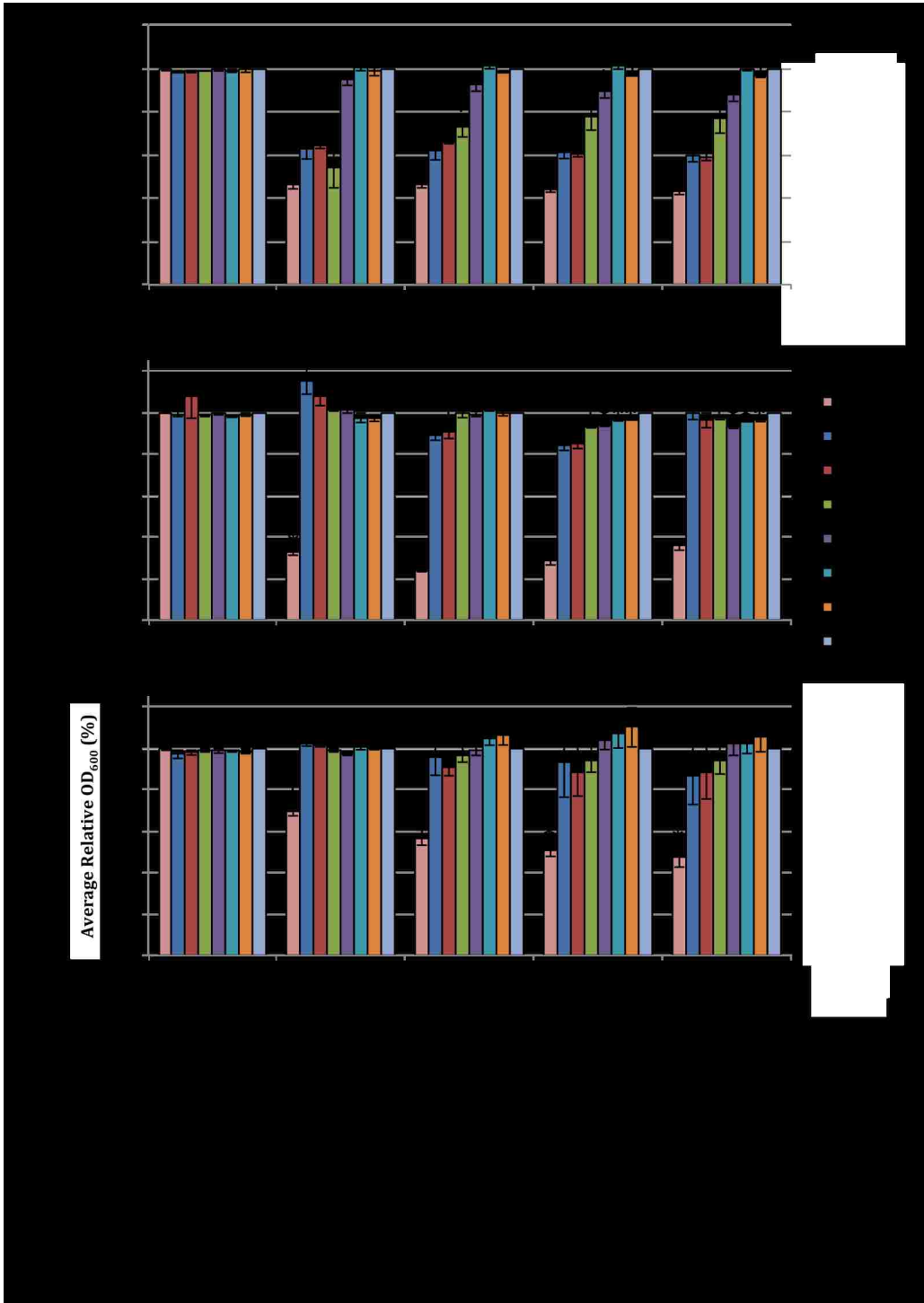
3.3.2. *In vitro* Testing of Abaecin for Antimicrobial Activity

Abaecin was the next AMP to be tested using the ZoI assay. *E. coli* did not exhibit a zone of clearance (Table 5). This result is surprising because previous reports indicate that *E. coli* is susceptible to abaecin (Casteels et al., 1990). When the *P. alvei* was grown in the presence of abaecin, as expected no zones of clearance were observed. Similarly, when *P. larvae* was challenged with abaecin no zones of clearance were observed, which indicates *P. larvae* is not susceptible to abaecin under these conditions.



| | Average (mm) ± Standard Error | | |
| --- | --- | --- | --- |
| | Distilled Water | Cm | Abaecin |

When abaecin was tested in the 96-well plate assay against *E. coli* (Fig. 6a), a substantial and statistically significant percentage decrease in relative $OD_{600}$ was observed only for the 200 and 100 μg/ml concentrations at 6 h, 9.2 ± 1.7% and 5.8 ± 0.9%, respectively. Based on these results, *E. coli* is susceptible to abaecin. At the remaining time points, although statistically significant changes were observed for other concentrations, they did not meet the threshold 5% percentage decrease. Taken together these results suggest that abaecin may have a growth phase dependent effect on *E. coli*. *P. alvei* treated with abaecin (Fig. 6b) resulted in statistically significant percentage decreases in relative

45
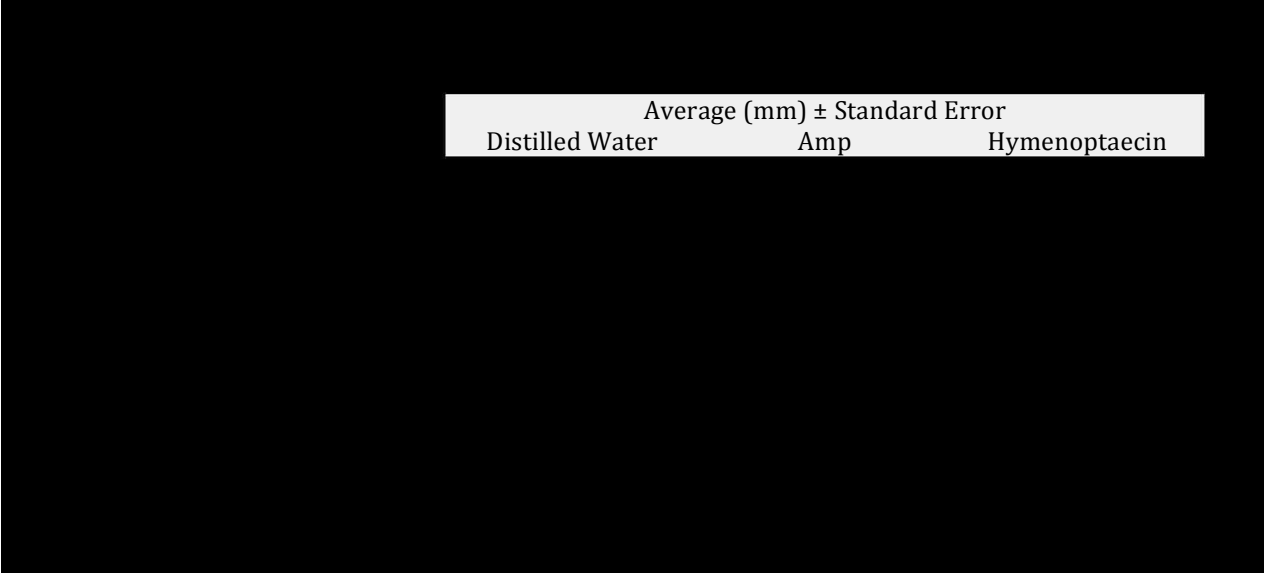
OD$_{600}$ readings between 12 and 24 h for multiple concentrations tested when compared to the [0] control. At 12 h, the 200 μg/ml concentration of abaecin resulted in a substantial and statistically significant decrease in relative OD$_{600}$ of 16 ± 4.3%. At 18 h, the concentrations ranging from 200 to 1 μg/ml produced statistically significant and substantial changes in relative OD$_{600}$, 16 ± 1.0% to 5.5 ±1.8%. However, at 24 h, a percentage decrease in relative OD$_{600}$ of 7.3 ± 1.4% was observed for only the 100 μg/ml concentration. These data suggest abaecin is active against *P. alvei* and may also have a growth phase dependent effect. These results suggest that abaecin at concentrations greater than or equal to 100 μg/ml are active against *P. alvei*.

P. larvae* treated with abaecin (Fig. 6c) displayed statistically significant changes in OD$_{600}$ between the 6 and 18 h time points for various concentrations, yet none of these changes met the threshold requirements for substantial percentage decrease. These data indicate that abaecin is not effective against *P. larvae* in the liquid culture assay. Based on the results of the ZoI and 96-well plate assays, although the results obtained for the representative Gram-negative and Gram-positive organisms did not agree with one another, the results for the test subject did agree: *P. larvae* was not found to be substantially susceptible to treatment with abaecin in either assay.

Time (h)
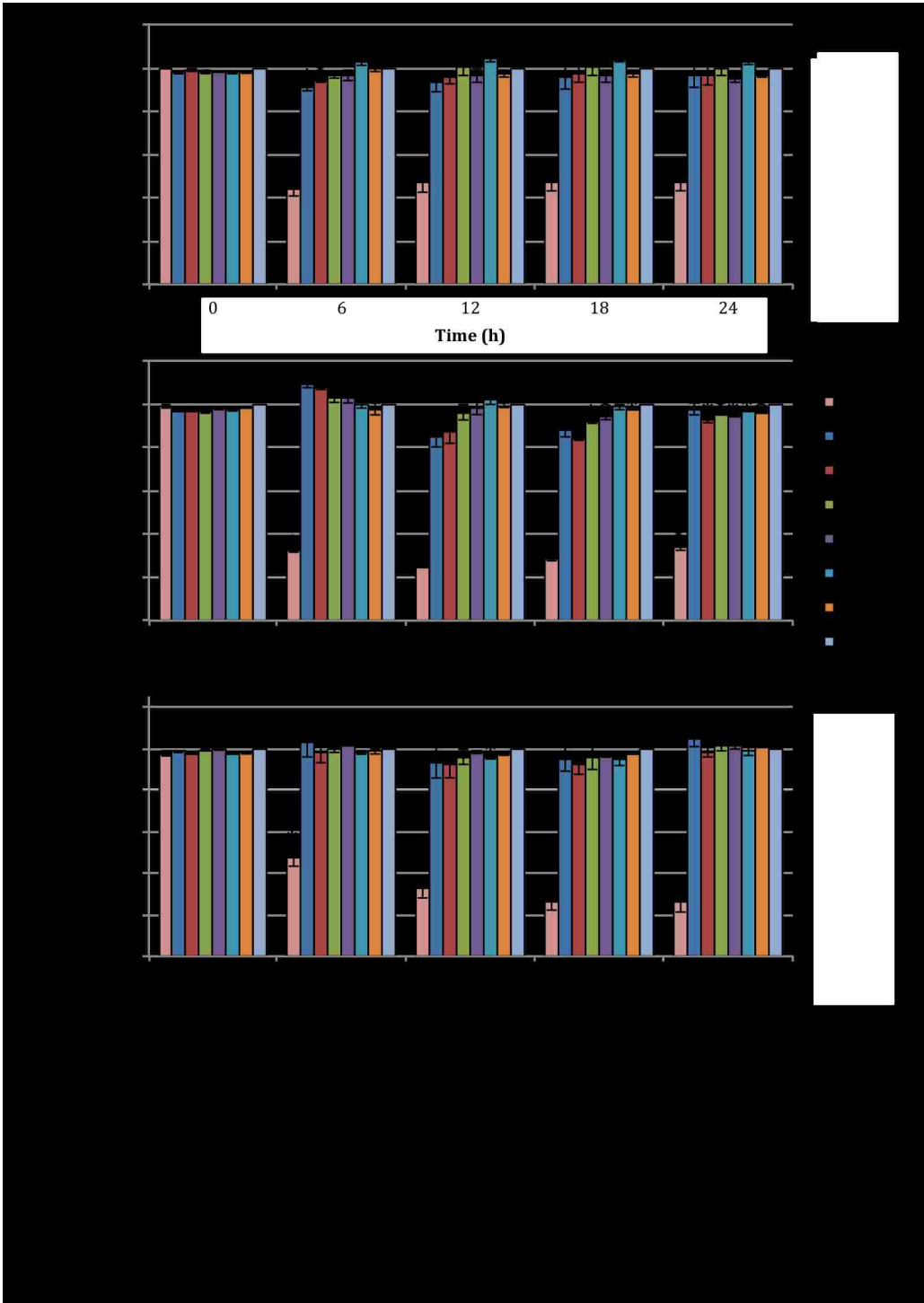
### 3.3.3. *In vitro* Testing of Hymenoptaecin for Antimicrobial Activity

Hymenoptaecin was the third AMP to be tested using the ZoI assay. Unexpectedly, *E. coli* did not display zones of clearance (Table 6), suggesting that hymenoptaecin is not active against *E. coli* in this assay. As expected *P. alvei* did not exhibit zones of clearance when exposed to abaecin. Similarly, when *P. larvae* was challenged with hymenoptaecin no zones of clearance were observed. Consequently, these data demonstrate that *P. larvae* is not susceptible to hymenoptaecin in the ZoI assay.



| | Average (mm) ± Standard Error | |
|---|---|---|
| Distilled Water | Amp | Hymenoptaecin |

Hymenoptaecin was then tested using the 96-well plate assay. *E. coli* displayed a statistically significant decrease in $OD_{600}$ when incubated with 200 µg/ml hymenoptaecin from the 6 h time point and onwards. This decrease approached that seen when *E. coli* was incubated with the antibiotic control, ampicillin (Fig. 7a). Even when *E. coli* was treated with 100 µg/ml of hymenoptaecin, $OD_{600}$ readings were similar to those of the ampicillin control through 12 h, and gave statistically significant readings throughout. At 24 h, the 200 and 100 µg/ml concentrations of hymenoptaecin displayed 67± 2.8% and 37 ± 13% decreases in relative $OD_{600}$, respectively, when compared to the [0] control. The remaining

concentrations also all produced statistically significant percentage decreases at 24 h that ranged from 14 ± 4.1% to 6.4 ± 0.5%. These data indicate that hymenoptaecin is active against *E. coli* at all concentrations tested. *P. alvei* (Fig. 7b) had statistically significant decreases in relative $OD_{600}$ readings for various concentrations between 12 and 24 h. At 12 h, the concentrations ranging from 200 to 10 µg/ml resulted in statistically significant percentage decreases in relative $OD_{600}$ readings ranging from 16 ± 4.8% to 7.8 ± 0.8%. At 18 h, the concentrations ranging from 200 to 10 µg/ml resulted in substantial and statistically significant percentage decreases in relative $OD_{600}$ readings ranging from 23 ± 1.1% to 11 ± 0.8%. Finally, at 24 h, the percentage decrease in relative $OD_{600}$ that were substantial and statistically significant ranged from 17 ± 0.2% to 5.8 ± 0.8% for concentrations ranging from 200 to 1 µg/ml. Consequently, the highest decrease in relative $OD_{600}$ readings was observed at 18 h, which may be indicative of a growth phase dependent effect. According to these results, *P. alvei* is susceptible to hymenoptaecin.

Finally, *P. larvae* samples (Fig. 7c) experienced no statistically significant decreases in relative $OD_{600}$ that met the threshold requirements for any of the concentrations of hymenoptaecin tested throughout the course of the assay. Therefore, when considering the ZoI and 96-well plate assays, the results of both assays indicate that hymenoptaecin is not active against *P. larvae*.

Interestingly, in the 96-well plate assay that tested hymenoptaecin, the samples treated with 200 and 100 µg/ml hymenoptaecin had higher relative $OD_{600}$ readings than the other concentrations used at 0 h, regardless of the organism tested. I initially thought that the $OD_{600}$ was being altered by the addition of the high concentrations of the AMP, 100 and 200 µg/ml. To determine whether the $OD_{600}$ was altered by the addition of the AMP, tests were run comparing the AMP in media vs. media treated with the solvent, water (discussed below). The results demonstrate that the optical density of the media containing the AMP

was increased. Therefore, I concluded that either hymenoptaecin could have been precipitating out of solution. Furthermore, hymenoptaecin in combination with the cells could have caused hymenoptaecin to precipitate out of solution, resulting in increased $OD_{600}$ readings, or that the light scattering ability of AMP-treated cells was altered by the membrane disrupting capabilities of hymenoptaecin. Changes in cell structure often lead to changes in optical density, like those seen for the 200 and 100 µg/ml treated samples. Although lack of time prohibited investigation, microscopic investigation would have addressed this issue.

Average Relative OD$_{600}$ (%)

Time (h)

51
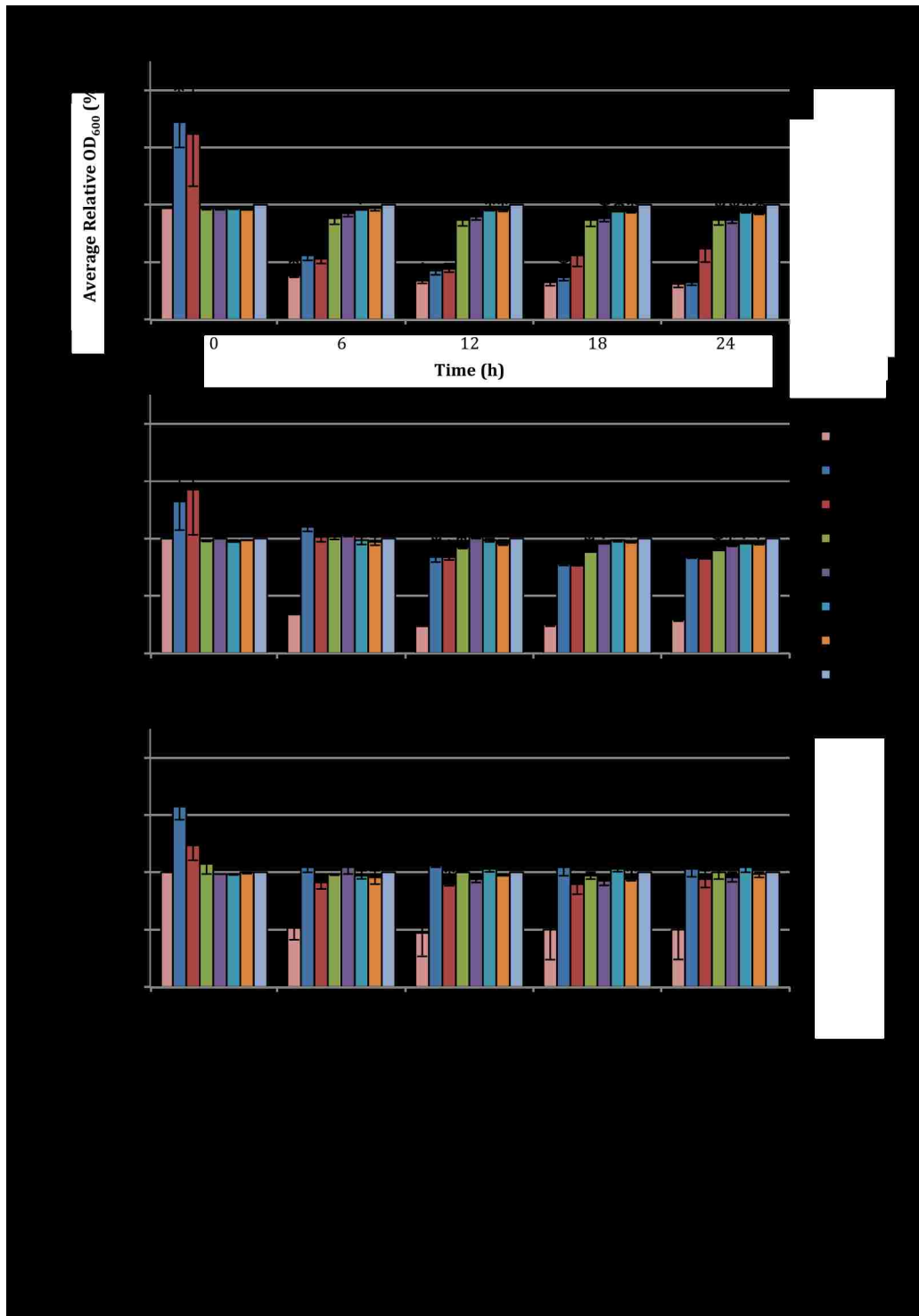
3.3.4. *In vitro* Testing of Defensin 1 for Antimicrobial Activity

Testing of defensin 1 began with the ZoI assay. When challenged with defensin 1, no clearance was observed on plates bearing *E. coli* (Table 7). Zones of clearance were observed for *P. alvei*; however, they were too small to measure accurately. Taken together these results are consistent with the proposal that defensin 1 is primarily active against Gram-positive organisms (Bachanová et al., 2002; Ilyasov et al., 2013; Ilyasov et al., 2012). In support of this, when *P. larvae* was challenged with defensin 1, zones of clearance measuring 6.67 mm ± 0.21at 48 h were detected. This result indicates *P. larvae* is susceptible to honeybee defensin 1 in the ZoI assay.

| | Average (mm) ± Standard Error | | |
| Distilled Water | Amp | Defensin 1 |

Defensin 1 was then tested against all 3 organisms using the 96-well plate assay (Fig. 8). As expected, *E. coli* (Fig. 8a) did not produce statistically significant percentage decreases in relative $OD_{600}$ readings that met the threshold requirements for any of the concentrations of defensin 1 tested. These data suggest that defensin 1 is not active against *E. coli*. *P. alvei* (Fig. 8b) had statistically significant percentage decreases in relative $OD_{600}$ readings for the 200 and 100 µg/ml concentrations of defensin 1 at all time points. At 24 h, the statistically significant and substantial percentage decreases in relative $OD_{600}$ were 53 ±

7.3%, 68 ± 2.4%, and 7.9 ± 0.9% for the 200, 100, and 1 μg/ml concentrations of defensin 1, respectively, when compared to the [0] control. Interestingly, the 100 μg/ml concentrations consistently produced the greatest percentage decrease from 6 h and onwards. It is possible that the 200 μg/ml concentration of defensin 1 could have saturated the media, leading to AMP aggregation and their ultimate precipitation out of solution (tested below). An alternative possibility could have been that the cellular structure of *P. alvei* could have been altered resulting in an increase of the $OD_{600}$ readings for samples treated with the 200 μg/ml concentration of defensin 1. Regardless, these data indicate defensin 1 is active against *P. alvei* in these assays.

At 6 h, *P. larvae* (Figure 8c) challenged with defensin 1 exhibited a statistically significant decrease in relative $OD_{600}$ of 17 ± 3.2% for the 100 μg/ml concentration. However, at 18 and 24 h, only the 10 μg/ml concentration produced statistically significant percentage decreases in relative $OD_{600}$ of 17 ± 4.6% and 26± 8.1%, respectively.  It remains unclear why the 100 μg/ml concentration only produced statistically significant results at 6 h, when the 10 μg/ml concentration of defensin 1 was capable of killing *P. larvae* at 18 and 24 h. Nevertheless, I conclude that defensin 1 is active against *P. larvae* in the liquid culture assay. Overall, the data collected, for the ZoI and 96-well plate assays demonstrate that *P. larvae* is susceptible to defensin 1 in both the semi-solid and liquid culture assays. To ensure that defensin 1 was still intact after storage at -20 °C, experiments were also run to test whether the integrity of defensin 1 was maintained. The results indicated that defensin 1 indeed maintained antimicrobial activity as shown in Appendix A.
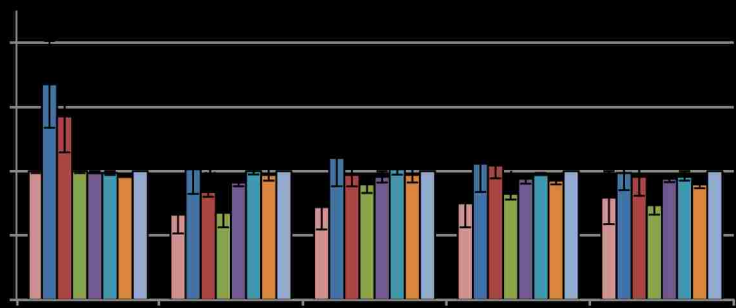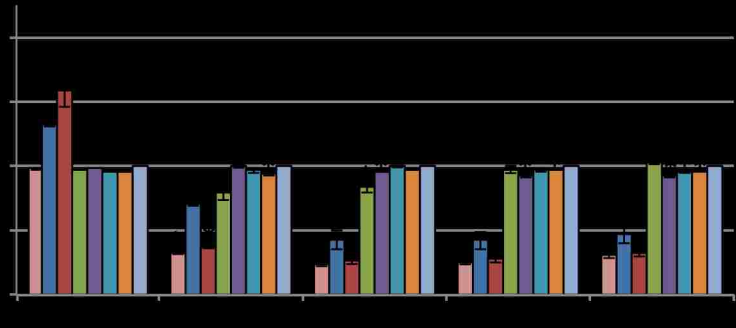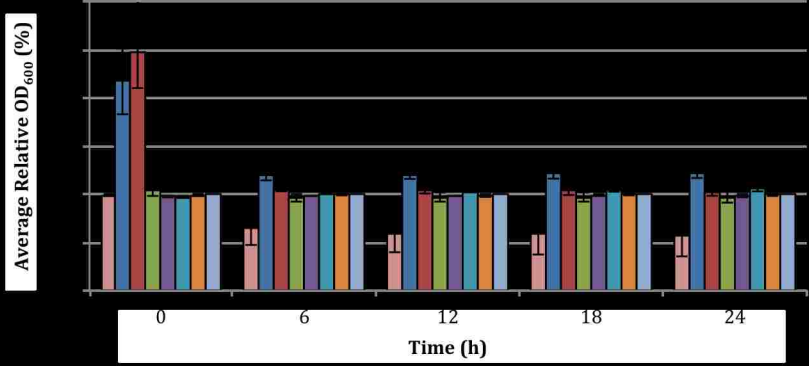
3.3.5. *In vitro* Testing of Defensin 2 for Antimicrobial Activity

Defensin 2 was initially tested using the ZoI assay. *E. coli* did not display zones of clearance when challenged with defensin 2 (Table 8). *P. alvei*, challenged with defensin 2 displayed no zones of clearance. When the growth of *P. larvae* was challenged with defensin 2, no zones of clearance were produced demonstrating that *P. larvae* is not susceptible to defensin 2 in the ZoI assay.

| | Average (mm) ± Standard Error | | |
| --- | --- | --- | --- |
| | Distilled Water | Amp | Defensin 2 |

Finally, defensin 2 was tested against all three organisms using the 96-well plate assay (Fig. 9). Surprisingly, statistically significant decreases in relative $OD_{600}$ were detected for *E. coli* (Fig. 9a) tested against honeybee defensin 2 at various concentrations from 6 h onwards relative to the [0] control. At 6 h, substantial and statistically significant percentage decreases in relative $OD_{600}$ were observed for the concentrations between 200 and 1 μg/ml that ranged from 24 ± 2.2% to 7.4 ± 2.0%. At 12 h, the 200, 100, 10, and 1 μg/ml concentrations resulted in statistically significant percentage decreases in relative $OD_{600}$ that ranged from 33 ± 5.4% to 11 ± 3.6%. Next, at 18 h, all statistically significant concentrations, 200, 100, and 1 μg/ml, resulted in relative $OD_{600}$ percentage decreases of 35

± 5.0%, 34 ± 3.1%, and 12 ± 4.0%, respectively. Finally, at 24 h, the percentage decrease for the 200 and 100 µg/ml concentrations were 33 ± 5.1% and 32 ± 3.5%, respectively. Based on these data, defensin 2 is active against *E. coli* in these assays. Defensin 2 was next tested against *P. alvei* in the 96-well plate assay (Fig. 9b). Substantial and statistically significant percentage decreases in relative $OD_{600}$ were observed at 12 and 18 h for the 200, 100, 10 and 1 µg/ml concentrations (where applicable). At 24 h, the percentage decrease in relative $OD_{600}$ were substantial and statistically significant for all concentrations tested, except 0.1 µg/ml, and ranged from 20 ± 2.1% (200 µg/ml) to 5.6 ± 1.3% (0.01 µg/ml). These results indicate that defensin 2 is active against *P. alvei*.

P. larvae* (Fig. 9c) treated with the 100 µg/ml concentrations of defensin 2 produced substantial and statistically significant percentage decreases in relative $OD_{600}$ readings between 12 and 24 h. The percentage decreases remained similar between 21 ± 3.6% and 20 ± 3.9% throughout. At 24 h, the 200 µg/ml concentration of defensin 2 also produced a substantial and statistically significant percentage decrease in $OD_{600}$ of 6.6 ± 1.9% compared to the [0] control. Overall, these data indicate that *P. larvae* is susceptible to defensin 2 in the liquid culture assay at 200 and 100 µg/ml. Interestingly, though the lower concentration (100 g/ml) produced the bigger antimicrobial effect. For example, at 24 h, decreases in $OD_{600}$ of 6.6% and 20% were observed for the 200 and 100 µg/ml concentrations, respectively. Overall, considering the results of the two assays, *P. larvae* is susceptible to defensin 2 in the liquid culture assay but not in the ZoI assay. Possible reasons for this will be explored in the discussion section.
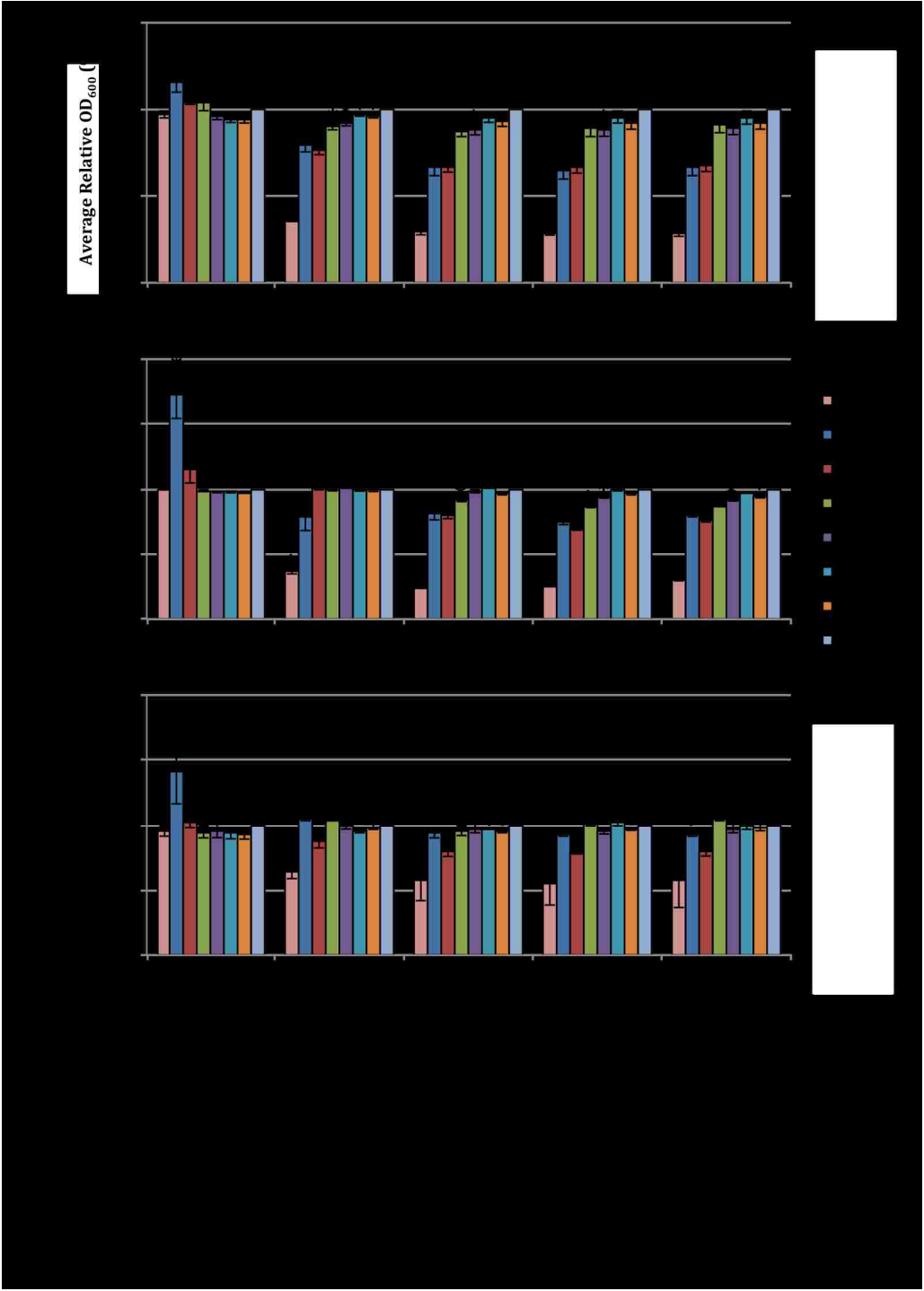
3.3.6. *In vitro* Testing of Individual AMPs for Precipitation

At the 0 h time point for the 100 and 200 µg/ml concentrations of hymenoptaecin, defensin 1, and defensin 2, I observed an increase in optical density relative to the [0] control. This anomaly required further investigation. As stated previously, the most probable cause for these data was that the AMPs were precipitating out of solution. Therefore, the optical densities of wells containing media and individual AMPs at varying concentrations were measured using the same test conditions as described for the 96-well plate assay (section 3.2.4.). These experiments revealed consistent $OD_{600}$ readings for apidaecin and abaecin (data not shown). In contrast, hymenoptaecin, defensin 1, and defensin 2, each showed an increase in optical density greater than the [0] control at the 0 h time point (Fig. 10). However, with prolonged incubation the optical density of wells containing 100 and 200 µg/ml concentrations of hymenoptaecin decreased to levels equivalent to those containing the [0] control (Fig. 10a). Interestingly, for defensins 1 and 2 (Fig. 10b and 10c) the increase in optical density observed at the 0 h time point decreased but remained higher than the [0] controls. These data suggest that defensins 1 and 2 at the 100 and 200 µg/ml concentrations may precipitate out of solution at 0 h and potentially throughout the assay, leading us to treat the data collected with these concentrations of defensin 1 and defensin 2 cautiously. To further investigate whether defensins 1 and 2 are indeed precipitating out of solution at the 100 and 200 µg/ml concentrations follow up centrifugation and microscopy studies should be used.
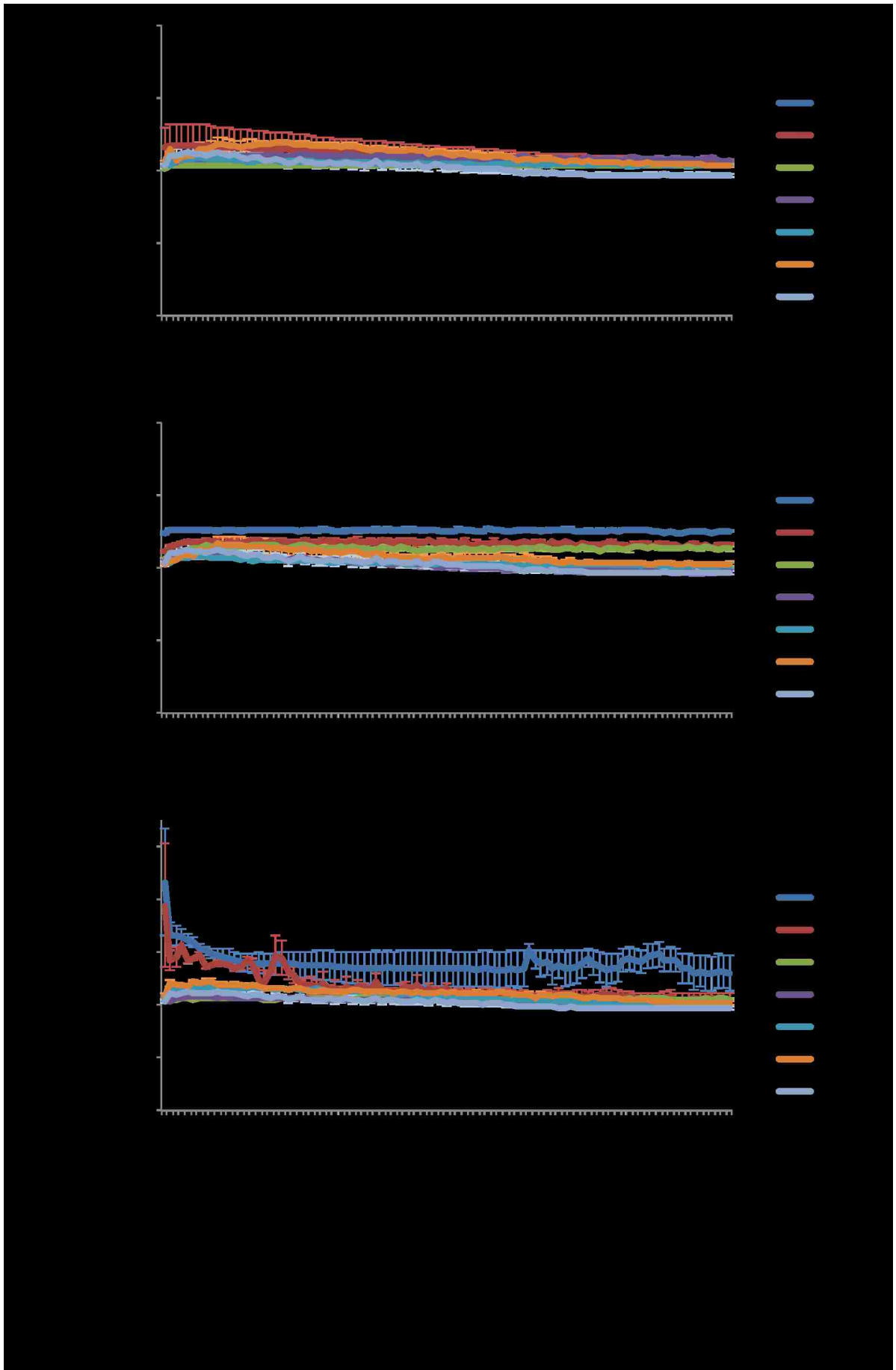
3.3.7. 96-well Plate Assay for AMP Combinations

Next, I wanted to test whether a combination of AMPs could increase the antimicrobial effect on the growth of vegetative *P. larvae* cells *in vitro.* Defensin 2 was chosen to be the AMP that would be tested in combination with the remaining AMPs because it yielded consistent overall decreases in $OD_{600}$ based on the final results of the single AMP testing in the liquid culture assay for the 100 μg/ml concentration (section 3.3.5.). The 96-well plate assay was chosen because it is a quantitative method and enabled me to perform calculations that were not possible on those performed on semi-solid media (data not shown). Throughout the assay, no statistically significant ($p < 0.01$) decreases in $OD_{600}$ were detected for any of the combinations when compared to the control treated with defensin 2 only, therefore the results indicate that none of the combinations tested were better than using defensin 2 alone.

However, when compared to the [0] control, at 6, 12, and 18 h statistically significant decreases in $OD_{600}$ of *P. larvae* were observed for defensin 2, as well as the combinations of defensin 2 with either apidaecin or abaecin for (Figure 11a). These statistically significant differences are also represented as a percentage decrease in $OD_{600}$ readings in Figure 11b. For all time points, except 18 h, the combination of defensin 2 plus apidaecin yielded the most growth inhibition.

**Time**
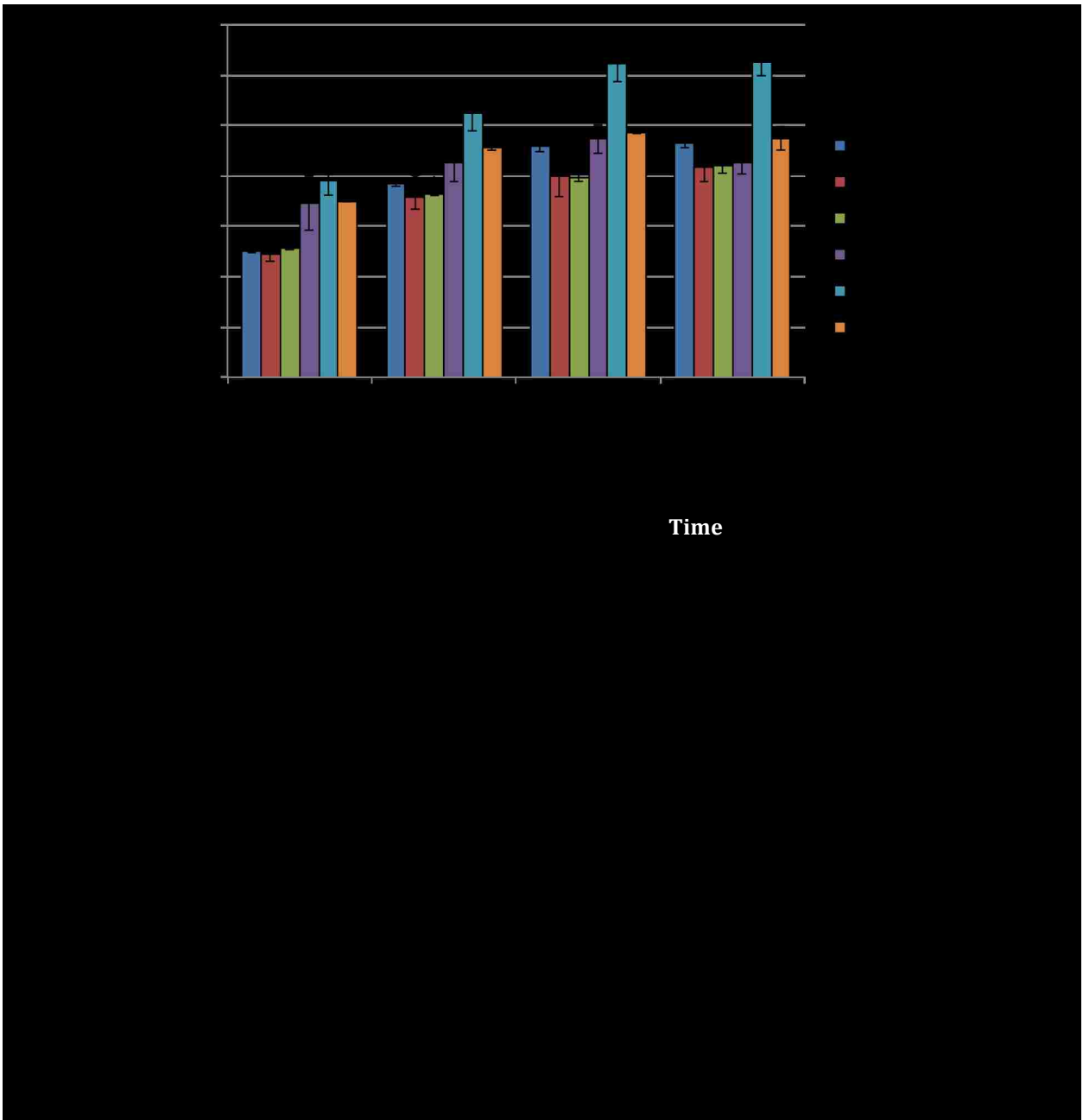
One anomaly in these data (Figure 11) is the combination of defensin 1 and defensin

2. When tested together, the $OD_{600}$ readings increased for the *P. larvae* samples (teal bars in

Figure 14a; negative percentages in Figure 11b). Two possible explanations for this have

been mentioned previously (section 3.3.3.). Briefly, defensins 1 and 2 could have been

aggregating in solution resulting in the precipitation of the AMPs. Another possibility could

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have been that the cellular structure of *P. larvae* could have been altered, resulting in an increase in the $OD_{600}$ readings for the samples treated with the combination of the defensins 1 and 2.

3.4. Discussion

The purpose of this research was to determine if there are any naturally occurring, honeybee AMPs capable of inhibiting the growth of vegetative *P. larvae* cells *in vitro*. Two methods were employed for the AMP testing: the qualitative ZoI assay and the quantitative 96-well plate assay. Each assay was established to test the antimicrobial susceptibility of vegetative *P. larvae* cells *in vitro* (see Chapter 2). The purpose of using both methods was to test the AMPs under two different conditions and to find which compound, if any, demonstrated antibacterial activity in both assays. Only defensin 1 displayed substantial and statistically significant antimicrobial activity against *P. larvae*, in both the semi-solid and liquid culture assays. However, in the 96-well plate assay defensin 2 also displayed substantial and significant antimicrobial activity, as shown in Table 9. I therefore chose to revise my criteria for an active compound to include any AMP that displayed activity in a single assay. Consequently, based on the results of my experiments, both defensin 1 and 2 were considered candidates for an alternative treatment for AFB.

| | Zone of Inhibition Assay Results | | | 96-well Plate Assay Results | | |
|---|---|---|---|---|---|---|
| AMP | *E. coli* | *P. alvei* | *P. larvae* | *E. coli* | *P. alvei* | *P. larvae* |
| Abaecin | - | - | - | + | + | - |
| Defensin 1 | - | + | + | - | + | + |

Next, I wanted to investigate whether different combinations of AMPs would inhibit *P. larvae* growth more than a single AMP based on my findings. For this work, double AMP combinations that included defensin 2 were evaluated because this AMP has not been as well characterized as defensin 1 against *P. larvae.* In addition, defensin 2 is located in the hemolymph where the pathogen would at some point encounter it. The major finding of these combinatorial 96-well plate assays showed that none of the combinations tested displayed more statistically significant antimicrobial activity than defensin 2 alone. Nevertheless, when all treatments were compared to the [0] control, the strongest combination of AMPs was defensin 2 paired with apidaecin. This was surprising since apidaecin was originally thought to be primarily active against Gram-negative organisms and acts via a bacteriostatic mechanism. One possible explanation could be that apidaecin acts on the *P. larvae* cells first, leaving the cells in a state of non-growth, which leaves them more susceptible to the action of defensin 2, which is characterized as permeating the cell membrane, resulting in more efficient lysis of the cells.

While it is clear that the conditions found in my *in vitro* assays do not even attempt to approximate the conditions where AMPs are naturally found and active in the honeybee, it is interesting to note that at least for some of the AMPs their activity differed depending on whether they were tested in semi-solid or liquid assays. I speculate that certain AMPs may work best under conditions that mimic the environment in which they are found in the honeybee and honeybee larva. Following this logic, apidaecin, abaecin, hymenoptaecin, and defensin 2 should work best in the liquid culture assay because they are found circulating throughout the hemolymph of the honeybee; whereas defensin 1 should work best in semi-solid media because it is found in viscous substances, such as honey and royal jelly. This is supported by the results obtained with abaecin, hymenoptaecin, and defensin 2 (summarized in Table 9), but not with apidaecin and defensin 1, which worked in both assays  (as seen in Table 9). Thus, the correlation between the natural environmental condition of the AMPS and the activity of the AMPs tested validates the two-method *in vitro* approach that I have taken to assess the antimicrobial activity of these five AMPs against *P. larvae*.

Representative organisms *E. coli* (Gram-negative) and *P. alvei* (Gram-positive) were used throughout my experiments because each AMP I tested had previously been characterized as primarily active against either Gram -positive or -negative organisms. Expected activity for each AMP and organism can be seen in Table 10. Results consistent with the literature were found for apidaecin tested against *E. coli* and defensin 1 tested against *P. alvei* (as seen in Table 9) (Bulet et al., 1999; Casteels et al., 1989).   However, hymenoptaecin inhibited the growth of *E. coli* in the liquid culture assay only. Contrary to previous findings, abaecin demonstrated an inhibitory effect on the growth of *E. coli* only at 6 h in the liquid culture assay suggesting a growth phase dependent effect (Casteels et al., 1990).  Surprisingly, my experiments revealed that defensin 2 was active against *E. coli* in

the liquid culture assay which removes defensin 2 from the Gram-positive specific categorization.  As for *P. alvei,* all three AMPs described as Gram-negative specific displayed activity against *P. alvei* in the liquid culture assays. Once again, my results emphasize that honeybee AMP activity categorizations are only generalizations and cannot be accurately applied to all organisms. Furthermore, the results of my experiments demonstrate the importance of testing these AMPs against *P. larvae* in spite of previous reports of honeybee AMP activity categorizations.

| AMP | Expected Zone of Inhibition Assay Results | | | Expected 96-well Plate Assay Results | | |
|---|---|---|---|---|---|---|
| | *E. coli* | *P. alvei* | *P. larvae* | *E. coli* | *P. alvei* | *P. larvae* |
| | | | | | - | |
| Abaecin | + | - | - | + | - | - |
| | + | | | | - | |
| Defensin 1 | - | + | + | - | + | + |
| | | + | + | - | | |

For all AMPs tested, the results of the ZoI and 96-well plate assays illustrate that the categorizations of AMP activities do not always hold true and should be tested regardless. As demonstrated in Table 10, not all of the expected results were observed. Thus, these results suggest that not all categorizations should be weighted so heavily during the experimental design process. Another interesting observation was that strain-to-strain

differences existed in my experiments. For example, during assay development apidaecin was tested using the ZoI assay against *E. coli* strain DH10B. Routinely, no zone of clearance was observed in the ZoI assay. This result led me to switch to *E. coli* strain MC4100. A distinct zone of clearance was observed consistently with comparable measurements between assays for MC4100 (representative data in Appendix). Therefore, some functional difference exists between *E. coli* strains DH10B and MC4100. My work clearly demonstrates that not all organisms fit the mold and that each test subject should be considered as a separate entity.

Overall, the significant findings of my work were that both defensins 1 and 2 displayed antimicrobial activity against *P. larvae*. However, the antimicrobial activity of defensins 1 and 2 against *P. larvae* was perhaps not too surprising because both defensin1 and 2 were previously characterized as primarily active against Gram-positive organisms (Bulet et al., 1999; Ilyasov et al., 2013; Ilyasov et al., 2012). Importantly though, defensin 1 had never been tested against *P. larvae* using the methods used here, and defensin 2 has never been directly tested on *P. larvae*. Furthermore to the best of my knowledge the other AMP used in this study; apidaecin, abaecin and hymenoptaecin, had not been directly tested on *P. larvae*. My research is therefore novel, but my findings are consistent with work of others

In conclusion, despite several conflicting data sets, I have found that vegetative *P. larvae* cells are most susceptible to defensins 1 and 2 *in vitro*, using the semi-solid and liquid culture assays. To that end, defensin 1 and defensin 2 have the potential to be used as alternative treatments for AFB. Upon further investigation, it was found that defensin 2 in combination with apidaecin also has the potential to be used as an alternative treatment for AFB. Although significant growth inhibition of *P. larvae* was not observed when compared to defensin 2 alone, combining these two AMPs could potentially be used to boost production of AMPs circulating throughout the hemolymph of a healthy larva, thus helping

to fight replication of vegetative cells in case a larva becomes infected. Future work would focus on *in vivo* studies using these AMPs on larvae with active *P. larvae* infections. Initial experiments would consist of feeding infected and non-infected larvae royal jelly supplemented with the AMPs in the controlled environment of the lab. These studies could be further expanded by infecting the non-infected larvae royal jelly supplemented with the AMPs and *P. larvae* spores, then observing those results. Depending on the results of initial tests, these treatments could then be tested at the colony level and potentially applied into the infected hive in an aerosolized form.

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CHAPTER 4

DISCUSSION

4.1. Conclusions

Worldwide accounts of American Foulbrood Disease (AFB) have been recognized since the 19th century, and it has been documented since the beginning of the 20th century (White, 1906). During the last seventy years, there has been a steep decline in the commercial honeybee population in the United States of America, where a contributing factor is AFB. The only accepted treatment for the disease is to burn the infected hives and associated equipment due to the resilient spores formed by the causative agent of AFB, *Paenibacillus larvae* (Arbia & Babbay, 2011; Genersch, 2010). These spores are the infectious unit and are extremely difficult to eradicate. Beekeepers have tried to treat their infected hives with antibiotics, such as oxytetracycline, however, antibiotic resistance has developed (Miyagi, Peng, & Chuang, 2000). Therefore, the need for an alternative treatment method against *P. larvae* is substantial. Based on previous reports, antimicrobial peptides (AMPs) are ideal candidates for an alternative treatment (Boman, 2003; Nathan, 2004). My area of research is novel in that it focuses on the potential use of active, honeybee antimicrobial peptides as an alternative treatment for AFB.

4.1.1. Methods Development

In order to test the susceptibility of vegetative *P. larvae* cells to active, honeybee AMPs, I wanted to establish two *in vitro* methods. Ultimately, the goal of establishing two methods would allow me to compare the results of the two assays and determine which of the AMPs could be used as an alternative treatment method in future experiments. The two methods: a zone of inhibition (ZoI) assay and a microtiter (96-well plate) assay were adapted from previous methods or developed *de novo* for testing antimicrobial

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susceptibility, respectively (Davis & Stout, 1971; de Graaf et al., 2013). Generally, these methods are employed to conduct minimum inhibitory concentration (MIC) assays by using decreasing amounts of antimicrobial compound. However, the downside to using these methods is that they require a large volume of antimicrobial compound. Contrary to antibiotics, AMPs are high in molecular weight (>500 Da), found in relatively small volumes and are expensive to synthesize.

The first step for developing these methods was to find media suitable for testing AMPs and that allows reliable *P. larvae* growth. *S*everal media types have been previously identified: Brain Heart Infusion (BHI) broth; Mueller-Hinton broth supplemented with pyruvate, yeast extract, and phosphate (MYPGP); and nutrient broth (Gende, Eguaras, & Fritz, 2008). I originally tested *E. coli* and *P. larvae* in BHI broth, a modified BHI broth (1% MgCl (w/v) and 1% CaCl (w/v)), and nutrient broth. Consistent growth and/or antimicrobial activity was not observed using any of these media. In addition, I consistently observed the formation of clumps when growing *P. larvae*. Therefore I chose to use a new liquid medium, 3X R2B, for several reasons: 1) it has a similar composition to MYPGP but unlike MYPGP it is commercially available, allowing growing conditions to be more reproducible, 2) it allows the growth of *P. larvae* without the formation of clumps and 3) it contains no sodium chloride which has been shown to impede the function of certain AMPs (Casteels et al., 1990; Casteels et al., 1989).

Next, Gram positive- and negative- control organisms were selected based on the literature available for these AMPs (Casteels et al., 1990; Casteels et al., 1989, 1993). Initially *E. coli* strain DH10B and *B. subtilis* strain YB955 were used, however, no antimicrobial activity of apidaecin was detected against DH10B in either semi-solid or liquid culture assays. This lack of activity was likely caused by the fact that DH10B is a heavily altered genetically strain that is likely to carry mutations that lead to changes in the

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cell envelope. This was important to consider because it has been shown that changes to the cell envelope can promote the resistance of bacteria to certain AMPs (Groisman, Parra-Lopez, Salcedo, Lipps, & Heffron, 1992). I remedied this situation by testing apidaecin on *E. coli* strain MC4100 and *Shigella flexneri* strains 2457T, and a virulence plasmid cured derivative (BS103) in the ZoI assay (representative data of *E. coli* strains DH10B and MC4100 in Appendix). *B. subtilis* originally served as the positive control and data was collected; however, this data were rejected because the strain used displayed a strange growth pattern occurring in the 96-well plate assays data analysis (representative data in Appendix). I concluded that the observed pattern was caused by autolysis (Jolliffe, Doyle, & Streips, 1981; Young, Tipper, & Strominger, 1964). Because this effect confounded my results and their interpretation, a new Gram positive-control organism was chosen, *P. alvei*.

The first protocol is a modified zone of inhibition assay. This qualitative assay has conventionally been conducted using disks containing an antimicrobial compound of interest (Davis & Stout, 1971), but this method was not suitable for testing functional honeybee AMPs against *P. larvae* due to their high molecular weight. Preliminary tests using the disk diffusion method for the antibiotic, chloramphenicol, produced zones of clearance when placed on bacterial lawns of *E. coli* and *P. larvae*. In contrast, preliminary results using the disk diffusion method with AMPs displayed no zones of clearance. The paper disks potentially could have impeded efficient diffusion of the high molecular weight AMPs into the agar matrix. Ultimately, a hole punched through the agar provided the ideal method of delivery to the agar matrix and consistently allowed zones of clearance to be measured for susceptible organisms (representative data in Appendix).

The second method is an adaptation of a minimum inhibitory concentration (MIC) assay, in which a 96-well plate is used (de Graaf et al., 2013). This assay provided a quantitative method of testing multiple concentrations of the low abundance AMPs against

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*P. larvae* simultaneously. Generally, the 96-well plate assay method is used to determine an MIC of a compound, or the concentration at which growth is completely inhibited. However, this method was utilized to identify concentrations at which *P. larvae* was in any way susceptible to honeybee AMPs.  Originally, I had intended to take the experiment further by i) plating treated cells from the 96-well plate to count colony forming units (CFU) and ii) taking direct counts using a Petroff Hausser Chamber. However, these assays provided inconsistent results and proved to be unsuccessful. Some of the challenges were that the direct count assays were difficult to perform due to long chains of cells. Furthermore, it was not possible to determine whether the cells being viewed under the microscope were alive or dead using this specific method. Thus, using the spectrophotometer was deemed the most appropriate method, but it still had its limitations.

The two *in vitro* methods described in this work will allow others to test the antimicrobial activity of relatively high molecular weight and low abundance compounds, such as AMPs against *P. larvae*. The qualitative ZoI assay allows a visual measurement of the susceptibility of microorganisms without the use of paper disks, which prevent the diffusion of AMPs into the semi-solid agar matrix. The qualitative microtiter plate assay allows for direct measurement of AMPs while using very small amounts of the otherwise expensive compound. Furthermore, the use of a commercially available medium conserves valuable time in the lab. Overall, these methods can assist others in the field of *P. larvae* research, but they do have their limitations. For instance, during the development of these methods it became clear that different assay conditions can influence the activity of honeybee AMPs, that not all conditions allow the antimicrobial activity of a given honey bee AMPs to be detected and that precipitation of the high molecular weight AMPs may occur at high concentrations in the 96-well plate assay. These will be important considerations for those wishing to translate *in vitro* findings to *in vivo* settings.

4.1.2. Identification of AMPs Active Against *P. larvae*

The goal of this portion of my research was to find whether naturally occurring

honeybee AMPs can prevent the growth of *P. larvae in vitro*. A decrease in *P. larvae* growth

was observed when testing apidaecin and abaecin in the liquid culture assay, except those

results did not meet my criteria that I had defined as an active compound. While defensin 1

was the only AMP to inhibit *P. larvae* growth in the ZoI assay, both defensins 1 and 2

displayed a substantial and statistically significant decrease in *P. larvae* growth in the 96-

well plate assay. I therefore conclude that *P. larvae* is susceptible to both defensins 1 and 2.

The antimicrobial activity of defensins 1 and 2 against *P. larvae* was perhaps not too

surprising, because both defensins 1 and 2 were previously characterized as primarily

active against Gram-positive organisms (Bulet, Hetru, Dimarcq, & Hoffmann, 1999). While

defensin 2 has never been directly tested on *P. larvae,* defensin 1 was shown to be active

against *P. larvae* in an unusual assay involving a polyacrylamide gel (Bachanová et al.,

2002). Importantly though it had never been tested against *P. larvae* using more traditional

methods growth assays like those employed in this work.  Consequently, my research is

novel, but my findings are consistent with the work of others.

Naturally, the next step was to test the AMPs in combination pairs against *P. larvae*.

Based on the results of the single AMP testing, I chose to focus on defensin 2 for these

assays, because prior to this work defensin 2 had never been directly tested on *P. larvae*.

Defensin 2 was used in combination with the remaining AMPs.  The results indicated that no

significant inhibition of growth occurred when defensin 2 was used in combination with the

other AMPs when compared to defensin 2 alone. Yet, the combination that inhibited *P.*

*larvae* growth the most of all was defensin 2 and apidaecin when compared to the water-

treated control.

While defensins 1 and 2 do not present a new selection pressure for *P. larvae* when used at physiological concentrations, if used prophylactically or as treatments at super-physiological concentrations, a new selection pressure is being introduced. However, there are two features of defensins that make resistance in *P. larvae* unlikely to occur. First, these AMPs are cationic and hydrophobic and are attracted to the negatively charged cell envelope. Second, defensins work via non-specific disruption of the bacterial cell membrane. Since, it is quite difficult to isolate mutants with alterations in membrane composition, because these mutations frequently decrease the viability of these mutants, making them less competitive in nature (Boman, 2003), the likelihood of *P. larvae* developing resistance to defensins 1 and 2 is remote.

In summary, I have found that *P. larvae* is susceptible to both defensin 1 and defensin 2 *in vitro*. Consequently, I propose that defensins 1 and 2 are candidates for an alternative treatment for AFB. Subsequent combination assays demonstrated that the combination of defensin 2 and apidaecin displayed significant growth inhibition of *P. larvae,* although this combination did not yield significantly more killing of *P. larvae* than defensin 2 alone, under my assay conditions. My studies therefore provide insight into which natural AMPs are the most active against *P. larvae* and reveal that combinations of AMPs do is not necessarily result in more killing of *P. larvae* than single AMPs.

4.2. Future Directions

Having found that defensins 1 and 2 provide the most antibacterial activity against *P. larvae,* the next step would be to conduct *in vivo* testing of the AMPs on infected honeybee larvae. Honeybee larvae, infected and non-infected, could be fed royal jelly supplemented with active AMPs, defensins 1 and 2 in particular. If this research proves successful, it could then be moved to a series of hives in their natural settings. The treatment could potentially

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be administered to individual cells of frames containing honeybee larvae. A few things need to be considered for this testing. For example, the storage solution (25% acetonitrile for storage, but approximately 1% at active concentrations) for defensin 2 could have the potential to kill the larvae. Therefore, the appropriate controls need to be considered. Another consideration is that the peptides could potentially be denatured during the treatment regime.

Another future direction would be to test the AMPs on the infective unit of AFB, the *P. larvae* spore. Specifically, testing would concentrate on spore outgrowth, or the process of the germinated spore replicating into a vegetative cell. It is possible that the AMPs may inhibit the replication of the infected spore based on other research findings (Brötz & Sahl, 2000; Gut, Prouty, Ballard, van der Donk, & Blanke, 2008). Finally, if the AMPs prove to be ineffective, research could be done to assess compounds created by the honeybee microbiome. Previous findings have demonstrated that honeybee midguts can inhibit the growth of vegetative *P. larvae* spores *in vitro* (Riessberger-Gallé, von der Ohe, & Crailsheim, 2001), so pursuing this avenue of research could prove fruitful.

Finally, given more time, I would explore the literature to see if there are any other suitable candidate molecules that could be used to kill or inhibit the growth of *P. larvae*. Initial searches revealed a honeybee peptide called apisimin. This serine-valine-rich peptide seemed appealing at first because it is found in royal jelly and could have polyfunctional properties, including antimicrobial properties. However, upon further investigation, it was found that apisimin is potentially involved in the activation of several cellular processes (Bıliková, Hanes, Nordhoff, & Saenger, 2002). More recently, 4 new honeybee AMPs were isolated from royal jelly. These AMPs, known as jelleines I-IV were shown to have antimicrobial effects on both Gram-negative and Gram-positive organisms (Fontana et al., 2004). Coincidentally, none of these AMPs have been characterized against *P. larvae*, raising

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the possibility that one of these AMPs could be more potent against *P. larvae*. To conclude,

while multiple avenues are being explored to find a solution to the global epidemic that is

AFB, this research focuses on using five naturally occurring honeybee AMPs and the

findings of my *in vitro* assays indicate that the use of AMPs to control and treat AFB has

potential and could be pursued further.

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APPENDIX

SUPPLEMENTAL INFORMATION


A.1. METHODOLOGY

A.1.1. Growth Conditions of *P. larvae* Strains

Frozen glycerol stocks of *P. larvae* strains NRRL B-2605 and B-3554 (see Table 3) were initially thawed and used to inoculate a 50 ml Pyrex flask containing BBL™ Brain Heart Infusion (BHI) broth [Becton, Dickson and Company (BD); Maryland, USA] or BHI modified to have a final concentration of 1 mM of $MgCl_2$ and 1 mM $CaCl_2$ (mBHI) using a 1:20 dilution factor. Samples were incubated in an environmental shaker (MaxQ 4000; Barnstead Lab Line) at 37 °C with rotational shaking at 100 rpm. Incubation times varied from 16 to 40 h. These media could not be used to test the AMPs because it contains NaCl which has been shown to impede the function of AMPs (Casteels et al., 1990; Casteels, Ampe, & Jacobs, 1989).

An updated protocol of preparing *P. larvae* strains involved scraping a frozen glycerol stock from the -80 °C freezer using a sterile wooden stick. The inoculum was placed in 5 ml 3X R2B broth (EMD Chemicals, Inc.; New Jersey, USA) in a culture tube (16 × 150 mm; VWR®) which was placed in an environmental shaker (MaxQ 4000; Thermo Scientific) at an ambient temperature of 37 °C with rotational shaking at 325 rpm for approximately 16 h overnight. The overnight culture was sub-cultured using a 1:20 ratio in 5 ml fresh 3X R2B broth and grown using the same growth conditions previously described. If larger aliquots of *P. larvae* were needed, a flask containing 20 ml of 3X R2B broth was inoculated with 1 ml of the original overnight culture initially described and placed in an environmental shaker (MaxQ 4000; Barnstead Lab Line) at an ambient temperature of 37 °C with rotational shaking at 100 rpm.

A.1.2. Determination of Colony Forming Units

      Frozen glycerol stocks of *P. larvae* were thawed and placed in 20 ml BHI at a 1:20 ratio. Cultures were grown in same conditions as described above for 3X R2B broth. Spectrophotometric readings at 600 nm ($OD_{600}$) using the DU® 520 UV/Vis spectrophotometer (Beckman Coulter) were taken when the culture was within linear range of the machine (0.14 – 1.0 ABS). Samples from the spectrophotometric cuvette were taken (Fig A1, a and b) and placed on a Petroff Hausser Chamber (Electron Microscopy Sciences; Pennsylvania, USA). The results were photographed using an Olympus BX-51 microscope (Olympus America Inc.; New York, USA) and calculated using the following formula:

Number of cells per milliliter = Number of cells counted × dilution (if used) × 50,000

A one microliter sample was also taken and serially diluted ten-fold, $10^{-3} – 10^{-7}$. One hundred microliters of each dilution was inoculated on either an R2B agar plate or BHI agar plate. The plate was sealed with Parafilm® and incubated for 72 h. Colony counts were taken at 24, 30, 48, and 72 h (Fig, A1c).
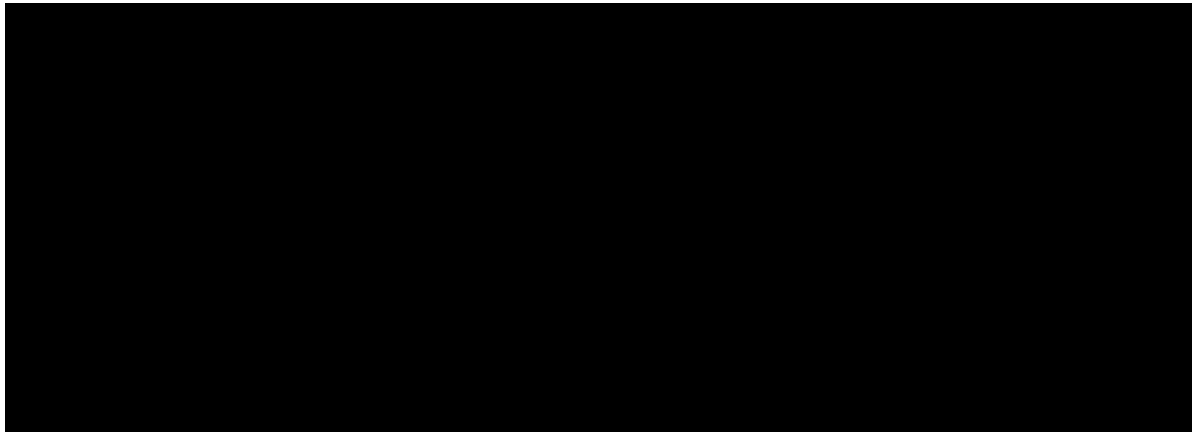
A.1.3. Zone of Inhibition Assay

      Originally, 5 μl of either apidaecin solution, chloramphenicol, or sterile distilled water were placed on the surface of a diffusion disk that would contact the agar. Treated disks they were left to dry in a Petri dish for 2 h at room temperature, approximately 25 °C, in a vacuum hood. A list of the honeybee AMPs used and their sequences are listed in Table A1. Cells were spread across the surface of R2B agar to create a bacterial lawn, and disks were placed on the agar surface (Davis & Stout, 1971; Jorgensen & Ferraro, 2009). The

plates were sealed with Parafilm® and incubated at 37 °C for 48 h. Measurements were

taken at 24 and 48 h. The revised protocol is located in Chapter 2.
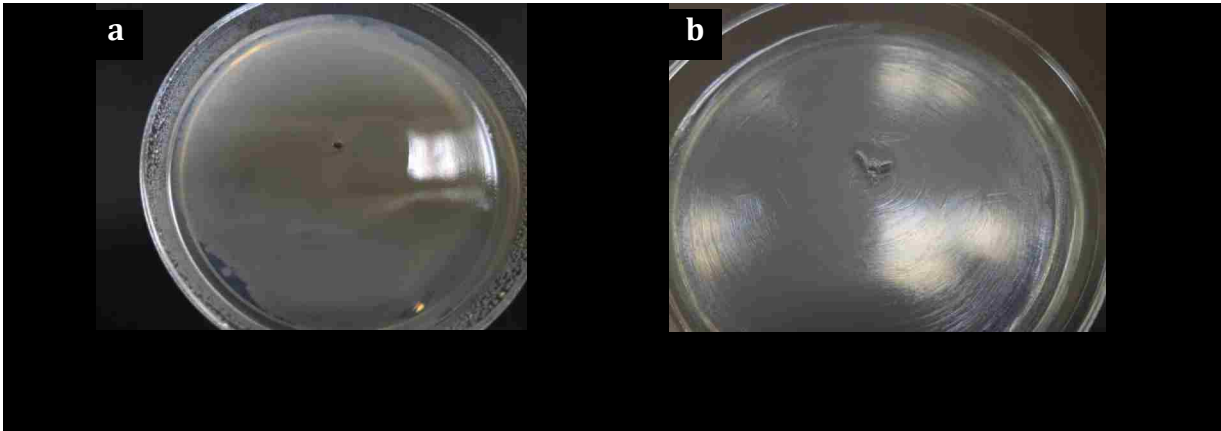

A.1.4. 96-well Plate Assay

The initial protocol required the cells, *E. coli* or *P. larvae*, to be incubated in a

tabletop shaker at 37 °C with rotational shaking at 100 rpm (Incubating Mini Shaker; VWR

International, Visalia, CA). The plate was then read at 0, 1, 2, 3, 4, 8, and 24 h in a plate

reader (SpectraMax Plus 384 Microplate Reader; Molecular Devices, Sunnyvale, CA). Refer

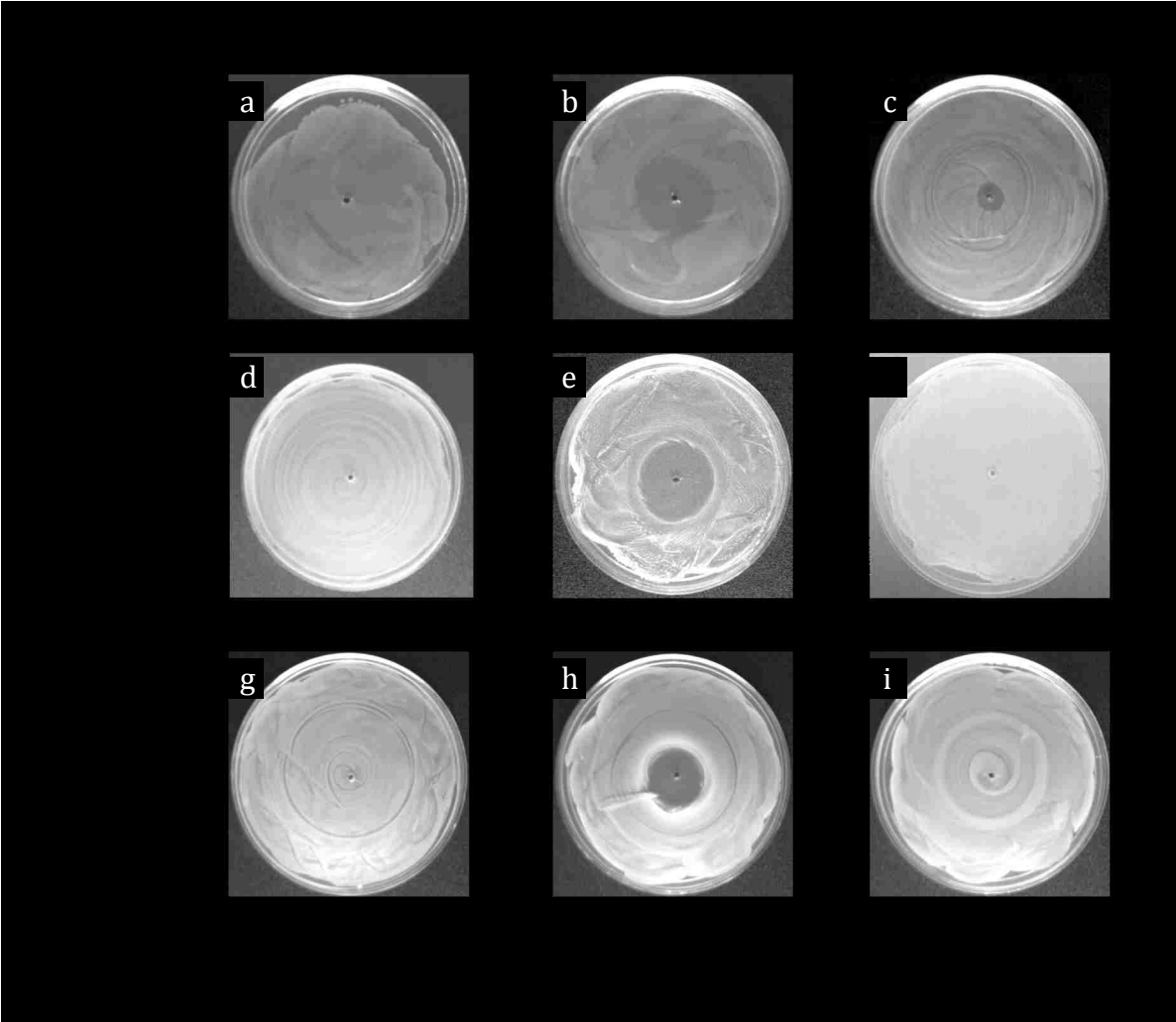to Chapter 2 for the revised protocol.

A.2.1. Initial growth characterization of *P. larvae*



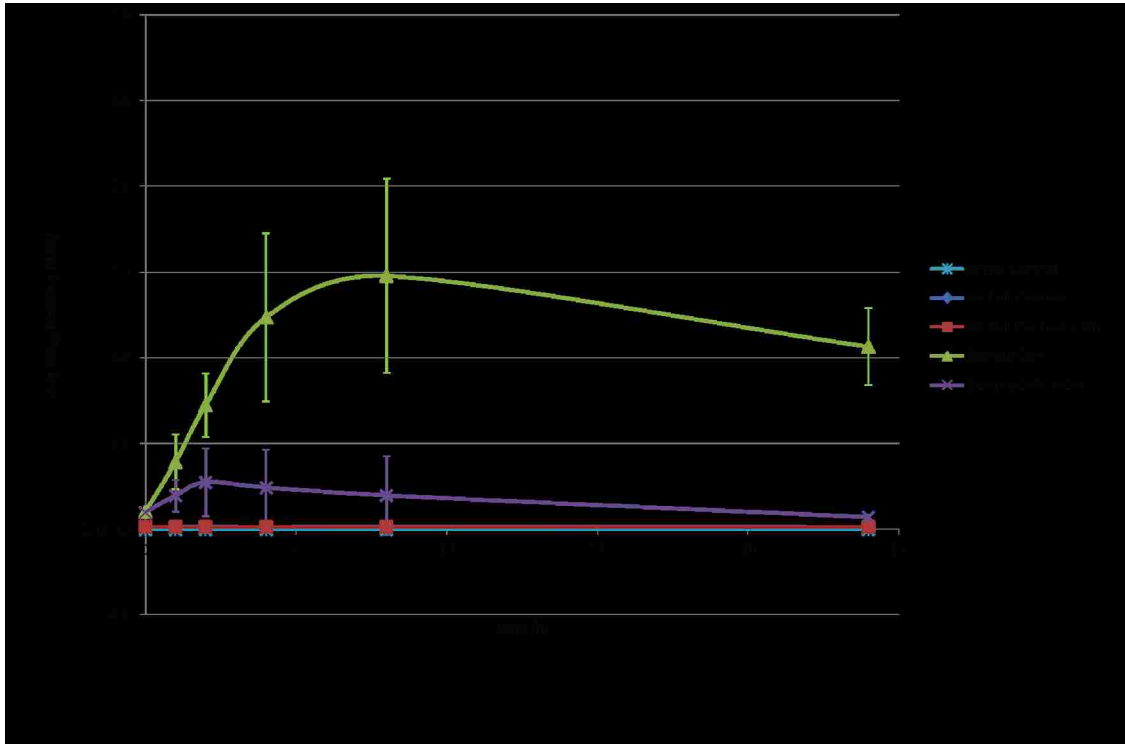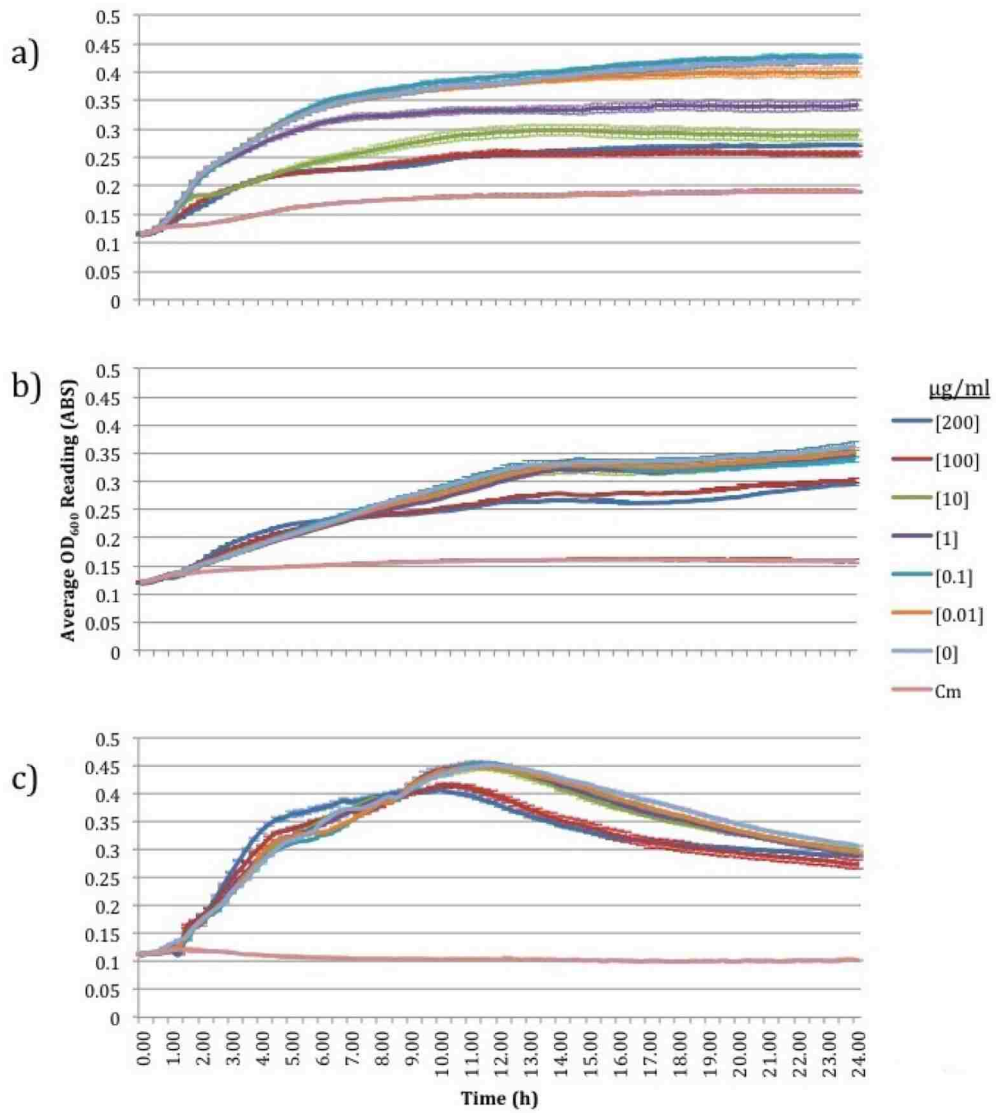| | | Strain and Media Type | | | |
|---|---|---|---|---|---|
| | | NRRL B-2605 | | NRRL B-3554 | |
| Time (h) | Dilution Factor | R2B agar | BHI agar | R2B agar | BHI agar |

A.2.2. Zone of inhibition testing

A.2.3. 96-well plate assay results

A.2.4. Testing Structural Integrity and the Activity of Defensin 1 After Storage

Defensin 1 was active against *P. larvae* in both the ZoI assay and the 96-well plate assay. However, the ZoI assays were done approximately 2-3 months before the 96-well plate assays; therefore, I wanted to test whether the activity of the compounds had declined during storage at -20°C. Defensin 1 has three disulfide bridges that are necessary for the activity of this compound, so I chose to compare the activity of the defensin 1 that had been stored at -20 °C to defensin 1 that had had its essential disulfide bonds broken (modified defensin 1) (Figure A7)*. P. alvei* was chosen as the test organism for this *in vitro* assay, because it was found to be susceptible to defensin 1 in the liquid culture assay (see section 3.3.4.).

Defensin 1 was denatured by incubating it in a 1% β-mercaptoethanol (BME) solution (v:v) (Calbiochem; Gibbstown, NJ) at 37 °C for 24 h (Raina & Missiakas, 1997). To ensure complete denaturation of disulfide bonds, samples were boiled for 5 minutes. Non-denatured defensin 1 (Def1) was used as the positive control. A culture of exponentially growing *P. alvei* cells was diluted to achieve an $OD_{600}$ of 0.200. The Each trial represents the average of 3 samples per condition. The experiment was replicated twice.

The defensin 1 treated sample produced a statistically significant ($p < 0.01$) decrease in the $OD_{600}$, but the modified defensin 1 and BME control did not (Figure A7). Consequently, these results demonstrate that at least some of the antibacterial activity of defensin 1 was retained during its storage at -20°C.
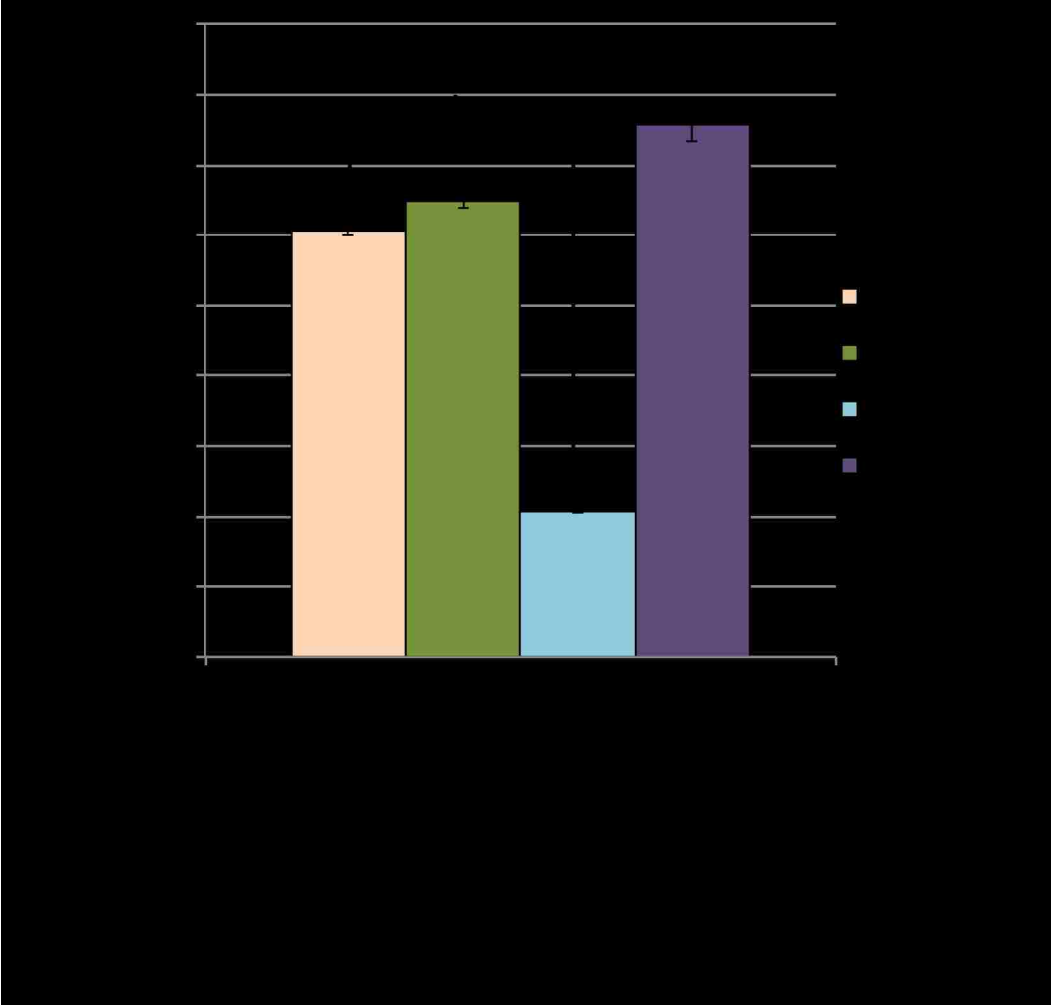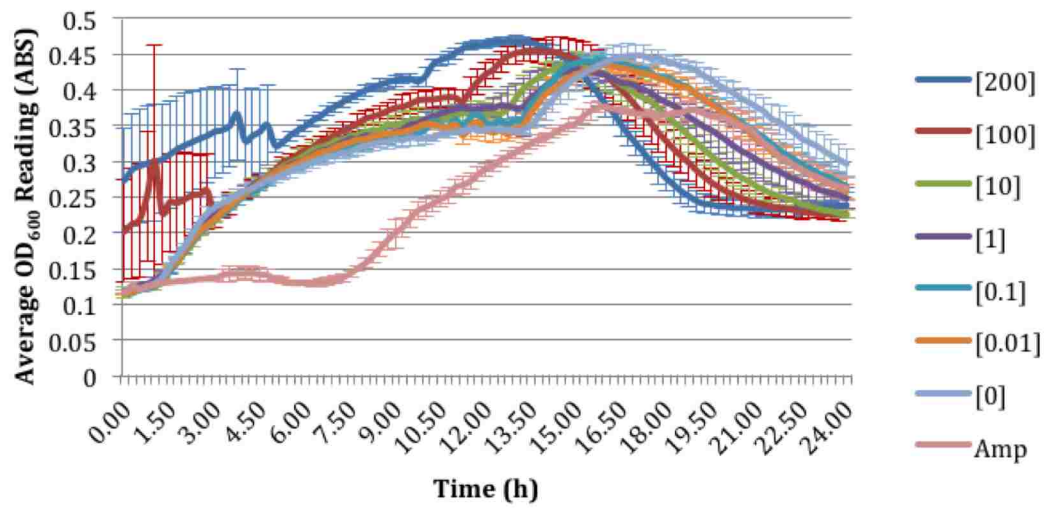
decrease in $OD_{600}$ begins to occur at approx. 14h, which is consistent with autolysis - a trait known to be exhibited by this strain (Jolliffe, Doyle, & Streips, 1981; Young, Tipper, & Strominger, 1964)

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## Curriculum Vitae

### Employment History

UNLV, School of Life Sciences; July 2011-present
· Position of Teaching Assistant for Microbiology Laboratory sections
 · Entailed teaching a variety of microbiological techniques (i.e. Litmus Milk test, Nitrogen Fixation, Antibacterial Susceptibiity, etc.)
· My thesis project entails using naturally occurring peptides as an antimicrobial agent
 · Successfully created methodology for killing a honeybee pathogen
 · Testing of potential treatments for a honeybee pathogen
 · Working on a practical application of treatments on honeybee larvae
· General laboratory duties

Allele Biotech; Technical Admin Support; July 2010 – 2011
· In charge of editing weekly blogs and website content
· Editing and designing Technical Data Sheets
· Uploading and creating descriptions of products onto newly launched website
· DNA (oligo) post-synthesis processing duties

Viacyte, Inc.; Stockroom Intern; July 2010 – May 2011
· Performed tissue culture experiments in response to naturally occurring hormones
 · Isolated RNA from various tissue culture samples
 · qPCR on Corbett Robots and BioRad CFX96
· Some experience in experimental design
· Organized stockroom and kept labs stocked with essential supplies
· Managed inventory and ordered lab supplies

Innovive, Inc.; Marketing Intern; January 2009 – March 2009
· Distributed samples to various clients
· General administrative duties
· In charge of memberships to different associations and event planning
· Learned new databases quickly and learned to use them efficiently

Hollister Co.; Part-Time Impact; July 2005 – July 2010
· Assisted in planning of organizational projects
· Assisted in managing inventory
· Helped customers in a friendly and approachable manner

## Education

University of Nevada, Las Vegas; 2012-present
Master's of Science, Microbiology (in progress)
Vice President of the American Society for Microbiology, UNLV Student Chapter (Dec 2012-Dec 2013)

University of California, San Diego; 2008-2011
Major - General Biology
Minor - Psychology

Southwestern College, 2005-2008
Associate in Arts; Transfer Studies
Member and officer of joint associations: Society of Hispanic Professional Engineers/Society of Women Engineers

## Awards and Nominations

· 2015 Nomination to the National Society for Leadership and Success
· AAAS Membership through the AAAS/Science Program for Excellence in Science
· NSF Travel Grant for travel to 2013 Wind River Prokaryotic Conference
· GPSA Grant for travel to 2013 Wind River Prokaryotic Conference
· 2nd Place Awarded at GPSA Graduate Student Research Forum
· NSF Travel Grant for travel to 2012 Wind River Prokaryotic Conference
· GPSA Grant for travel to 2012 Wind River Prokaryotic Conference

## Presentations

· Poster presentation at the Annual Graduate and Professional Student Association Research Forum (29 March 2014) – "Growth Inhibition of *Paenibacillus larvae* Using Honeybee Antimicrobial Peptides"
· Talk and poster presentation at the 57th Annual Wind River Conference on Prokaryotic Biology (5-9 June 2013) – "Testing Honeybee Antimicrobial Peptides to Counter the Growth of the Causative Agent of American Foulbrood Disease, *Paenibacillus larvae*"
· Poster presentation at the 52nd Annual Meeting of the Arizona/Southern Nevada Branch of the American Society for Microbiology (13 April 2013) – "Testing Honeybee Antimicrobial Peptides to Counter the Growth of the Causative Agent of American Foulbrood Disease, *Paenibacillus larvae*"
· Poster presentation at the Annual Graduate and Professional Student Association Research Forum (16 March 2013) – "Microbial Biology as the Basis for New Treatments for American Foulbrood"
· Poster presentation at the 51st Annual Meeting of the Arizona/Southern Nevada Branch of the Poster presentation at the 1st Annual STEM Summit (14 January 2013) - "Microbial Biology as the Basis for New Treatments for American Foulbrood"
· Poster presentation at the 56th Annual Wind River Conference on Prokaryotic Biology (6-10 June 2012) – "Developing a Method to Test Honeybee Antimicrobial Peptides Against *Paenibacillus larvae*"

· American Society for Microbiology (21 April 2012) – "Developing a Method to Test Honeybee Antimicrobial Peptides Against *Paenibacillus larvae*"

## Publications

· Protocols to test the antimicrobial activity of antimicrobial peptides against the honey bee pathogen *Paenibacillus larvae.* 2015. Journal of Microbiological Methods. *In Press*

## Educational Outreach

· UNLV Student Chapter of ASM "Outreach Activity at the Las Vegas Natural History Museum" 28 April 2013
· UNLV Student Chapter of ASM "Outreach Activity at the Las Vegas Natural History Museum" 24 March 2013

## Skills

· Antimicrobial susceptibility assays, SDS PAGE, qRT-PCR, tissue culture
· Primary concentration working with RNA at Viacyte
· Aseptic techniques in the lab and working in hoods
· Proficient in MS programs
· Internet web design