Growth Inhibition of *Staphylococcus epidermidis* using Hemolymph Extracted from *Manduca Sexta*

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Abstract

The immune system of tobacco hornworms (*Manduca sexta*) can serve as a model of the mammalian immune response and a potential source of novel antibiotics (Fig. 1). Forty-five tobacco hornworms were reared, and 13 were chosen randomly for inoculation with gram negative bacteria, while 12 received no injection and served as the control. After 24 hours, hemolymph was extracted from both groups. A microplate assay was performed to determine whether immune challenged individuals displayed a better capacity to inhibit growth of *Staphylococcus epidermidis* compared to control individuals. Previous research suggested that antimicrobial peptides from insects are better at inhibiting gram negative bacterial growth than gram positive bacterial growth. Our results do not support this research as the immunized hemolymph did inhibit growth of the gram positive bacteria *S. epidermidis*. This necessitates further studies directly comparing inhibition of gram positive and gram negative bacteria.

Introduction

A prevalent issue in healthcare around the world is the existence of antibiotic-resistant bacteria (Adegoke *et al.* 2017). Much research has gone into finding novel antibiotics that can fight these resistant and very dangerous microbes (Moloney 2016, Ohlsen 2009). One mode of investigation is looking at antimicrobial peptides (AMPs) produced by insects. Insects are abundant, relatively low-cost to raise, and have short life spans allowing for efficient examination (Reinecke *et al.* 1980). The more known about AMP production in insects, the more knowledge can be applied to development of new antibiotics that have the potential to fight antibiotic-resistant bacteria. Insects such as large moths allow great observation and collection of large amounts of hemolymph, the equivalent of blood in a human. Hemolymph exposed to bacteria should contain AMPs that will fight the bacteria more quickly and efficiently at a second exposure. To test this, the effects of hemolymph taken from both infected and uninfected tobacco hornworms on growth of gram positive bacteria (*Staphylococcus epidermidis*) were analyzed. We hypothesized that *S. epidermidis* growth will not have significant inhibition, per previous research. However, it is also hypothesized that the hemolymph from immune challenged tobacco hornworms will be more efficient at bacterial growth inhibition than the control group.

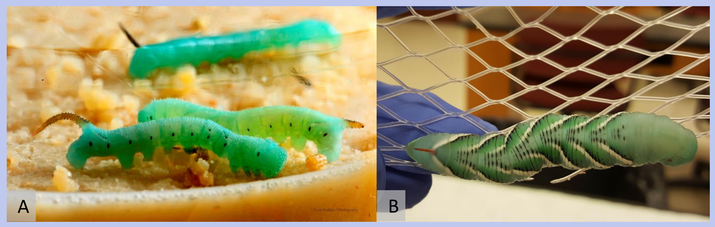

Figure 1. (A) Tobacco hornworm (*M. sexta*) first instar (B) *M. sexta* fifth instar

Methods

Rearing the Colony

- ~100 tobacco hornworm eggs were obtained from Carolina Biological Supply Company and reared in clear plastic cups with food layered on the bottom (Fig. 1)
- Rearing chambers were monitored closely and kept under light conditions for 12 hours
- 45 tobacco hornworms were reared until their fifth instar

Insult with Bacteria Cocktail

- Randomly selected for either the control group (12 total) or the experimental group (13 total)
- Placed in the refrigerator for 45 minutes to chill, cleansed in ethanol, and dried
- Experimental group was insulted with 20µL bacteria cocktail while the control group received nothing
- Kept for 24 hours in rearing chambers labeled with their respective treatment

Hemolymph Extraction

- Placed in the refrigerator for 45 minutes, cleansed in ethanol, and dried
- Sterile scissors were used to partially sever each individual's 2nd proleg, and hemolymph was collected into 2 centrifuge tubes, each with ~2g PTU
- Stored in the freezer until needed for the microplate assay

Microplate Assay

- 96-well plate contained a negative control, positive control, and the 2 hemolymph groups (Fig. 3)
- Allowed to run in a microplate reader for 24 hours at 30°C.
- Growth curves based on optical density were measured using a microplate reader and analyzed using t- tests.

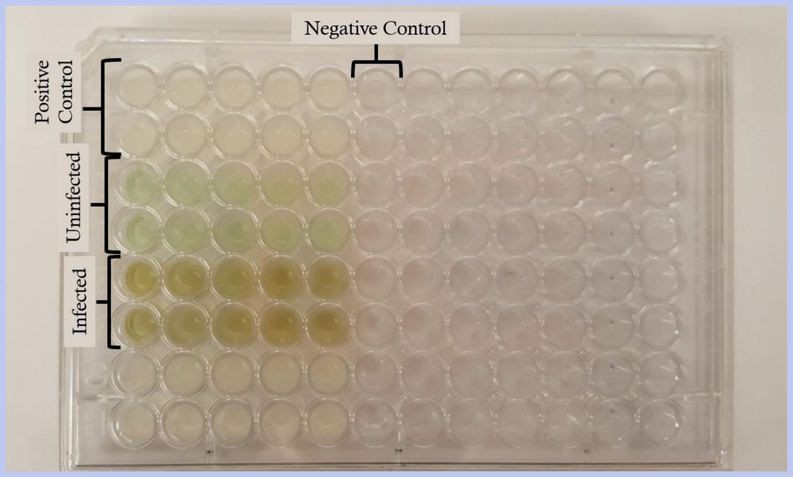

Figure 3. Microplate Assay of *S. epidermidis* with phosphate buffer saline (PBS), *S. epidermidis* with uninfected hemolymph, *S. epidermidis* with infected hemolymph, and sterile PBS as the negative control

Results

- The negative control did not exhibit any bacterial growth and both the uninfected and infected hemolymph growth curves showed limited *S. epidermidis* growth (Fig. 4)
- Statistical analysis comparing the maximum optical densities of the positive control to both hemolymph groups demonstrated that both uninfected and infected hemolymph exhibited significant growth inhibition of *S. epidermidis* (Fig. 5)
- The negative control and infected hemolymph were not significantly different (Fig. 5).

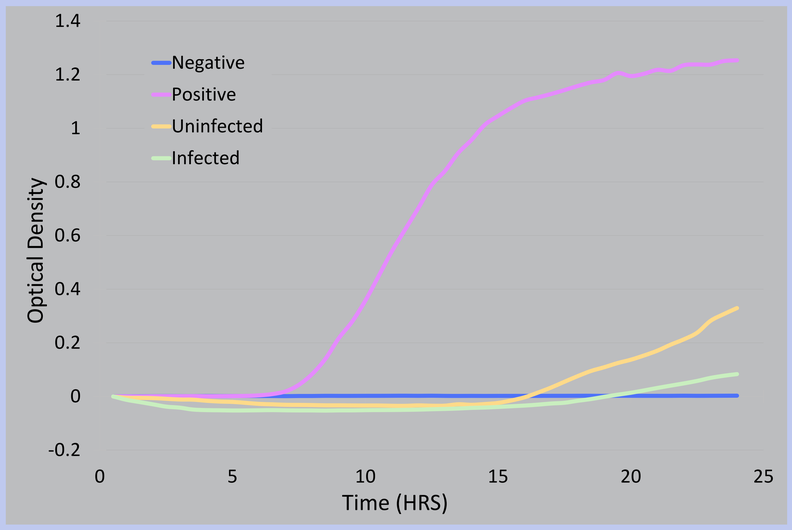

Figure 4. Optical density readings of sterile PBS (-) and *S. epidermidis* growth in the presence of PBS (+), uninfected hemolymph, and infected hemolymph over a 24 hour period

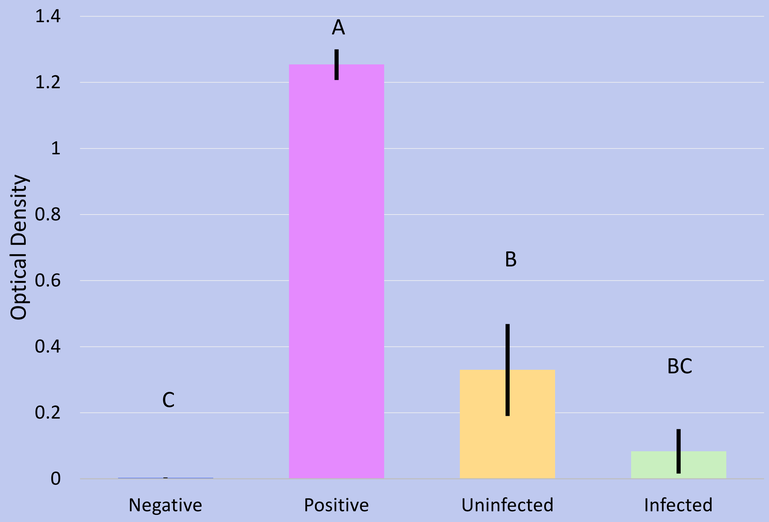

Figure 5. Maximum optical densities analyzed using t tests

Discussion

Growth curves from both uninfected and infected hemolymph indicate *S. epidermidis* was greatly inhibited. The hypothesis that hemolymph from *M. sexta* will not significantly inhibit growth of *S. epidermidis* can be rejected. After receiving an insult with the bacteria cocktail, AMP synthesis began in the infected individuals and provided them with the means to combat bacterial growth when exposed a second time (Trauer and Hilker 2013). Previous research suggests AMP activity remains unchanged in unchallenged individuals (Haines *et al.* 2008). Therefore, it is unknown why the uninfected hemolymph significantly inhibited bacterial growth, as those individuals received neither an immune challenge nor a mechanical insult.

Future research should focus on other Lepidopteran species, such as *Actias luna*, that have not been studied as well as *M. sexta*. Great care should be taken with them throughout development to ensure cross-contamination does not occur between reared individuals, thereby preserving the integrity of our results. From there, *A. luna* hemolymph could be analyzed for any possible AMP production.

Literature Cited

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