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How Polymicrobial Infection Impacts *Acinetobacter baumannii* Virulence in the Host Organism *Drosophila melanogaster*

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Abstract

The emergence of antibiotic resistant *Acinetobacter baumannii* is raising considerable alarm within the medical community. Because *A. baumannii* is becoming a common member of the human microflora, the potential for *A. baumannii* infections to occur among healthy individuals is likely to rise. The goal of this research is to determine if the virulence of *A. baumannii* strengthens during simultaneous infection with other common microbes of the human flora – namely *Staphylococcus epidermidis* and *Bacillus subtilis*. Subcutaneous intrusions of these microbes individually may not weaken an individual with a competent immune system; but exposure to several microbes at the same time may afford *A. baumannii* an opportunity to establish an infection that is difficult to resolve. We challenged *Drosophila melanogaster* through singular and polymicrobial microinjection using the microbes mentioned above. We evaluated the microbial cell density at 1 day, 1 week, and 2 weeks post-infection to determine if the microbes established a persistent infection. Additionally, we monitor the mortality rate of the infected *D. melanogaster* against a control population to determine if the infection caused death. We concluded that the virulence and any changes in the pathogenicity of microbes is based on individual characteristics of pathogens. Therefore, the difference in virulence must continue to be studied, as the diversity of microbes and hosts provides a platform for a wide variety of outcomes.

Keywords: Polymicrobial infection, *Acinetobacter baumannii,* virulence

1. Introduction

1.1.Initial Concept

Acinetobacter baumannii is a pathogen of peculiar interest to the public. It is a member of the ESKAPE pathogen community (*Enterococcus aureus, Staphylococcus aereus, Klebsiella pneumonia, Acinetobacter spp., Psuedomonas aeruginosa, Enterobacter spp*.), is known to develop resistance to antibiotics and can be spread through improperly sterilized equipment, open wounds from surgery, and prolonged hospital stays.[1,2] *A. baumannii* has been appearing more frequently in soldiers treated in hospitals in Iraq and Kuwait.[3] Infection with this bacteria has a wide variety of symptoms, but two patients demonstrated fatal necrotizing fasciitis.[4] Both cases occurred in immunosuppressed patients and had a polymicrobial microbiome. The polymicrobial infections and their resulting fatalities led to a hypothesis that the virulence of a specific pathogen may become more potent when a host becomes infected with multiple pathogens. Therefore, an experiment was undergone in order to study the hypothesis: If an organism is infected with *A. baumannii* and another microbe, then *A. baumannii* will be more virulent than as a monomicrobial infection.

1.2 Selection of Microbial Organisms

Upon developing our initial concept and hypothesis, we queried various microbial journals to determine likely microbial candidates that are common among human flora. We determined that microbial candidates required certain characteristics to support our hypothesis. First, we determined that these candidates needed to have pathogenic capabilities that could potentially work in conjunction with *A. baumannii* in a polymicrobial inoculation. Second, the desired microbes needed to have a high chance of being present on or near subcutaneous lacerations, enabling invasion of exposed epithelial cells. Therefore, we selected the strains *Bacillus subtilis* and *Staphylococcus epidermidis*, which are both common microbes found on humans.[4,5]

1.2.1 Current Understanding of microbes

The pathogen, *A. baumannii*, is a gram-negative coccobacillus that can sometimes present challenges in de-staining, leading to misidentification of the pathogen as gram-positive. As stated previously, it is a pathogen stirring concern due to the resistance developed by strains and the infection rate of immunocompromised patients. It is known to grow on human skin and has been correlated with aquatic environments.[6] *B. subtilis* is also a gram-positive bacteria and can be found in both aquatic and dry environments, and is common with the gastrointestinal (GI) tract of animals. Its spores can be found in soil at high concentration.[4] *S. epidermidis* is an interesting pathogen. It is very common on human skin and has adapted little virulence to remain on human skin. However, recent virulent strains have been found to infect patients in hospitals, including up to 22% of bloodstream infections in the ICU.[5] These pathogens were all selected as they are common to humans and are well-researched.

1.2.2 Significant Information about the Host

Drosophila melanogaster served as the host organism for this experiment. We selected *D. melanogaster* because several published studies link genetic familiarities between its innate immune system and that of humans. The innate immune system of *D. melanogaster* consists mostly of two pathways for resolving infection: the Toll pathway and the immune deficiency (Imd) pathway.[7] These responses both release anti-microbial peptides, or AMPs, and vary as to the stimuli that trigger them.[7] The Toll pathway has been studied and shows that humans react similarly to flies with certain infections.[8] Because of this similarity in an immune response to pathogenic infection, *D. melanogaster* is an ideal model to understand simple mechanics of pathogens.

2. Methods

2.1 Model Organism

The use of *D. melanogaster* and the experiment was approved by the Department of Chemistry and Life Science research program. We used Carolina wild type drosophila (product #172100) as the host organism. We maintained all fly cultures in clear plastic fly vials and fed them a typical fly diet by using Carolina Formula 4-24 plain drosophila medium (product #173200) and its recommended preparation. We placed a small amount of baker's yeast on top to help the flies be more active and productive. We transferred flies to new vials with fresh food every two weeks to reduce death among a population due to a breakdown in food composition.

2.2 Preparation of the Microbes

The microbes used in this project were acquired from BEI Resources. We used the individual strains *A. baumannii* (NR-13374), *B. subtilis* (NR-607), and *S. epidermidis* (NR-45862). Only the *A. baumannii* strain was resistant to ampicillin. We cultured each individual microbial strain on a Luria Broth (LB) agar plate at 37°C overnight. Plates were stored in a refrigerator until required for use. To prepare the strain for infection, an isolated colony was transferred from the plate and placed in a sterilized glass tube containing 3.0 mL of LB. The tube was placed in a shaking incubator at 37°C overnight to allow for the most optimal growth at OD600. 1.0 mL of this media was transferred into a1.5 mL Eppendorf tube and then centrifuged for 2 minutes at 800rpm. The supernatant was removed, leaving only the pellet. Another 1.0ml of the cultured media was transferred to the same Eppendorf tube with the pellet

and once again centrifuged for the same time and speed, with the supernatant removed and pellet remaining. The process was repeated a final time for the remaining liquid culture (~1.0ml) The final pellet was re-suspended in 0.2 mL of LB for microinjection.

2.2.1 microinjection

2.2.1.1 monomicrobial

Flies were transferred from the feeding vials to sterile empty vials. The vial containing the flies was placed in a Styrofoam container of ice and anesthetized for approximately 30 minutes. Once anesthetized, approximately 5 flies were transferred to a sterilized petri dish on ice as a holding area (5 flies is a safe number to have in an open, ice-chilled petri dish before the flies recover from the effects of being anesthetized). A single fly was transferred from the petri dish and placed on a clear glass slide under a dissecting microscope. A .125mm ultra fine dissecting needle (Roboz Surgical Instruments) was dipped into the prepared solution containing the suspended microbes to contaminate the needle. The needle was then injected into the lateral thorax of the fly. The fly was then added to a new tube with fly food and allowed to recover from the anesthetic. This process was done for each individual population of flies. For polymicrobial infection, two separate needles were used, and each fly was infected twice. For the control populations, we used a sham injection method where the flies were anesthetized in the same manner and injected with a sterile solution of LB media, using a sterilized dissecting needle.

2.2.1.2 polymicrobial infection

This process followed the same process for monomicrobial infection with a few exceptions. All flies of a population were infected with a single microbial strain before another strain. This ensured that flies were in fact being injected with a significant dose of each microbial strain. After being microinjected with the first strain, the flies were transferred to a separate, sterile vial tube that was also on ice, instead of being transferred to the vial containing food. Once the flies of that population were microinjected, the process was repeated a second time using the other microbial strain. Additionally, a new dissecting needle was used to once again prevent contamination and questionable infectious results. Once each fly was microinjected with the second strain, it was transferred to the vial containing fly food and allowed to recover from being anesthetized.

2.2.2 homogenization and serial dilution

After infection, fly populations were selected to be tested for infection at 1 day, 7 days, and 14 days post-infection. The homogenization process begins by first anesthetizing the flies with ice as previously stated, and then externally sterilized in a 50 mL solution of bleach and cool deionized water (10 mL: 40 mL). Following this, the flies were air dried for approximately 3 minutes, and they were blotted with a towel to ensure no excess fluid remained. Each fly was placed in 100 μL of sterilized water in a 1.5 mL Eppendorf tube. The flies were then homogenized with sterilized pellet pestles. A new, sterile pestle was used on each fly to avoid contamination. The homogenized solution was then used in a serial dilution, with 20 μL of solution moved to each well containing 80μL of sterile water for a total of 5 dilutions.. Once diluted, 5μL from each column was transferred to LB agar plates. For polymicrobial infections, the microbes were plated on ampicillin and non-ampicillin plates, with a sample from the original sterilized glass tube and added at the bottom of each plate to use as a means for identification. Ampicillin was used to select for *A. baumannii.* To confirm the speciation of all colonies, a sample was isolated and tested with MALDI-TOF and its corresponding database. This was done after analyzing the colony through visual observations of color and size.

3. Results

3.1 Monomicrobial and Polymicrobial Infection on D. melanogaster populations

We measured the effects that the monomicrobial infection of each strain had of the fly populations and compared these results to those of polymicrobial infections. *A. baumannii* and *B. subtilus* were the only strains that measured any growth of infection upon infection. We did not observe any growth and promulgation of the *S. epidermidis* strain in

any population of flies and the control populations demonstrated no noticeable infection nor impact of the sham injections. (see Figure 1). For the monomicrobial infection phase, we observed a decrease in the fly population infected from Day 1 to Day 14 for *A. baumannii* and *B. subtilus*. *A. baumannii* infected 90% of the population at Day 1, but decreased to infecting only 30% by Day 7 and 0% by Day 14. *B. subtilus* infected nearly 80% at Day 1, but then decreased to about 18% by Day 7, and finally less than 10% by Day 14. (see Figure 1A).

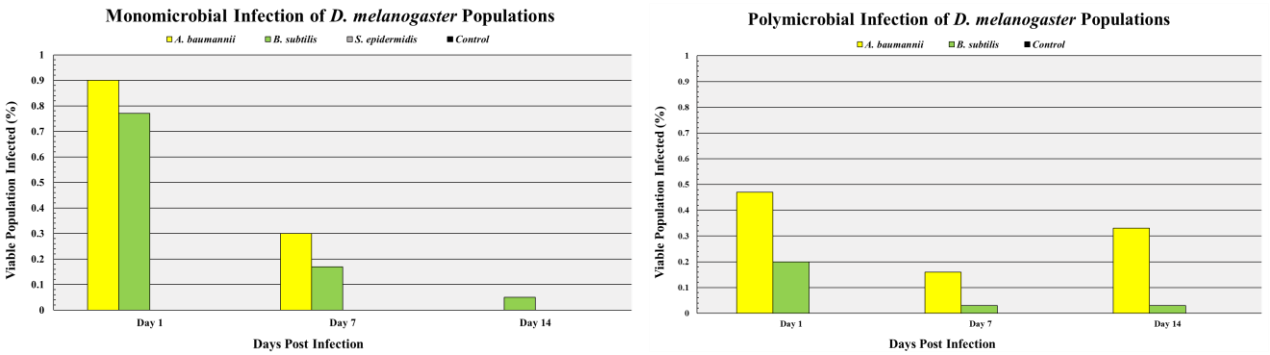

Figure 1: *Viable Fly Population Infected with Pathogens.* A) Monomicrobial Infection: Comparison of the viable fly populations infected with monomicrobial strains. Only *A. baumannii* and *B. subtilis* infected the flies with a certain degree of confidence. (n=30 for *A. baumannii*, S. *epidermidis*, and *Control* for Day 1, 7, and 14. n=30 for *B. subtilis* for Day 1, n=24 for Day 7, and n=20 for Day 14). B) Polymicrobial Infection: Comparison of the viable fly populations infected with the polymicrobial strains, *A. baumannii* and *B. subtilis*. $P < 0.05$ for Day 1 and Day 14 compared to monomicrobial infection. (n=30 for Day 1, n=24 for Day 7, and n=18 for Day 14. n=30 for *Control* for Day 1, 7, 14).

When we conducted the polymicrobial infection phase, we noticed a significant change in the infected fly population from the monomicrobial infection. Neither When used together for infection, neither *A. baumannii* nor *B. subtilis* infected above 50% of the population, as *A. baumannii* was isolated from just under 50% of population and *B. subtilus* isolated from 20% of the population. Less than 20% of the Day 7 fly population was infected with *A. baumannii* or *B. subtilus*. However, we did observe *A. baumannii* in over 30% of the Day 14 fly population, which was significantly higher than the infected population under monomicrobial conditions (see Figure 1B). We did not undertake to conduct polymicrobial infections with S. epidermidis due to the negative results using it in monomicrobial infections.

3.2 Monomicrobial and Polymicrobial Infection Density

We compared CFUs to analyze the infection density of each pathogen. We observed a higher cell density of *B. subtilus* to *A. baumannii* under monomicrobial infection (see Figure 2). *B. subtilus* had a higher mean cell density in both the Day 1 and Day 7 populations, with nearly a 20-fold increase and 10-fold increase respectively. We did not observe any cell density of *A. baumannii* at Day 14 and too few specimens of *B. subtilus* at Day 14 (see Figure 2A). Additionally, as noted earlier, *S. epidermidis* did not manifest any observable infection and therefore we could not distinguish or count CFUs.

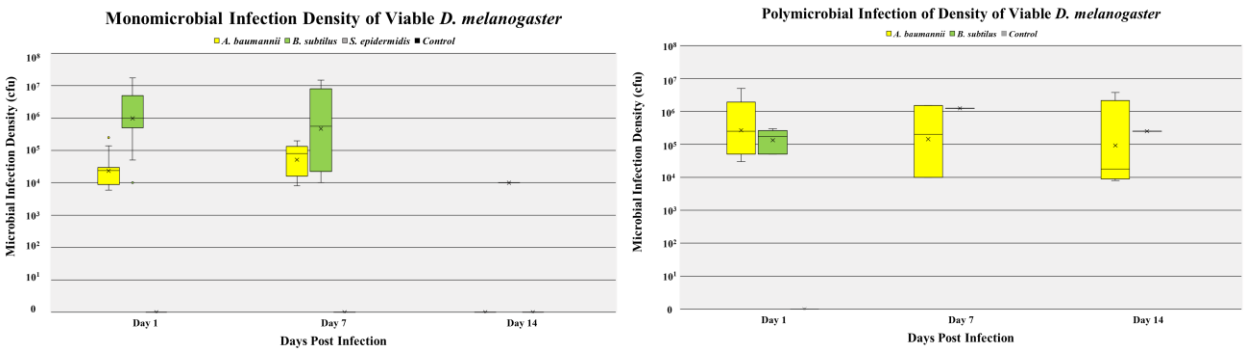

Figure 2: Cell Density of Monomicrobial and Polymicrobial Infection. A) Monomicrobial Infection. *B. subtilis* maintained the highest cell density between the three monomicrobial strains. S. epidermidis did not infect beyond any confidence that it ever entered log phase upon infection. B) Polymicrobial Infection. *A. baumannii* maintained the highest cell density in the polymicrobial infection compared to *B. subtilis*, as well as manifesting CFU throughout the duration of the experiment. $P<0.05$ of *A. baumannii* cell density of Day 1 and Day 14 populations between monomicrobial and polymicrobial infections.

We observed a significant difference when we analyzed the CFU of the pathogens during polymicrobial infection. The mean cell density of *A. baumannii* increased about 10-fold in the polymicrobial infection, whereas the mean cell density of *B. subtilus* decreased by almost 20-fold. Additionally, the mean cell density remained the same for *A. baumannii* throughout the duration of the experiment for each population that manifested infections (see Figure 2B).

3.3 Survival Rate of D. melanogaster with Monomicrobial and Polymicrobial Infections

The monomicrobial and polymicrobial infections did yield fatal results. We focused on the Day 14 population of flies because this population would produce the best effects that the infections had on the flies. *B. subtilus* proved to be the only pathogen that affected the survivability of the flies (see Figure 3). For the monomicrobial infection, 10 flies (~30% of the population) died between days 4 and 9, with 7/10 infected with *B. subtilus*. Flies that were infected with *A. baumannii* or *S. epidermidis*, did not die and instead maintained a survival rate comparable to the control population (see Figure 3A). After day 9, the population infected with *B. subtilus* did not experience a further decline in survivability.

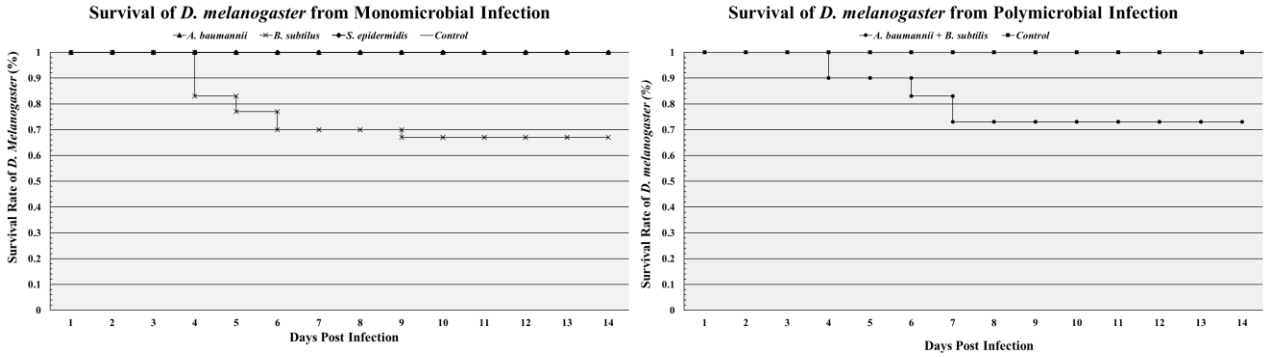

Figure 3: *Survival Rate of D. melanogaster Day 14 Population.* A) Monomicrobial Infection: *B. subtilis* was the only bacterial species that killed individual flies. Deaths occurred within the first 6 days, with 7/10 being infected. B) Polymicrobial Infection: Flies from the Day 14 population did die from the polymicrobial infection. However, dead flies had a negligible density of *A. baumannii* ($<10^3$) when compared to the cell density of *B. subtilis*. (n = 30, $P<0.05$ for monomicrobial and polymicrobial)

When flies were infected with *B. subtilus* and *A. baumannii*, a survival curve similar to the monomicrobial survival curve was very distinguishable (see Figure 3B). Between days 4 and 7, a total of 8 flies died with 7/8 infected with *B. subtilus*. However, only 2/8 were also infected with *A. baumannii*. Similar to the monomicrobial infection, the survival rate remained steady after Day 7 for the duration of the experiment with no more observable deaths.

4. Discussion & Conclusion

Our results indicate that the virulence of *A. baumannii* is not impacted by polymicrobial infections, thus disproving our original hypothesis. We observed that in the monomicrobial infection phase that *D. melanogaster* was able to eliminate *A. baumannii*, likely due to an immune response. Similarly, *D. melanogaster* was able to cure itself of the other pathogens *B. subtilus* and *S. epidermidis*, even though the former did have lethal results shortly after inoculation.

However, during the polymicrobial infection, it appears that *A. baumannii* did benefit from co-infection with a pathogen such as *B. subtilus*. Although our data does not support that *A. baumannii* impacted the survival of *D. melanogaster*, it does support that *A. baumannii* can establish a persistent infection in the host organism with a co-pathogen. The fact that the number of flies infected with *A. baumannii* and the increase of its cell density in the polymicrobial infection is significantly higher than in the monomicrobial infection leads us to this conclusion.

We did not anticipate the lack of results with *S. epidermidis*. This species is ubiquitous in numerous environments and is related to other staphylococcus species that are quite virulent. However, this supports the current understanding that *S. epidermidis* is less virulent than other staphylococcus species. Additionally, we could not establish confirmation that *S. epidermidis* was not already in the host's body as a communal symbiont of the host's gut. Therefore, we determined to move beyond this for the polymicrobial infections.

Putting this research into context, a study on periodontitis, or gum disease, studied the synergistic relationship

between *A. baumannii* and *Porphyromonas gingivalis*. *P. gingivalis* is commonly found in tooth plaque, but when there is a higher concentration of it, *A. baumannii* flourished as well. The RNA produced by each bacterium was analyzed, and proteins affecting adhesion, biofilm formation, and protein secretion were mutated and adapted by both species, indicating a synergistic relationship. This is related to the paper as it puts *A. baumannii* in context, and demonstrates a possible method for *A. baumannii*'s increased traits—the bacteria evolves and changes the protein composition to alter the expression of major extracellular responses.

This research experiment and its results presents a platform for future research. The next step is to analyze and test more microbes with *A. baumannii*, to see if similar trends exist throughout a variety of different microbes. Perhaps a different combination can react synergistically or otherwise. We hope to further understand how polymicrobial infections may impact human health as our microbiome continues to change.

5. Acknowledgements

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