Examining the Antibiotic Effects of Hemolymph from Immune Challenged Squash Bugs (*Anasa tristis*)

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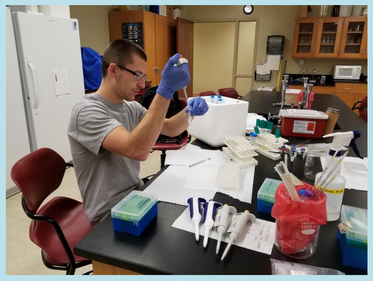
Abstract

Insects represent a promising source for novel antimicrobial peptides for combatting the growing epidemic of antibiotic resistant bacteria. The antibiotic properties of squash bug (*Anasa tristis*) hemolymph were tested against *Escherichia coli* in a growth inhibition assay. *Anasa tristis* groups were either immunized with a mixture containing *E. coli* and *Staphylococcus epidermidis* or un-immunized. Hemolymph from both groups was extracted and tested against *E. coli* growth in a microplate assay. Growth curves revealed *E. coli* grew more in the presence of immunized and un-immunized hemolymph than the positive control. Growth curves between immunized and un-immunized hemolymph were not significantly different. Overall, no significant antibiotic activity in *A. tristis* hemolymph was observed. Instead, the hemolymph increased *E. coli* growth, possibly due to the total nutritional value of the insect hemolymph.

Introduction

- Antibiotic resistance is a major threat to patient health.
  - ~2 million people per year in the U. S. become infected with antibiotic-resistance bacteria (Centers for Disease Control and Prevention 2018).
- Natural sources are being investigated to find novel antibiotics.
  - Insects produce bactericidal and bacteriostatic antimicrobial peptides (AMPs) that affect many bacteria (Lauth et al. 1998).
  - Herzner et al. (2013) isolated a new AMP from a parasitoid wasp that is effective against strains of antibiotic resistant *Staphylococcus aureus.*
- This experiment examined the common squash bug (*Anasa tristis)* for novel AMP production.
  - The induced immune response of *A. tristis* was tested against the growth of the Gram-negative species *E. coli*.
- We hypothesized that *A. tristis* hemolymph would inhibit *E. coli* growth.

(A)



(B)

Figure 1. (A) *Anasa tristis* nymphs resting on bean leaves within the environmental chamber. (B) Collection of *Anasa tristis* hemolymph post centrifugation.

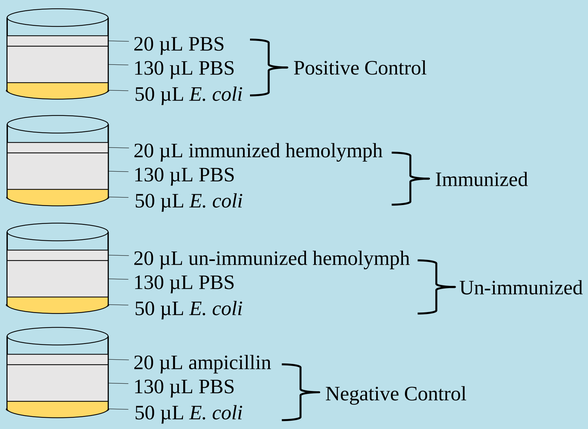


Figure 2. Microplate wells with added treatment group volumes.

Methods

Rearing *Anasa tristis*

- Populations were collected near Jackson, TN.
- They were housed in an environmental chamber, kept at a constant temperature and photoperiod, and fed squash leaves and fruit [Figure 1(A)].

Experimental Groups

- Twenty *A. tristis* adults were randomly assigned to 1 of 2 groups: 10 immunized with a mixture containing *E. coli* and *S. epidermidis* and 10 un-immunized.
- Nutrient broth cultures for *E. coli* and *S. epidermidis* were made from stock plates and incubated for 24 hours at 37°C at 200 rpm.
- Both cultures were mixed together and a dipped inoculation needle punctured the immunized group's abdomens.

Hemolymph Extraction

- Both groups had their legs and antennae severed with sterile scissors, heads decapitated, and bodies placed in 1-mL microcentrifuge tubes.
- Bodies were centrifuged for 10 minutes at 10,000 rpm.
- Hemolymph (supernatant) was collected from each tube and transferred to 1-mL microcentrifuge tubes [Figure 1(B)].

Microplate Assay

- 96-well plate contained four treatment groups tested in triplicate (Figure 2).
- Microplate incubated for 16 hours at 37°C with continuous shaking at 200 rpm. Optical density (OD) readings were taken every 30 minutes.

Statistical Analysis

- An ANOVA verified if statistically significant differences occurred within the data for average maximum OD ($\alpha \leq 0.05$).
- A Student's t-test verified statistically significant differences occurred when comparing two specific groups ($\alpha \leq 0.05$).

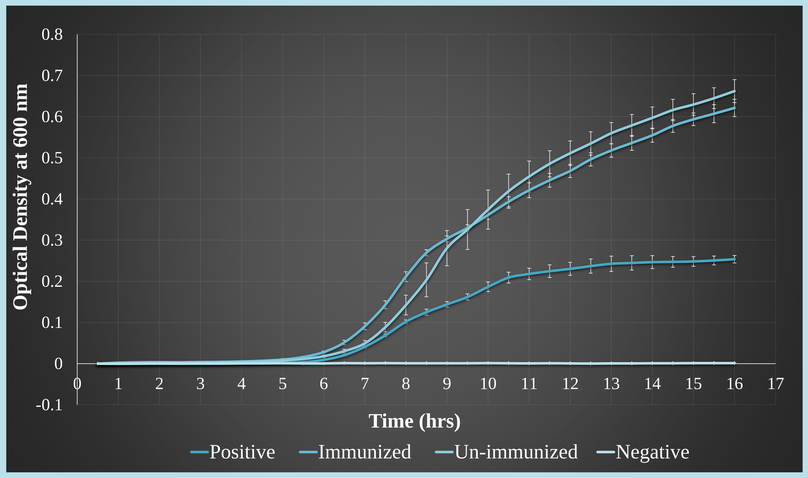


Figure 3. Average optical density measurements of *Escherichia coli* in the presence of immunized and un-immunized *Anasa tristis* hemolymph, positive control (phosphate buffered saline), and negative control (ampicillin) for 16 hours in a microplate reader.

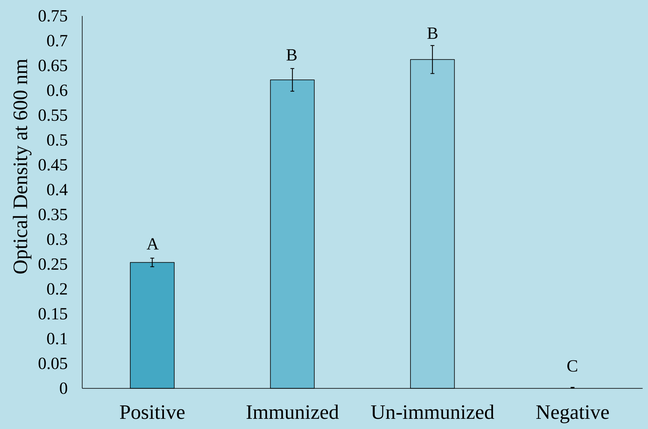


Figure 4. Average maximum optical density measurements for *Escherichia coli* in the presence of immunized and un-immunized *Anasa tristis* hemolymph, positive control (phosphate buffered saline), and negative control (ampicillin).*
*Statistically significant differences are shown with opposing letters with $\alpha \leq 0.05$.

Results

Growth Curves

- *E. coli* growth was higher in the immunized and un-immunized groups than the positive control after 16 hours (Figure 3).

Average Maximum Optical Density

- Average maximum OD's for the positive control, immunized, un-immunized, and negative control were 0.253, 0.583, 0.663, and 0.003, respectively.
- There was a significant difference between the positive control and both immunized and un-immunized groups, but there was no significant difference between the immunized and un-immunized groups (Figure 4).
- The positive control, immunized, and un-immunized groups were all significantly different than the negative control (Figure 4).

Discussion

No Antibiotic Activity was seen in *A. tristis* Hemolymph

- An activated immune response may not have occurred despite mechanical injury with an inoculated needle.
  - No visible melanization was seen on any of the immunized group's abdomens.
  - No insects died 24 hours post inoculation.
  - A cytometer was not used to verify if any activated plasmocytes were present.
- Hemolymph itself may have provided nutrients for *E. coli* to growth better.
  - Follow-up study showed *E. coli* grown in immunized *A. tristis* hemolymph had a similar average maximum OD compared to *E. coli* grown in tryptic soy broth (TSB) (Table 1).

Future Studies

- A cytometer can verify that an immune response occurred by counting plasmocytes present.
- A larger population size would help for greater hemolymph volumes to conduct more replicated measurements.

Table 1. Comparison of average maximum optical densities (OD) for 50 µL of *Escherichia coli*, 130 µL of phosphate buffered saline (PBS), and either 20 µL of immunized *Anasa tristis* hemolymph, un-immunized *A. tristis* hemolymph, PBS, nutrient broth (NB), or tryptic soy broth (TSB) in a microplate assay.

Content Added	Average Maximum OD
Immunized Hemolymph	0.583*
Un-immunized Hemolymph	0.663
PBS	0.254
NB	0.339
TSB	0.562*

* No statistically significant difference between the two values with $\alpha = 0.2355$.

Conclusion

1. We reject our hypothesis stating the hemolymph would inhibit *E. coli* growth.
2. This increased growth is possibly due to the total nutritional value of *A. tristis* hemolymph.

Literature Cited

Centers for Disease Control and Prevention. 2018. About Antimicrobial Resistance. Available at: https://www.cdc.gov/drugresistance/about.html

Herzner, G., A. Schlecht, V. Dollhofer, C. Parzefall, K. Harrar, A. Kreuzer, L. Pilsl, and J. Ruther. 2013. Larvae of the parasitoid wasp *Ampulex compressa* sanitize their host, the American cockroach, with a blend of antimicrobials. Proceedings of the National Academy of Sciences 110:1369-1374.

Lauth, X., A. Nesin, J. Briand, J. Roussel, and C. Hetru. 1998. Isolation, characterization and chemical synthesis of a new insect defensin from *Chironomus plumosus* (Diptera). Insect Biochemistry and Molecular Biology 28:1059–1066.

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