Background

Bacterial species in the gut can exert an effect on different host fitness traits such as development, death, and fecundity. The magnitude of this effect has not yet been resolved, particularly the degree to which individual bacterial species versus microbiome community interactions shape host fitness. In the early 20th century, it was postulated that germ-free flies have a longer lifespan than those with a populated microbiome [1]. However, variation in life history is coupled with fitness trade-offs; therefore, an increase in lifespan (thus, an improvement in fitness) is accompanied by a decrease in another fitness trait [2]. For example, an increase in lifespan may result in a decrease in fecundity. But what wields a greater effect on host fitness traits – standalone bacterial species or bacterial interactions? And regarding interactions, is it lower or higher order interactions (in other words, the level of diversity of the microbiome) that result in a greater affect? It is important to determine these distinctions in order to further understand how bacterial communities shape the physiology and fitness traits of the host.

The five species of bacteria that were studied were *Lactobacillus plantarum (LP), Lactobacillus brevis (LB), Acetobacter pasteurianus (AP), Acetobacter tropicalis (AT), and Acetobacter orientalis (AO)*. Each is a core bacterial strain in the gut microbiome of the fruit fly *Drosophila melanogaster* and generated 2^5 possible bacterial combinations that a fruit fly could have in the study. Fruit flies normally have a relatively low bacterial diversity in their microbiome, thus rendering it to be a robust model.

One aspect of the study aimed to measure the differential abundances of the 32 possible combinations of the five species of bacteria. The intent of this was to determine the effect that the presence and absence of different bacterial species as well as how the relative abundances of the bacterial species that were present would have on the fruit fly's fitness phenotype. How does the relative abundance of each bacterial species change as diversity of the microbiome increase? How does the total abundance fluctuate when bacterial diversity increases or decreases? The flies for this part of the study were prepared by treating 5-7 day old germ-free flies with one of the 32 pre-determined bacterial combinations. These flies were then transferred to fresh food daily. There were 2 biological replicates of these flies with each biological replicate having 12 males and 12 females.

Another region of interest was if the fitness phenotype of fruit flies with more than one bacterial strain colonizing its microbiome could be predicted using single species and pairwise modeling predictions. For example, a single-species mixing model would predict the fitness phenotype measurement of a fly with *LP*, *AO*, and *AT* by averaging the individual species measurements for *LP, AO,* and *AT*. Essentially, a higher diversity microbiome is broken down into its individual species components. A pairwise-species mixing model would predict the fitness phenotype measurement of a fly with *LP*, AO, and *AT* by averaging the measurements of flies with existing pairs of species, *LP* and *AO*, *LP* and *AT*, and *AO* and AT. The researchers utilized a multivariate linear regression model to determine if there were any lower or higher order bacterial interactions between the five bacterial species. They found out that the microbial interactions had a similar level of effect as single species. For example, they discovered that the mean lifespan of a fly with a microbiome free of any bacterial species was 53 days. *AO* decreased mean lifespan by 10 days while a pair of microbial interactions can decrease mean lifespan by 8 days. Therefore, the goal of pursuing the single species and pairwise models was to further explore microbiome interactions and determine if an individual species or pair of bacterial interaction would better predict and account for variability in fitness diversity and host physiology.

All data used for this project was stored in csv files that was acquired from the Dryad Digital Repository. Mean colony-forming unit (CFUs) counts were provided for each of the 24 flies in both of the biological replicates for each bacterial combination. Experimental measurements for mean fecundity, mean development time, mean lifespan, and mean CFUs were also provided for each bacterial combination.

Data Analysis and Structure

For figure 2, I prepared the data by transforming the raw data for all of the four host fitness traits and its corresponding standard error (SE) into Numpy arrays. Binary code notation was used to indicate the bacterial combination and was converted into an array as well. For example, '10110' meant that *LP, AP*, and *AT* were present in the microbiome. The order of the binary notation always corresponded to *LP, LB, AP, AT,* and *AO* in that order. For figures 2A, 2C, 2E, and 2G, I plotted the experimental measurements and SE for each bacterial combination for each fitness trait.

For the single species prediction model, I averaged the corresponding diversity measurements of a single bacterial species. For example, to predict the phenotype of flies with a microbiome with the combination *LP, AT*, and AO, individual measurements of species *LP, AT*, and *AO* were averaged. For any binary notations that had more than one 1 in its binary notation, I would use the index of these 1's in the binary notation as an index of the corresponding experimental single species measurement to find the predicted average.

For the pairwise species prediction model, I averaged the corresponding diversity measurements of a pair of bacterial species. For example, to predict the fitness phenotype of flies with a combination of *LB, AP*, and *AO* present (with its binary notation being 11001), I averaged the measurements for that same fitness phenotype for when *LB*, and *AP* were

present (11000), *LB* and *AO* were present (10001), and *AP* and *AO* were present (01001). For each of the four phenotypic fitness traits, I considered the bacterial combinations that had 3 or more species. For these combinations, I determined if 1 was present in its binary notation. If it was, I indexed the position of that 1 out of the five possible positions so I could later determine which of the 5 bacterial species was present based on the position of that 1 in the binary notation. Using the indices of the 1 in each binary notation, I found all of the possible combinations of the pairs of bits that make up the given indices. For example, if the indices were [0, 1, 2] for one bacterial combination (11100) that you want to predict, the possible inner pairs that could be used to predict are [(0, 1), (0, 2), (1, 2)]. This means that the binary code is 11000, 10100, and 01100 and that the measurements of the pairs LP and LB, LP and AP, and LB and AP were used to predict. Using the list of inner pairs, I was able to add all of the correct pairs of measurements together to get my prediction. To further elaborate, while looping through all of the binary notations, for the inner pair in the aforementioned list (0, 1), if the bit in the binary notation in positions 0 and 1 were both 1, then I would use the fitness measurement for that binary notation towards my prediction. The higher the level of bacterial diversity, the more pairs of species I would use in my prediction. I would perform this loop for all of the possible pairs for every bacterial combination, thus resulting in a total of 32 predictions for each of the four fitness traits. Both single and pairwise predictions were computed and plotted in a single function. It was carried out in a single function to prevent the passing back and forth of variables and axes.

For both single and pairwise predictions, I found the 95% confidence intervals by calculating the variance of the SE of all of the single species and pairwise measurements used for each prediction. In a separate function from the one that generated the predicted measurements, I compared each prediction to its corresponding experimental measurement. I plotted these difference between the experimental and corresponding predicted measurement as well as the error bars as 95% confidence intervals for each data point. For statistics, I made a separate function to determine how many predictions for both of the models were captured within the 95% confidence intervals and performed Fisher's exact test for each model for all fitness traits (this was not graphed, but you can see it printed in my script or reported in the conclusion).

For all graphs, I pre-determined the x-axis coordinates to prevent any overlapping of data points to make the plots more readable. I also created a function to create each of the 32 unique pie chart markers using specific pre-determined colors and ratios of each color. The ratio of each section of the pie marker is representative of the species present for each data point. For the first column of graphs, I overlaid them over the original marker generated by matplotlib. For the second column of graphs, I presented them along the x-axis (here, each pie marker corresponds to the confidence interval directly above it) using the y-min. For the top-most legend, I had a separate class that created specific patches of colored circles that I added to the legend. For the legend above figure A, I read a png image of the 5-colored pie chart that I had saved and used annotation Bbox to upload it. All eight graphs were subplots of a single figure and I gradually filled the figure with a pair of subplots (experimental measurement and its corresponding prediction error plots) at a time in a loop.

For figure 3B, to find the abundance of each species for each bacterial combination, I found the mean bacterial load of each of the five species as well as the total mean bacterial load for each combination (each combination consisted of 2 biological replicates on a food treatment in which they received fresh food everyday intermixed with 2 other biological replicates on another food treatment that I did not use). I appended each bacterial species' mean to a master array and then converted the array to a pandas data frame with all of the mean bacterial loads of each of the species. The total mean bacterial load was plotted on a logarithmic scale. The relative abundance of each species was shown on a linear scale. In order to preserve the linear scale of the relative abundances on a log10 plot, I converted the proportions into what it would appear on a log10 plot using the equation, p*Tot=10^x in which x is the pseudo CFU count of each species so that its proportion in the population appears as p of the stacked bar and Tot is the log10 of the total number of CFUs. Therefore, each bar in the stacked bar plot represents the linear proportional presence of each bacterial species for each combination, but the height of the bar represents the total abundance on a logarithmic scale. In a separate function, I calculated the standard error of the mean (SEM) of the total mean abundance and plotted these error bars. All of the data I used were part of biological replicates 2 and 3 (meaning that the flies were transferred to fresh food daily). I created a function to create each individual pie chart (each pie was different depending on the ratio of its sections and what bacteria was present) and saved each image as a png file. I created another function that read each png image and added it to an annotation Bbox. I passed along all of the colors I wanted for each pie chart to this function and appended each box underneath its intended bar on the plot beneath the x axis (the y coordinate was logarithmic). Similar to figure 3, I had a separate class that created specific patches of colored circles that I added to the legend.

Conclusion

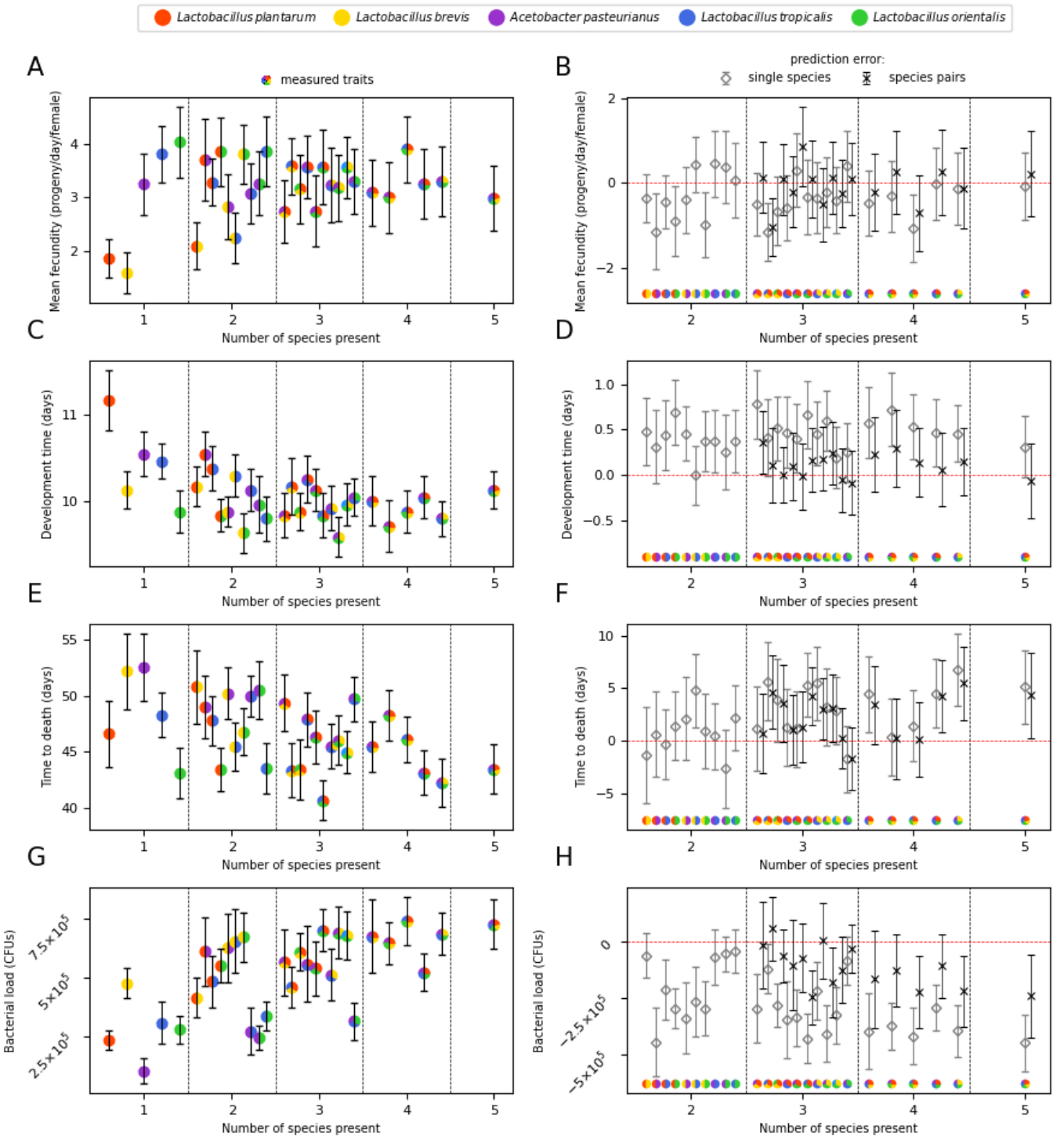
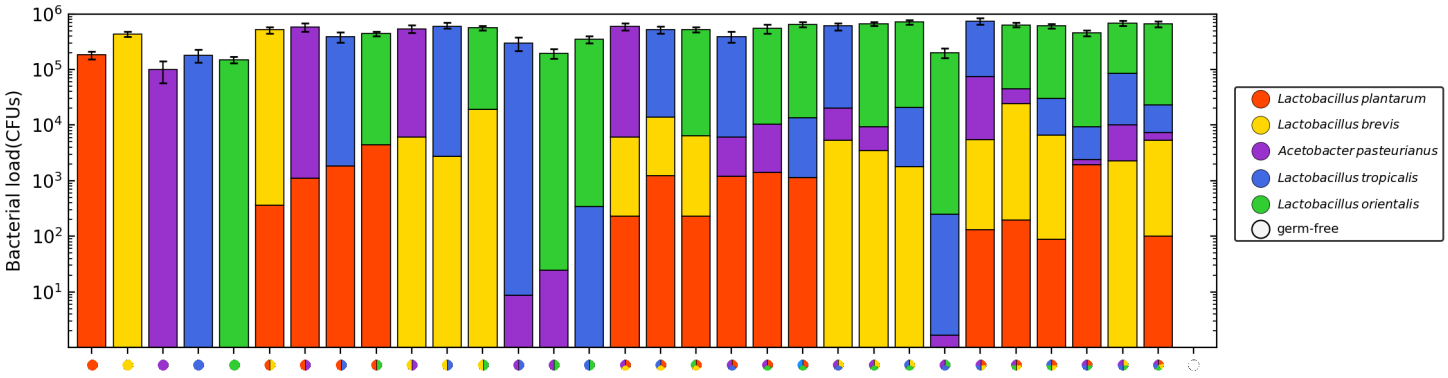


Figure 2 sought to understand how lower and higher order microbial interactions affect host fitness. Single species and pairwise modeling predictions used to evaluate higher diversity traits (bacterial diversity with 2 or more bacterial species present) using lower diversity measurements (bacterial diversity with 1 or 2 species present). Measurements for fitness traits (mean fecundity, development time, lifespan, and bacterial load) can be observed in reaching a convergence as the number of species increased (Fig. 2A, 2C, 2E, 2G). In order to elucidate if this gradual convergence was a result of a mixing effect of lower order microbiome interactions and if a higher order bacterial interaction could be predicted, I used a single-species mixing model and a pairwise-species mixing model to determine if the physiology of flies could be predicted using a single bacterial trait measurement and pairs of bacterial trait measurements, respectively. The error in the single species predictions are shown in gray, the pairwise predictions are shown in black.

The efficacy of each model's predictive ability was measured using 95% confidence intervals for each prediction's difference from the experimental measurement. For confidence intervals that did not include 0, it meant that the prediction for its associated phenotypic trait at the given bacterial diversity level was significantly different from the experimental measurement. Using this measure as predictive power, it was determined that across all four fitness traits, the single-species model correctly predicted 44% of traits (28 out of 64 traits total, 14 out of 16 for fecundity, 4 out of 16 for development, 9 out of 16 for lifespan, 1 out of 16 for bacterial load) and the pairwise-species model predicted 80% (51 out of 64 traits total, 15 out of 16 for fecundity, 15 out of 16 for development, 10 out of 16 for lifespan, 11 out of 16 for bacterial load). Fisher's exact test was used to examine if there was a significant association between the single species and pairwise species prediction model. For predicting mean fecundity, development time, lifespan, CFU count, Fisher's exact test resulted in p=1.0, n=16; p= 0.00017, n=16; p=1.0, n=16; and p= 0.00063, n=16, respectively. Only 16 bacterial combinations were used (bacterial diversity of 3 species or higher) in order to allow for comparison between single and pairwise species predictions. It can be concluded that the two models produced similar predictions for mean fecundity, lifespan, but the pairwise species model was a better predictor for development and bacterial load. This indicates variability in fly fitness may be more significantly affected by bacterial interactions in the microbiome.



For figure 3B, the goal was to demonstrate the mean microbiome (CFUs) for each bacterial species in each of the 32 bacterial combinations. The reasoning behind this was that if a particular bacterial species exerted an effect on one of the host's physiological fitness traits, then perhaps the abundance of that bacterial species should have a correlated affect as well. As evident in the figure, the more bacterial species that were present in the microbiome, the higher the mean bacterial load. However, as the number of bacterial species increased, the relative abundance of each species either remained constant or even decreased. Using the data generated from this figure, the researchers later discovered that the abundance of *LP* correlated with female fecundity and that the abundance of *AO* correlated with a shorter lifespan. The error bars are indicative of SEM.

A potentially interesting direction for this study to head towards could be to understand how the longevity and presence of these different bacterial species over time affect host fitness. Furthermore, the effects of diet and nutrition on microbiome composition could be another feasible route of research.

Overall, the results demonstrate that microbiome interactions and abundance result in a significant impact on the fly fitness genotypes and physiology as well as the overall microbiome composition. In particular, interacting bacterial species in the gut microbiome rather than singular species may be more intrinsic and important towards overall host physiology.

References

1. Steinfeld HM (1927) Length of life of *Drosophila melanogaster* under aseptic conditions. PhD dissertation, pp 1–47
2. Travers LM, Garcia-Gonzalez F, Simmons LW (2015) Live fast die young life history in females: Evolutionary trade-off between early life mating and lifespan in female Drosophila melanogaster. Sci Rep **5**:15469.