Oklahoma State University Honors Thesis

# Microbial interactions of necrophagous flies and their impact on bacterial transmission

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**ABSTRACT**

Flies are commonly found in environments that contain large quantities of microorganisms. However, they also frequently interact with humans, potentially initiating the transmission of diseases. In order to investigate the roles in which flies are involved in disease transmission, we used Illumina NGS MiSeq 16s targeted DNA sequencing and bioinformatic analysis to compare the microbiomes of flies and decomposing rat tissues over periods of time following exposure. Sequencing results were analyzed for presence of the 2017 World Health Organization (WHO) priority list antibiotic-resistant pathogens on Kraken database. Spread plate dilutions were also made to observe relationships between culturable bacteria on each of the samples. Sequence comparisons revealed variance in microbiomes between different time points, as well as between the flies and rat tissue. Higher varieties of bacteria were sequenced from the fly tissues than the rat tissues, while the rat tissue bacteria remained fairly constant in concentration ratios over time. Kraken database blast indicated the presence of at least 5 of the prioritized antibiotic-resistant pathogens on each of the samples. Spread plate dilutions showed larger amounts of cultured bacterial colonies on rat tissues than fly tissues, as well as an average decrease in bacterial concentrations over time. From this study, we concluded that the microbiomes of flies are significantly influenced by exposure to bacteria-rich food sources. We also saw evidence of antibiotic-resistant bacterial suppression on flies, though the means of suppression is still unclear.

## Introduction

Flies are commonly associated with the transmission of microorganisms and pathogens due to their physiology, feeding behavior, and life cycle. Decaying organic matter is utilized by flies to carry out their life cycle and obtain nutrients. For necrophagous flies, the decay of animal carcasses is the primary resource for which flies are able to feed upon and reproduce. However, these flies can also be found amongst human and animal dwellings, where they come into contact with various foods. Because of the high amount of microorganisms on decaying organic matter and fly physiological characteristics favorable for microorganism attachment, flies pose a potential risk of transmitting pathogens. Flies are currently known vectors of over 100 potentially pathogenic microorganisms that can cause more than 65 infectious diseases (Greenberg, 1965). Despite the high amount of interaction flies have with humans and animals, as well as the variety of environments in which flies can be found, fly-transmitted diseases are surprisingly not as prevalent as would be suspected.

The capability of flies to acquire and transmit pathogens is suspected to be largely due to various physiological structures specific to sarcophagous flies. The pulvilli, or pad-like structure between the tarsal claws, are known to assist flies with adhesion to smooth structures. However, Sukonaston *et al.* (2006) also found adhesive substances on the pulvilii that assist with adhesion of flies to smooth surfaces, and potentially the adhesion of bacteria to flies. Electron microsopy images reveal hair-like structures, or setae, that cover the entire body of the fly and collect bacteria. The tips of these hairs are even suspected to enhance bacterial adhesion because of their variance in tip structure. Often, the ends of setae that interact with surfaces are structured to increase the number of contact points of the fly. The labellum has also been identified as a major

contributing site to the acquisition and transmission of bacteria. This is partially because it is the part of the proboscis that interacts with surfaces for feeding, but the serrated inner structure of the labellum is responsible for the ability to grind against surfaces. Consequently, when flies use the hairs and serrated edges of the labellum, bacteria upon the surface are likely dislodged and congregated into the grooves of the labellum. Plus, electron microscopy images have been able to reveal bacterial adhesion to flies on various surfaces. These images, as well as recent advances in biotechnology and genetic sequencing have enabled further research into the transmission mechanisms of selected pathogens on flies between different surfaces, as well as the survivability of pathogens on fly surfaces.

Recent research has found that fly characteristics are beneficial for the survival of bacteria on the fly following initial exposure. Within the cycle of avian botulism outbreaks, the disease is known to take place in the bird carcass-maggot cycle. However, researchers were unsure whether adult flies were responsible for the outbreaks by transmitting *Clostridium botulinum* between carcasses, as well as how long the flies may be able to carry the pathogen. Anza *et al.* (2014) found that flies not only were transmitting the bacteria between carcasses, but also that the pathogen could be transmitted for up to 24 hours after feeding. A study by Wasala *et al.* (2013), introduced blow flies to *Escherichia coli* O157:H7 and then allowed to rest on spinach leaves. The flies were not only found to transmit this pathogenic strain of *E. coli* to the spinach leaves, the researchers also found traces of *E. coli* 0157:H7 on the flies up to 13 days following the initial inoculation. A similar study by Pace *et. al* (2017) also investigated the transmission of *E. coli* O157:H7, as well as *Salmonella enterica* to lettuce leaves. Comparisons between the acquisition and transmission of both pathogens by house flies and blow flies found that the acquisition and transmission amounts were variable between both the bacteria and the

flies. Jacques *et al.* (2017) attempted to attribute the variation of pathogens on flies to fly cleaning behavior. In this study, it was found that fly cleaning behaviors reduced the bacterial contamination on their bodies. However, they also found variations between different fly and bacterial species on the amount of bacteria that was removed following contamination. Another factor was the time which flies were given to clean themselves before they were retested for contamination quantities. Survival of bacteria on the surfaces of flies means that the transmissibility of pathogens is also possible for extended periods of time after the fly is exposed. These studies also identified that flies are capable of contaminating surfaces with pathogens and that further research is necessary for understanding of how contamination by the fly is accomplished.

To investigate the process by which flies transmit microorganisms, we chose to compare the microbiomes of flies exposed to decomposing rats with the microbiomes of flies left unexposed to this rich source of microorganisms. We also tested the microbiome of the rats before and after exposure to the flies to observe a potential change in bacterial flora on the rat. Knowledge of topics such as insect-microbe interactions contributes greatly to both microbial forensics, a steadily growing form of forensic analysis for criminal cases, and homeland security due to the health and environmental effects that could result if vectors are not understood. The goal for this project is to identify a pattern between flies and disease transmission through understanding of vector properties and microbial colonization succession. To do so, the first component of the study examines the microbial acquisition of the flies, whereas the second component analyzes the microbial acquisition of the carcasses. Illumnia 16s DNA sequencing allowed us to compare the bacterial communities of rat tissues with the communities on the flies, identify the changes in those bacterial communities over time, and identify antibiotic-resistant

bacteria genera on both the fly and rat samples that have been identified by WHO as needing further research. The results of this project will improve the understanding of the role of flies in transmission of microbes that potentially affect the health of humans and livestock and further our knowledge in decomposition ecology for improving forensic applications.

## Methods

### Sample Collection

Three whole frozen rats were acquired from ©Rodentpro.com and each placed in a tubberware container with a lid. These containers were then stacked in a five-gallon bucket and lidded. The bucket was placed outside in a partially sunny area in Stillwater, OK on October 27$^{th}$ and allowed to thaw and decompose for one week. Four metal insect cages had been cleaned and treated with 10% bleach. On November 2$^{nd}$, the bucket was retrieved and taken inside. Three cages were used to hold each of the flies exposed to rats, and another cage was prepared for the control flies. Each cage was supplied with containers of water and sugar that were placed in the same corners as the other cages. Flies from a two-week-old *Sarcophaga bullata* fly colony were vacuumed and separated into a maximum of 10-12 flies per group inside 50 mL sterile collection tubes and allowed to recover before placement into the cages. We collected a total of 95 flies, 20 flies for each of the rats, and another 35 flies for the controls.

Before placing the flies into the cages, tissue samples were collected from each of the rats. The storage containers were removed from the 5-gallon bucket and placed into a sterilized Laminar flow hood. With a sterile razor, we cut off tissue pieces from the same area of the lower abdomen, ensuring to penetrate the body cavity without harming the organs. Each of these samples was placed into sterile 5 mL microcentrifuge tubes and stored in a -80 ˚C freezer. Lids

were then replaced onto the containers. Treated tinfoil boats large enough for a rat were put into the center of three cages. Rats were deposited onto the tinfoil boats, making sure that no bodily fluids leaked onto the floor of the cages. Flies were then released into the same empty corner of each of the cages, with 20 for each of the rat cages and 35 control flies in the fourth cage.

Our testing procedure lasted for a total of 8 Days. The rats remained inside the cages with the flies for 72 hours, and on Day 3 the rats were removed, placed into lidded containers with foil lining, and put into the flow hood. Five flies were collected from each of the rat cages, and pooled together as one sample into sterile 15 mL collection tubes, and stored in a -80 ˚C freezer. Three groups of 5 flies were then collected from the control cage into sterile 15 mL collection tubes and stored at -80 ˚C. Following the same procedure as before, rat tissue samples were collected again from the same abdominal area in similar quantities and stored at -80 ˚C, and then the rats were properly disposed of. On Day 6, five flies were collected from each of the rat cages and stored at -80 ˚C. No flies were collected from the control cage. For Day 8, the flies were collected again from all of the cages in pools of 5 and stored at -80 ˚C.

**Sample Preparation**

All 21 samples were removed from the -80 ˚C freezer and put on ice. The 5 flies and rat tissues in each sample were then weighed and allowed to thaw. We calculated a 1:100 mass to volume ratio with 0.1% peptone water solution and placed both the buffer and samples into stomacher bags. The samples were ground for 15 seconds with a pestle and then put in an orbital shaker for 30 minutes at room temperature and 150 rpm. Stomacher bags were taken to the ventilation hood and the bacterial/tissue solution filtered from the larger pieces of sample tissues with the stomacher bag screen. 200 µL were initially extracted from each sample, placed in 5 mL

microcentrifuge tubes, and stored at -80 ˚C. The bacterial/tissue buffer solution was extracted under sterile conditions into two 15 mL vials and placed into the -80 ˚C freezer.

Later, after it was found that the vials had over expanded, both of the 15 mL vials for each sample were inverted into 50 mL tubes and allowed to thaw completely for about 2 hours. One of the two 50 mL tubes for each sample were then used to be shipped for 16s RNA sequencing, and the other was stored in the -80 ˚C freezer. The unstored 50 mL vials of solution were centrifuged for 5 minutes at 3,000 rpm to form a loose pellet. All of the supernatant fluid was then extracted until about 0.5 mL of solution remained. The remaining supernatant and pellet were vortexed to resuspend the pellet, and 500 µL of the sample was extracted using a micropipette with widened cut tips. The samples were stored in the -80 ˚C freezer until shipment for 16s RNA sequencing.

**Spread Plate Dilutions**

The 200 µL samples were removed from the freezer and placed in ice to thaw. Samples were vortexed and then we performed 5 dilutions of the samples (1:1; 1:10 ; 1:100 ; 1:1,000 ; 1:10,000) with deionized water using the spread plate technique on nutrient agar plates. 100 µL of the solution was spread on a plate (1:1 dilution), while the other 100 µL of solution was added to 0.9 mL of deionized water. The mixture was vortexed, then 100 µL was plated from the first mixture (1:10 dilution). Another 100µL of the mixture was added to 0.9 mL of deionized water, vortexed and 100 µL plated (1:100 dilution). This procedure was repeated two more times (1:1,000 and 1:10,000 dilutions). Plates were then incubated at 37 ˚C for 20 hours. Afterwards we removed the plates and recorded colony observations, colony counts and calculated the colony forming units (CFUs) per mL of sample and the log of the average CFU/mL per testing

group at each time. For colony counts, colonies were considered too numerous to count (TNTC) if the colony count exceeded 300, and too few to count (TFTC) if the colony count was below 30.

$$\text{CFU/mL} = \frac{\text{no. of colonies x dilution factor}}{\text{volume of culture plate}}$$

**16s DNA Sequencing & Analysis**

The 500 µL of each of the 21 sample solutions stored in the freezer were removed and shipped to Omega Bioservices per shipment protocols. Omega Bioservices performed the DNA extraction and conducted Illumina next generation 16s metagenomic sequencing. Sequence platform was MiSeq with a coverage per sample of 100,000 reads and a sequence read format of paired-end 300. The primer set used by Omega Bioservices amplified the V3 and V4 region of the gene. Sequencing data was transferred via Illumina BaseSpace web portal, where it was then downloaded for quality scoring and analysis.

After downloading the sequences we used FastQC (Andrews, 2010)-a modular set of analyses-to do quick quality control testing of our raw sequence data prior to further analysis. FastQC assessed the GC content, adaptor over-abundance, and over-represented sequnces, allowing for the estimation of PCR duplication. Due to a tile registration error on the reverse primer sequences, Omega Bioservices was willing to re-sequence our libraries. The re-sequenced data was quality scored again, and then concatenated with the former data sets and quality tested. We used Trimmomatic (Bolger *et al.* 2014) to trim the paired-end sequence reads, and then quality scored the trimmed reads with FastQC. Sequences were uploaded to Galaxy (Afgan *et al*. 2016; Blankenburg *et al.* 2014), an open source, web-based platform for data intensive biomedical research. On Galaxy, we used Kraken (Wood and Salzberg 2014) to assign

taxonomic labels from the Kraken bacterial database with the default job resource parameters. The results of Kraken were then run through Krona (Ondov *et al.* 2011) to create an interactive pie chart from the taxonomic profile hierarchy that demonstrated the proportions of bacteria identified by the Kraken database. We set the root basal rank at either the rat or fly tissue and then set the taxonomy ranks from the root to the genera of bacteria identified on the samples. These proportions were compared amongst the flies for different time points, as well as between the flies and the rats. The largest five percentages of bacterial genera identified per sample were compared amongst the different samples for determination of changes in the bacterial communities. Bacterial genera were included into the comparison only if they were at least 1% of the sample and were one of the top five bacterial genera. Bacterial genera that did not meet these limitations are notated as "other" bacterial genera. The WHO antibiotic-resistant bacteria were then blasted against Kracken database to determine the presence of these bacteria on the samples.

## Results

#### Dilution Spread Plates

For all of the samples, colonies were too numerous to count for the 1:1 and 1:10 dilutions (Table 1). The rat samples were also too numerous to count for the 1:100 dilutions. Based upon colony morphology, bacterial colonies appeared to consist of at least two different types of bacteria, though we did not attempt to identify them. No growth was observed for the majority of fly samples on Day 3, with most colony counts recorded for the 1:100 dilution. As reflected by Table 2, larger average log expressions of the CFU/mL were found in the rat tissues, followed by the control flies and then the flies exposed to rats. The rat tissues and control flies both had a

negative log trend (Figure 1), with a slightly steeper trend line in the rat tissues. The flies exposed to rats had an upward arcing log relationship, ending with a lower average log (CFU/mL) than the first testing period.

| *Samples* | *1:1* | *1:10* | *1:100* | *1:1,000* | *1:10,000* | *CFU/mL* | *Log(CFU/mL)* |
|---|---|---|---|---|---|---|---|
| *R1 D0* | TNTC | TNTC | TNTC | TNTC | 182 | $1.82 \times 10^{10}$ | 10.260071 |
| *R2 D0* | TNTC | TNTC | TNTC | TNTC | 112 | $1.12 \times 10^{10}$ | 10.049218 |
| *R3 D0* | TNTC | TNTC | TNTC | TNTC | 30 | $3.00 \times 10^{9}$ | 9.477121 |
| *R1 D3* | TNTC | TNTC | TNTC | 242 | TFTC | $2.42 \times 10^{9}$ | 9.383815 |
| *R2 D3* | TNTC | TNTC | TNTC | 288 | TFTC | $2.88 \times 10^{9}$ | 9.459392 |
| *R3 D3* | TNTC | TNTC | TNTC | TNTC | 74 | $7.40 \times 10^{9}$ | 9.869232 |
| *R1F D3* | TNTC | TNTC | 115 | TFTC | NG | $1.15 \times 10^{8}$ | 8.060698 |
| *R2F D3* | TNTC | TNTC | 47 | TFTC | NG | $4.70 \times 10^{7}$ | 7.672098 |
| *R3F D3* | TNTC | TNTC | 66 | TFTC | NG | $6.60 \times 10^{7}$ | 7.819544 |
| *C1 D3* | TNTC | TNTC | 126 | TFTC | NG | $1.26 \times 10^{8}$ | 8.100371 |
| *C2 D3* | TNTC | TNTC | 273 | TFTC | NG | $2.73 \times 10^{8}$ | 8.436163 |
| *C3 D3* | TNTC | TNTC | TNTC | 48 | TFTC | $4.80 \times 10^{8}$ | 8.681241 |
| *R1F D6* | TNTC | TNTC | 114 | TFTC | TFTC | $1.14 \times 10^{8}$ | 8.056905 |
| *R2F D6* | TNTC | TNTC | 125 | TFTC | TFTC | $1.25 \times 10^{8}$ | 8.096910 |
| *R3F D6* | TNTC | TNTC | 276 | TFTC | TFTC | $2.76 \times 10^{8}$ | 8.440909 |
| *R1F D8* | TNTC | TNTC | 51 | TFTC | TFTC | $5.10 \times 10^{7}$ | 7.707570 |
| *R2F D8* | TNTC | TNTC | 46 | TFTC | TFTC | $4.60 \times 10^{7}$ | 7.662758 |
| *R3F D8* | TNTC | TNTC | 26 | TFTC | NG | $2.60 \times 10^{7}$ | 7.414973 |
| *C1 D8* | TNTC | TNTC | TNTC | 41 | TFTC | $4.10 \times 10^{8}$ | 8.612784 |
| *C2 D8* | TNTC | TNTC | 26 | TFTC | TFTC | $2.60 \times 10^{7}$ | 7.414973 |
| *C3 D8* | TNTC | TNTC | 106 | TFTC | NG | $1.06 \times 10^{8}$ | 8.025306 |

TNTC = n > 300 TFTC = n < 30 NG = no growth

**Table 1:** Colony counts and calculated log (CFU/mL) for each of the samples.

| Testing Group | Day 0 | Day 3 | Day 6 | Day 8 |
|---|---|---|---|---|
| Control Flies | --- | 8.405925 | --- | 8.017688 |
| Rat Flies | --- | 7.960780 | 8.198241 | 7.595100 |
| Rats | 9.928803 | 9.570813 | --- | --- |

**Table 2:** Average log (CFU/mL) values for each of the testing groups collected for Day 0, Day 3, Day 6, and Day 8.

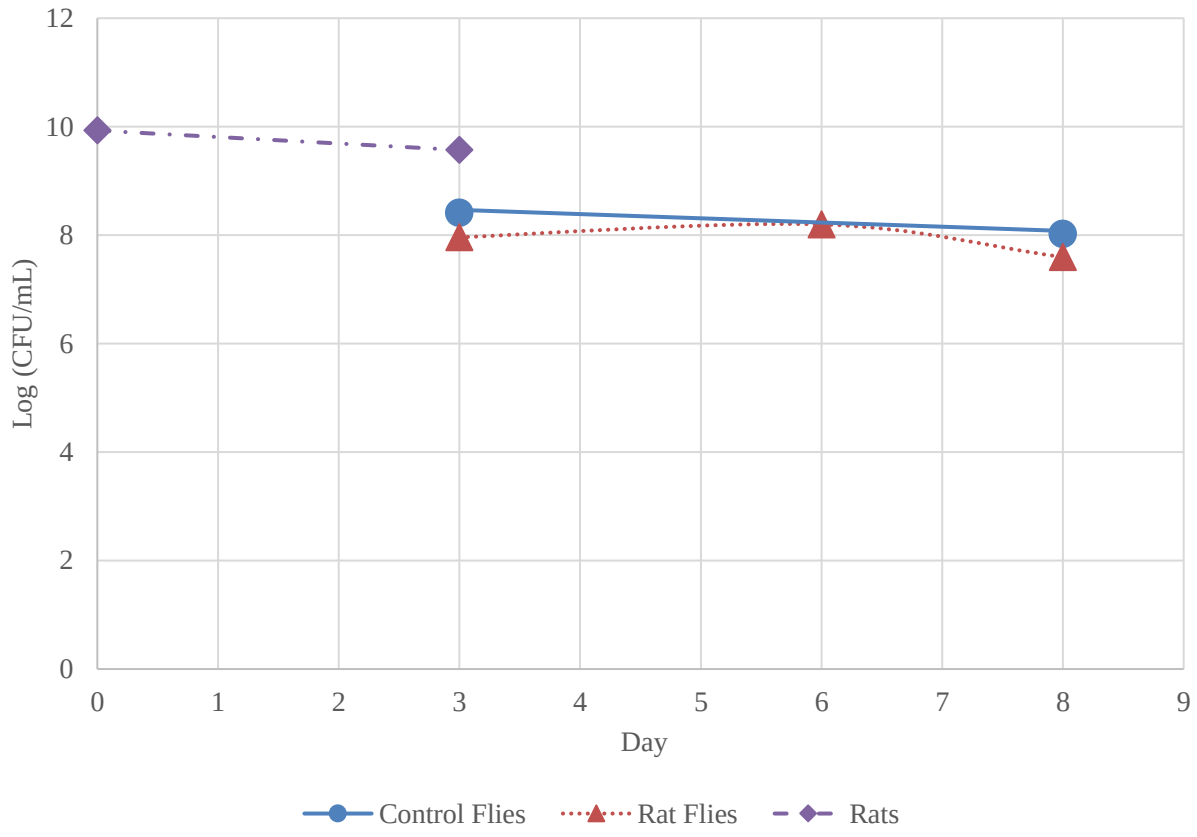


**Figure 1:** Log of average CFU/mL for flies exposed to rats, control flies, and rat tissues for Day 0, Day 3, Day 6, and Day 8.

**16s Metagenomic Analysis**

From our metagenomic analysis, we identified more than 60 different bacterial genera on each of the samples. Bacterial diversity fluctuated for each testing group between collection dates. Krona pie charts gave an overall indication of a higher diversity of bacterial genera for the flies versus the rat tissues. The largest 5 bacterial genera identified on flies exposed to rats were most often *Staphylococcus, Lactobacillus*, *Enterococcus, Enterobacter,* and *Pseudomonas*. For the control flies, the largest 5 bacterial genera were most often *Staphyloccus, Lactobacillus, Enterobacter, Pseudomonas,* and *Proteus* (Figure 2). The rat tissues did not always have 5 bacterial genera above 1%, with only a few larger genera and a variety of small percentages of other genera (Figure 3). The bacterial genera included into the top percentages for the rat tissues were primarily *Staphylococcus, Corynebacterium, Psychrobacter, Lactobacillus,* and *Geobacillus*. Despite the narrower variety of the rat tissue samples, the percentage of "other" bacterial genera in the sample was only a maximum of 38%. For the control flies, a maximum of 49% of the bacterial genera were "other" genera, while the flies exposed to rats had a maximum of 66% "other" bacterial genera. Rats 1 and 2 for Day 3 and Rat 3 for both Day 0 and Day 3 were the only samples not to have the full 5 bacterial genera listed due to the remainder of the identified genera being below 1% of the sample.

Each of the fly samples had differences between their 5 largest bacterial genera for the time points, and also contrasted with each other. Figure 2 shows the large variation of bacterial population between each sample for the flies. On average, the flies exposed to rats had higher percentages of "other" bacteria for each of the time points than the control flies. Larger

variations of the top 5 bacterial genera were seen between each of the samples for Day 3 rather than on Day 6 or Day 8, but Day 8 had higher variety of bacterial genera overall from the bacterial genera of the "other" category. The collected flies from the rats on Day 3 were the most dissimilar in their top 5 bacterial percentages than any of the other test dates, increasing in similarity over time. On Day 8, all of the flies exposed to rats were found to have *Acinetobacter* as one of their five larger genera percentages. All of the fly samples contained *Lactobacillus* as one of the largest five bacterial genera in the samples, and fluctuated in varying percentages of the sample over each of the time points. Most fly samples also contained *Staphylococcus* as one of the five larger genera, which generally decreased in each of the samples over time. *Pseudomonas* also existed as one of the five larger bacteria genera percentages in most of the samples, though constant relationship was observed for the samples.

Between Day 0 and Day 3, the percentage of "other" bacterial genera decreased for each of the rat tissue samples (Figure 3). The variety of bacterial genera in these rat tissues also decreased between Day 0 and Day 3. For rats 1 and 2, which started with *Geobacillus* on Day 0, no longer had a quantity of *Geobacillus* above 1% of the sample by Day 3. Rat 2 also did not show a significant percentage of Enterococcus on Day 3 as it had on Day 0. Rat 1 was the only rat found to have *Lactobacillus*, although its percentage decreased between Day 0 and Day 3. Percentages of *Corynebacterium* increased for all rat tissues on Day 3 versus Day 0. *Psychrobacter* percentages increased for Rat 1 and Rat 2 between Day 0 and Day 3 as well, with no signs of *Psychrobacter* for Rat 3. *Staphylococcus* did not show a determinable growth relationship for the rat tissues between Day 0 and Day 3. Rat 2 was the only rat tissue to have at least 1% of *Enterococcus*, but this percentage was not shown for Day 3.

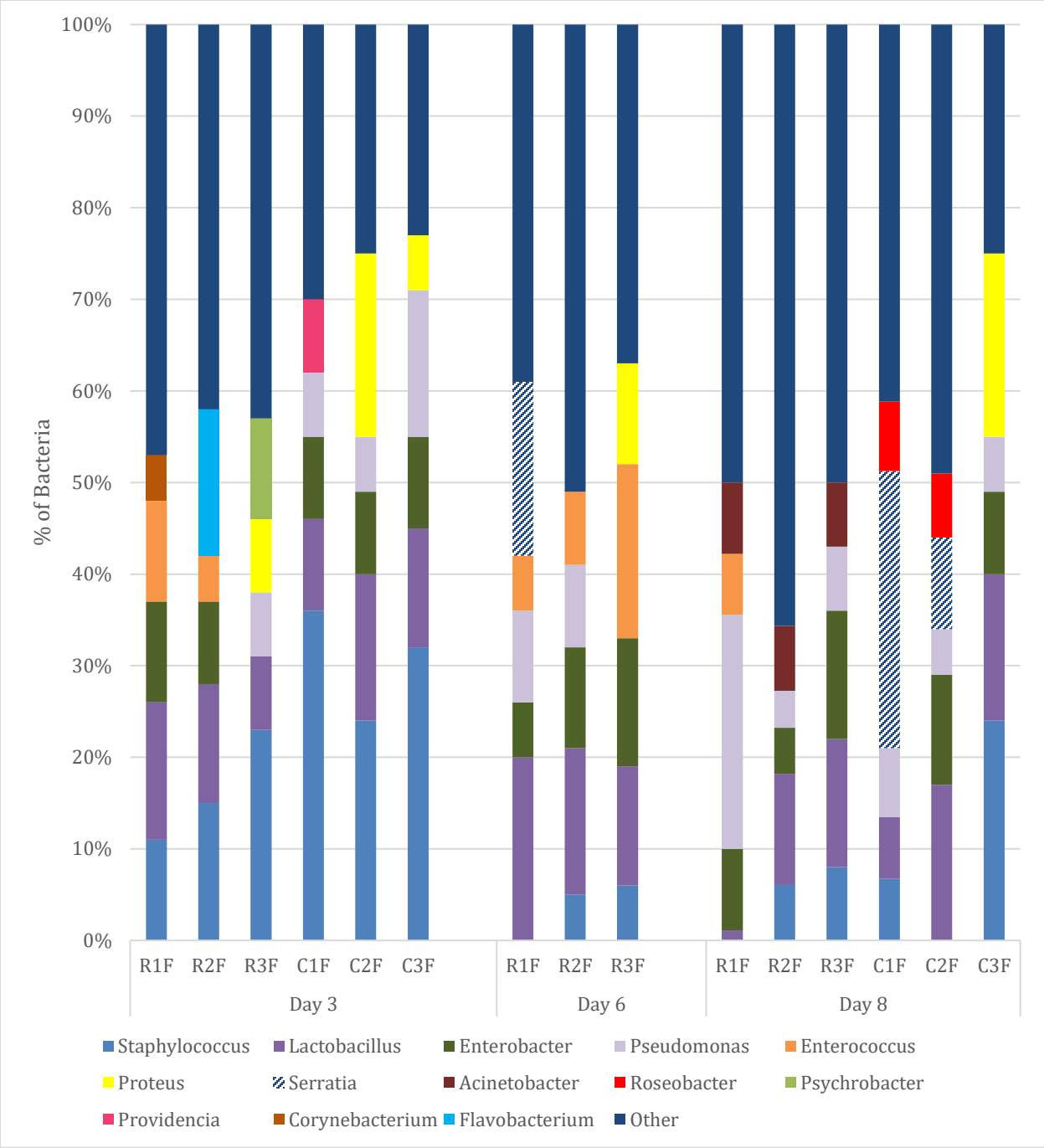


**Figure 2:** Percentage of bacteria identified as the five largest bacterial genera from fly samples on Day 3, Day 6, and Day 8.

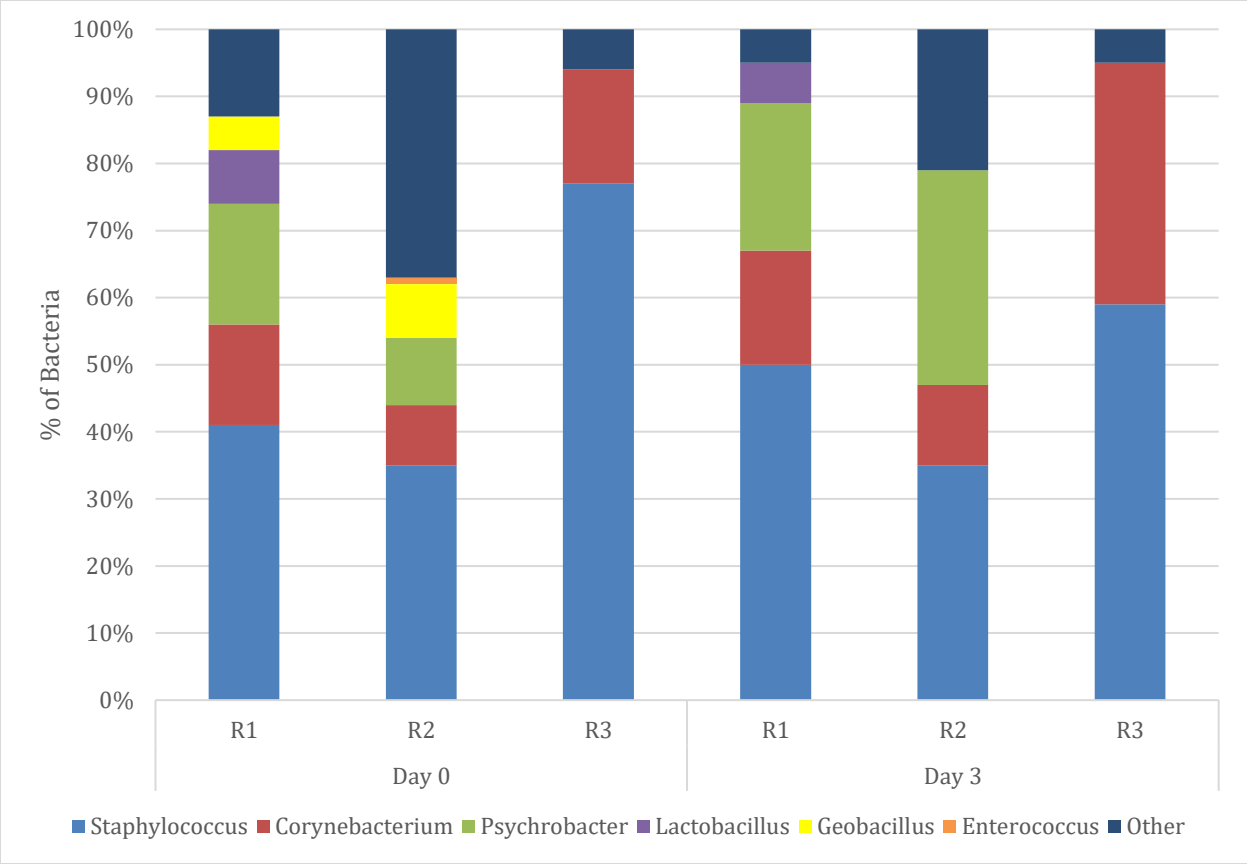


**Figure 3:** Percentage of bacteria identified as the five largest bacterial genera for rat tissue samples for Day 0 and Day 3.

Of the 12 bacteria listed on the WHO antibiotic-resistant priority list, the Kraken database identified 11 bacteria from our samples (Determine how many for flies vs rats). For the fly samples, the largest percentages of antibiotic-resistant bacteria were Enterobacteriaceae, *Staphylococcus aureus*, and *Acinetobacter baumannii* (Figure 4). Most other antibiotic-resistant bacteria identified in the fly samples were all less than 1% of the bacteria Kraken identified. Total percentages of antibiotic-resistant bacteria identified by Kraken from the fly samples ranged from 26% - 48% of the sample bacteria. The percentage of antibiotic-resistant bacteria for the flies exposed to rats increased between Day 3 and Day 6, and decreased between Day 6 and

Day 8. Control fly percentages showed varying trends for the antibiotic-resistant bacteria identified between Day 3 and Day 8, with C1 slightly increasing, C2 decreasing, and C3 increasing. Overall, the control flies had higher percentages of Enterobacteriaceae for each time point, but had lower percentages of *Acinetobacter baumannii* and *Staphylococcus aureus*. The flies exposed to rats increased in percentage of Enterobacteraceae between Day 3 and Day 6, but then decreased between Day 6 and Day 8. For all fly samples, *Staphylococcus aureus* decreased over time. *Acinetobacter baumannii* was variable in growth for the control flies and increased over time for the flies exposed to rats.

Rat tissue samples consisted of lower varieties of the WHO antibiotic-resistant bacteria (Figure 5). The major percentages of antibiotic-resistant bacteria for the rat tissues were mostly *Staphylococcus aureus* and Enterobacteriaceae, with the other bacteria comprising less than 1% of the samples. The total percentage of antibiotic-resistant bacteria for the rat tissues on both days ranged from 19% - 42%. *Staphylococcus aureus* was the largest percentage of the antibiotic-resistant bacteria in the tissue samples, and found in all three rat tissues. Enterobacteriaceae was found on all of the rats for both Day 0 and Day 3. Enterobacteriaceae increased between Day 0 and Day 3, whereas *Staphylococcus aureus* varied in growth on samples between each day.

| | **Rat 1 Flies** | | | **Rat 2 Flies** | | | **Rat 3 Flies** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 3 | Day 6 | Day 8 | Day 3 | Day 6 | Day 8 | Day 3 | Day 6 | Day 8 |
| *A. baumanni* | 0.5% | 5% | 7% | 0.8% | 5% | 6% | 2% | 2% | 7% |
| *P. aeruginosa* | 0.003% | 0.03% | 0.05% | 0.009% | 0.005% | 0.004% | 0.006% | 0.003% | 0.01% |
| *Enterobacteriaceae* | 24% | 40% | 22% | 27% | 34% | 17% | 20% | 44% | 33% |
| *E. faecium* | 0.005% | 0.002% | 0.001% | --- | 0% | --- | 0% | 0.002% | 0.001% |
| *S. aureus* | 5% | 2% | 1% | 5% | 2% | 2% | 11% | 2% | 3% |
| *H. pylori* | 0.001% | --- | 0% | --- | 0% | --- | --- | --- | 0% |
| *Campylobacter* | 0.02% | 0.001% | 0.001% | 0.001% | --- | 0.03% | 0.005% | 0.001% | --- |
| *Salmonella spp.* | 0.1% | 0.1% | 0.2% | 0.1% | 0.09% | 0.06% | 0.02% | 0.1% | 0.2% |
| *N. gonorrhoeae* | 0.005% | --- | --- | --- | 0% | 0.03% | 0% | --- | 0% |
| ***S.*** *pneumoniae* | 0.02% | 0.002% | 0.001% | 0.003% | 0.005% | 0.01% | 0.001% | 0.005% | 0.004% |
| *H. influenza* | 0.003% | 0.001% | 0.002% | 0.004% | 0.001% | 0.001% | 0.003% | 0.001% | 0.004% |
| *Shigella spp.* | 0.08% | 0.07% | 0.10% | 0.1% | 0.08% | 0.03% | 0.02% | 0.1% | 0.1% |

*0% = <0.001% but still detected

**Table 3**: Percentages of bacteria for targeted WHO antibiotic-resistant bacteria on pools of flies exposed to rats for all time points.

| | **Control 1 Flies** | | **Control 2 Flies** | | **Control 3 Flies** | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 3 | Day 0 | Day 3 | Day 0 | Day 3 |
| *A. baumanni* | 2% | 1% | 0.9% | 2% | 0.9% | 0.9% |
| *P. aeruginosa* | 0.004 % | 0.02% | 0.003% | 0.01% | 0.003% | 0.003% |
| *Enterobacteriaceae* | 33% | 40% | 43% | 36% | 31% | 43% |
| *E. faecium* | --- | 0.001% | 0.001% | --- | 0.001% | 0.001% |
| *S. aureus* | 4% | 0.7% | 3% | 0.3% | 3% | 3% |
| *H. pylori* | --- | 0% | --- | 0.002% | --- | --- |
| *Campylobacter* | --- | 0.002% | --- | 0.008% | --- | --- |
| *Salmonella spp.* | 0.08% | 0.1% | 0.05% | 0.1% | 0.07% | 0.05% |
| *N. gonorrhoeae* | --- | --- | --- | 0.01% | --- | --- |
| ***S.** pneumoniae* | 0% | 0.001% | 0.001% | 0.003% | 0.001% | 0.001% |
| *H. influenza* | 0.001% | 0.001% | 0.003% | 0.008% | 0.002% | 0.003% |
| *Shigella spp.* | 0.05% | 0.1% | 0.06% | 0.1% | 0.04% | 0.06% |

*0% = <0.001% but still detected

**Table 4:** Percentages of bacteria for targeted WHO antibiotic-resistant bacteria on pools of control flies for all time points.

| | **Rat 1** | | **Rat 2** | | **Rat 3** | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 3 | Day 0 | Day 3 | Day 0 | Day 3 |
| *A. baumanni* | 0.02% | 0.06% | 0.02% | 0.07% | 0.07% | 0.02% |
| *P. aeruginosa* | 0.001% | 0.001% | --- | --- | 0.001% | --- |
| *Enterobacteriaceae* | 0.2% | 0.2% | 8% | 16% | 0.3% | 0.4% |
| *E. faecium* | 0.001% | --- | --- | --- | --- | --- |
| *S. aureus* | 18% | 33% | 10% | 12% | 41% | 26% |
| *H. pylori* | --- | --- | 0% | --- | 0% | 0% |
| *Campylobacter* | 0.002% | 0% | 0.001% | 0.001% | 0.002% | 0.001% |
| *Salmonella spp.* | 0.006% | 0.003% | 0.04% | 0.09% | 0.006% | 0.003% |
| *N. gonorrhoeae* | 0.001% | 0.003% | 0% | 0.001% | 0% | --- |
| ***S.** pneumoniae* | 0.003% | --- | 0% | 0.001% | --- | --- |
| *H. influenza* | 0.001% | 0.001% | 0% | 0.002% | --- | 0% |
| *Shigella spp.* | 0.006% | 0.002% | 0.04% | 0.1% | 0.005% | 0.004% |

*0% = <0.0005% but still detected

**Table 5:** Percentages of bacteria for targeted WHO antibiotic-resistant bacteria on rat tissues for all time points.

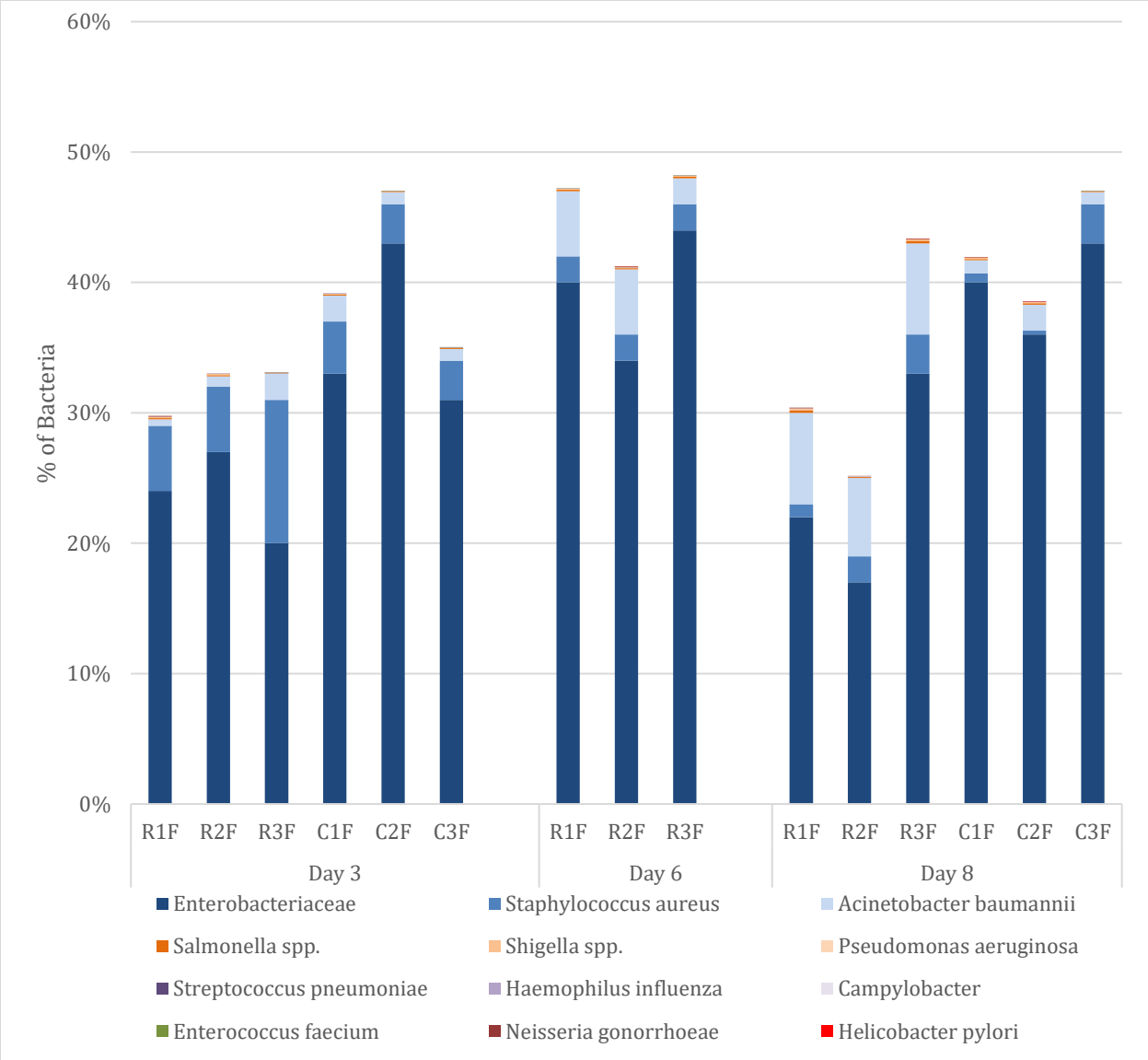


**Figure 4:** Percentage of bacteria identified as WHO antibiotic-resistant priority listed bacteria in fly samples from rat-exposed flies and control flies for Day 3, Day 6, and Day8.

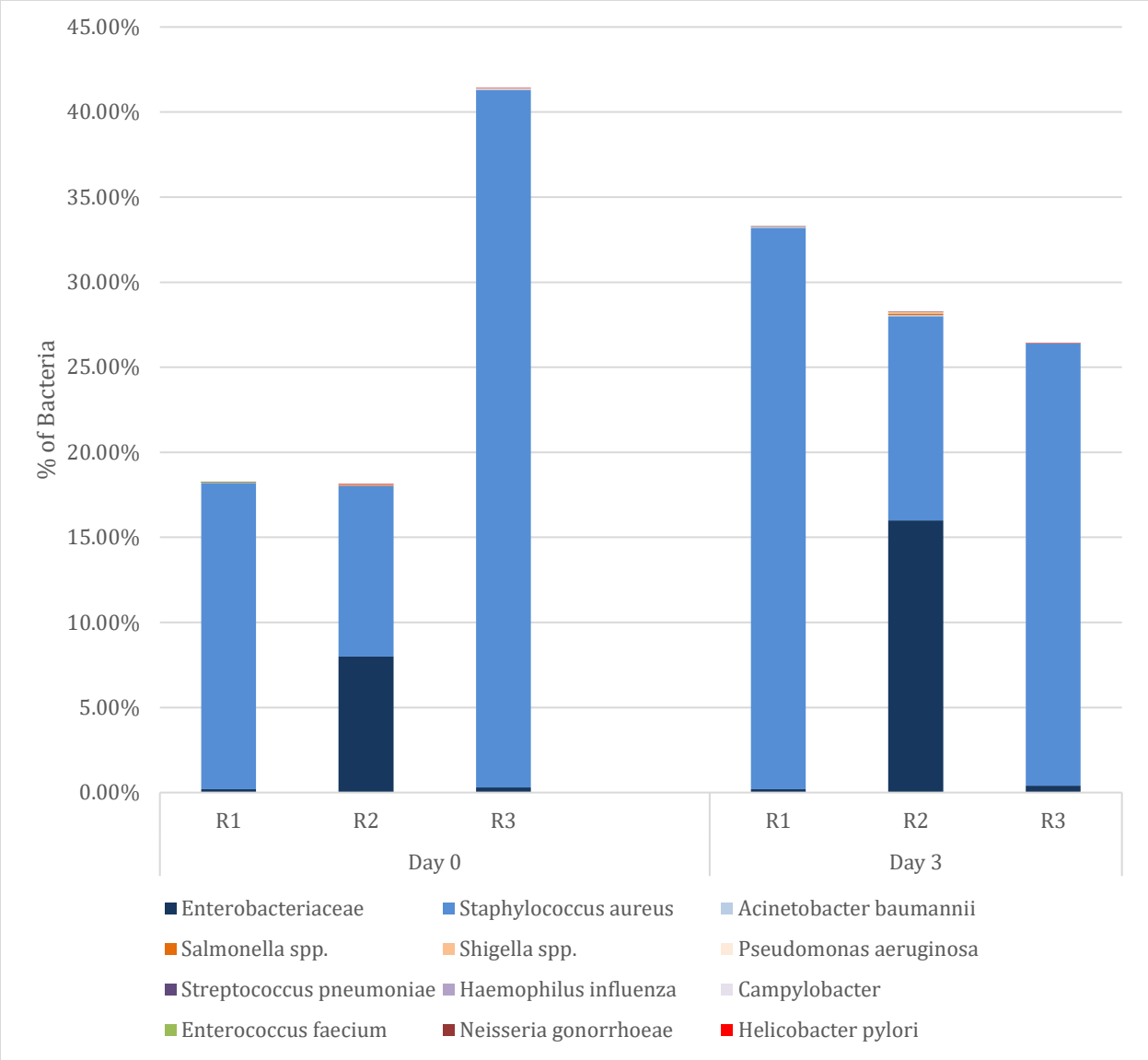


**Figure 5:** Percentage of bacteria identified as WHO antibiotic-resistant priority listed bacteria found in tissue samples collected from Rats 1, 2, and 3 for Day 0 and Day 3.

## Discussion and Conclusions

Our results indicate that the microbiomes of decaying organic matter and flies is altered by the interaction of flies feeding on the materials. The microbiomes of flies are also altered over time after a bacterial resource has been removed. We positively identified bacteria on both the

fly and rat samples that are both antibiotic-resistant and a research priority of the 2017 World Health Organization. Cultured bacteria from rat tissues expressed a decreasing bacterial density when collected at later dates. Fly samples also possessed a decreased bacterial density from the last day of collection versus the first day of collection.

From our plated dilutions, we saw a general decrease in bacterial densities over time, with a slight peak in colony densities for the flies exposed to rats. However, we only collected two rat tissue samples and two pools of control flies, whereas we collected three pools of flies exposed to the rats. Therefore, it is possible that the rat tissues and control flies could have had peaks in bacterial densities that we did not record. We believe that it is unlikely that the rat tissues would have peaked, though, because we collected the first and second tissue samples in a 3 day period, similar to that of the first two collected pools of flies exposed to the rats. If there were to be a peak in bacterial density for the rat tissues, we would have instead seen an increase in bacterial density between our collections on Day 0 and Day 3, instead of a decrease. We suspect the larger decrease in bacterial density over time for the rat tissue may have been because the body was beginning to dry out, therefore it may not have been as suitable of an environment for some bacterial growth. It is important to understand, though, that the plate dilutions are not an accurate reflection of the quantities of bacteria on the rats because it was an isolated section of the rat, plus it only allowed the growth of bacteria culturable at 37 ºC.

Our 16s DNA metagenomic analysis revealed a lower variety of bacterial genera between the rat tissues compared to the flies. We were also able to identify antibiotic-resistant from all of our samples that are considered to be a priority for research by the World Health Organization in 2017. Of the 12 priority listed bacteria, 11 were identified on our samples. The top WHO priority

listed bacteria found in our samples were *Acinetiobacter baumannii*, *Staphylococcus aureus*, and Enterobacteriaceae. While the relationships of the prioritized bacteria were sometimes indeterminable, we observed significant differences between bacterial percentages on the flies versus the rat tissues. There were also differences of prioritized bacteria percentages between the flies exposed to the rats and the control flies.

One advantage of this study is that the methods by which it was performed was highly representative of a natural environment compared to most fly microbial studies. Most often the flies are disabled from flight and/or restricted to specific areas. This likely distorts data from the natural means by which flies interact with bacteria. Instead, our study allowed the flies to move about in a fairly large environment without disabling their flight. Another reason why this experiment was more representative of natural fly interactions is because of the substance we exposed it to. In studies like Wasala *et al.* (2013), the flies are exposed directly to a bacteria in a concentration which exceeds that which the fly would likely come across in a natural environment. Studies like these show that flies are capable of transmitting pathogens in such manners, but it also brings to question the likelihood of the situation occurring in nature and the how significant the effect would be since the pathogen would likely be in lower quantities. While our study was more representative of the natural field, we did not perform any methods to ensure that all the flies interacted with the rats for a specific amount of time. In fact, it is possible that some of the flies did not interact with the rats at all, but instead acquired bacteria from the other flies either directly or from the cage surfaces, sugar, or water. But the differences in the microbiomes of the flies exposed to the rats versus the control flies suggest that even indirect exposure to the rat influenced the microbiomes of flies and that the flies were transmitting bacteria between each other.

We believe that the lack of microbial variety in the rat tissue versus those of the flies may be because of a few reasons. When the rats were brought inside from sitting outside for a week, we noticed that the body cavity was not ruptured as is characteristic of bodies that pass the bloat stage of decomposition. The bloat stage of decomposition is a result of the building of gasses within the body due to active decay of the body. As the body begins decomposition and starts to bloat, the chemistry of the body is changing. Typically, rats will pass the bloat stage of decomposition within one week, but this is also dependent on the temperature at which it is kept. Because we set out our rats at the end of October, it is likely that our rats did not reach the bloat stage of decomposition from the lower temperatures. Therefore, when we collected our tissue samples it was the first exposure of the internal cavity to the air. Plus, the internal organs may not have been decayed enough to begin leaking into the surrounding body fluid, which is especially important when considering the amount of bacteria within the digestive tract. Similarly, the area from which we collected the rat tissues was an isolated area of the rat, compared to using the entirety of the fly. Our fly samples contained the entire microbiome of the fly, whereas the rat samples were the bacteria found on a small area of the rat. To improve the sampling method, rat tissues could be taken from various areas of the body, much like human microbiome research in microbial forensic studies.

The fluctuations observed between samples on different dates indicates relationships between the bacteria-rich resources and the flies, especially when observing targeted bacteria. For example, the WHO priority listed bacteria were occasionally found to have specific relationships between the samples which could be further investigated in a more targeted study. *Staphylococcus aureus,* in particular was in much higher percentages on the rats than the flies, and the flies exposed to the rats had higher levels of *Staphylococcus aureus* than the control flies.

Once the rats were removed from the cages, the level of *Staphylococcus aureus* on the flies exposed to the rats decreased over time back to a level similar to the control flies by Day 8. While this may have been because of the introduced *Staphylococcus aureus* competition with the natural microbiome of the fly, it is also possible that the flies had a means of suppressing the growth of the excess *Staphyloccocus aureus* acquired from the rats.

It is difficult to determine the means by which the microbiomes of the flies and rat tissues were changing due to the design of our experiment. Our allowance of the flies to move about freely for extended periods of time without monitoring means that we are not sure with what the flies interacted. Because microbiomes of deceased animal hosts have been known to change over time during decomposition, we cannot be sure how much the flies contributed to the changing microbiomes of the rats (Pechal *et al.* 2013). As is evident by our control flies, the microbiomes of flies also change over time without the introduction of new bacteria-rich components. Therefore, the bacterial compositions of the samples could have been fluctuating because of the individual flies, the rats, exchange between the flies within the cage, acquisition of the bacteria from the bacteria-rich rats, or even competition between the bacteria. Future work could look further into the different relationships of targeted bacteria after initial exposure in more natural quantities over time. Another aspect is to characterize the means by which flies are capable of promoting or suppressing the growth of targeted bacteria within their microbiome. Due to the fluctuations of the bacteria over time, even for the control flies, it would be worthwhile to further investigate the microbiomes of flies with more frequent sampling dates.

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