

The Transience of the *C. elegans* Microbiome

Oscar Solberg, Dr. Kelly Salmon
Hanover High School (Hanover, NH)
New Hampshire Academy of Science (Lyme, NH)
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Abstract:

The *C. elegans* is a common and foundational model organism, specifically in the field of microbiome study, as their translucent anatomy, indiscriminate eating habits, and short life cycle make them an easily observable, low-maintenance subject for observation. As in humans, the microbiome has been seen to play an extremely important part in the workings of the *C. elegans*, and research on host-microbiome interaction has been quite common. However, there have been relatively few studies on the transience of the *C. elegans* microbiome. To attempt to gauge this, an experiment was performed in which *C. elegans* nematodes were fed teal fluorescent bacteria for three to six days, followed by orange bacteria for about 90 minutes, and then photographed to attempt to gauge the ratio of the two bacteria strains in the gut of the nematodes. During analysis, it was found that the ratio of the two bacteria strains varied very little over the course of the experiment, not only in the experimental group but also in groups exposed only to the teal bacteria or no bacteria at all. This suggests that the microbiome is a somewhat permanent construct. As the *C. elegans* has a roughly analogous physiology to humans, this data might inform the frequency with which microbiome treatments, such as fecal transplants, are performed.

Introduction:

The *Caenorhabditis elegans* species of nematode is undeniably one of the foundational model organisms in the life sciences. Its short life cycle, translucent anatomy, and indiscriminate eating habits (their primary diet being bacteria) land it a spot among the easiest organisms to maintain and experiment upon, particularly in the realm of microbiome research, or the study of microscopic organisms within the body. This recent addition to the *C. elegans*'s resumé has generally focused on host-pathogen interaction, yet the simple characteristics of the microbiome itself, such as its permanence, remain ambiguous. Studying this would not only provide greater insight into the *C. elegans* microbiome's characteristics, but could possibly even provide a gauge of the importance of *C. elegans* diet in a lab setting. In fact, due to their analogous physiology to humans, data collected on the *C. elegans* microbiome might even inform human health.

Past research into the "lifespan" of the *C. elegans* microbiome has revealed that not only does a microbiome exist in the digestive system of the *C. elegans*, but that it can remain in that system for a long period of time, even after the supply of bacteria is cut off, and even after the death of the *C. elegans*. What remains to be seen is whether or not the microbiome of the *C. elegans* can last through both the removal of its source, the passage of time, and the short introduction of new bacteria. That is what this experiment plans to address.

In this experiment, the transience of the *C. elegans* microbiome was experimented upon using two types of bacteria expressing different-colored fluorescent proteins. The translucent nature of the *C. elegans* anatomy and their indiscriminate feeding habits allowed these bacteria expressing fluorescent proteins to be ingested and for the fluorescence to be detected, and measured. By measuring the fluorescence intensity of each fluorescent protein, the abundance of each bacteria could be inferred, and a change in makeup of the *C. elegans* microbiome could allow quantitative observations to be made about

the transience of the *C. elegans*'s microbiome. Based upon background research on the microbiome, it is predicted that it will take more than the amount of time a microbiome took to establish to change its composition or break it down.

Materials:

This experiment required the use of 50 60 mm agar plates, 30 of which were unseeded nematode growth medium (NGM), 10 of which were seeded with a strain of teal fluorescent *E. coli* bacteria, 5 of which were seeded with non-fluorescent OP50 *E. coli* bacteria, and 5 of which were seeded with a strain of orange fluorescent *E. coli* bacteria. Fluorescent *E. coli* were kindly provided by the lab of Carey Nadell at Dartmouth College. It also required 375 wild-type *Caenorhabditis elegans*, a platinum pick for worm movement, and a bacti-cinerator III for pick sterilization. The imaging was done using a Nikon SMZ 1:10 stereozoom microscope, an Olympus Pen Lite E-PL7 camera with a microscope attachment, and a Nightsea fluorescence kit consisting of green and royal blue lights, emission filters, a visor for eye protection, and an SFA Light Base. The *C. elegans* were paralyzed for imaging using 2.5 mmol levamisole dissolved in M9.

Methods:

On the first day of the experiment, 375 wild-type *C. elegans* nematodes in the L4 stage were placed onto 15 agar plates. Five of these plates were seeded with 125 μ L non-fluorescent OP50 *E. coli* bacteria for the negative control group, and ten of them with 125 μ L teal fluorescent *E. coli* bacteria for the positive control group and experimental group. On the fourth day of the experiment, at 8:30 AM, the population of one plate of *C. elegans* from the positive control group ("positive control 1") was moved to an unseeded intermediary plate to allow the worms to shed bacteria from their cuticle. The same was done with one plate of *C. elegans* from the experimental group ("experimental 1"). At 10:00 AM, positive control 1 was moved to another unseeded agar plate to await imaging, while experimental 1 was moved to an agar plate seeded with 125 μ L orange fluorescent *E. coli* bacteria. At 11:30 AM, experimental 1 was moved to another unseeded intermediary plate. At 1:00 PM, experimental 1 was moved to an unseeded agar plate for imaging, as was one plate of *C. elegans* from the negative control group ("negative control 1"). At 2:00 PM, all three observation plates were photographed using the fluorescence setup. Photographs of the positive control 1 group were taken of each worm with both white light illumination and blue light illumination, while photographs of negative control 1 and experimental 1 were taken with white, blue, and green light illumination. Blue light illumination excites the fluorescence of the teal bacteria and green light illumination excites the fluorescence of the orange bacteria. The photos were taken with an exposure time of 2" (for white light illumination), 25" (for green light illumination), or 10" (for blue light illumination). The *C. elegans* were paralyzed using 500 μ L of 2.5 mmol levamisole, with appropriate amounts added to complete the paralysis of the worms as necessary. The photographed *C. elegans* and used plates were discarded following this procedure.

Over the following three days, the process of day two was repeated using one plate from each group each day ("positive controls 2-4", "negative controls 2-4", "experimentals 2-4"). Photographs from across the experiment were then compiled on a computer, and analyzed using FIJI (<https://imagej.net/software/fiji/>). In FIJI, polygons were drawn around worms in photographs using white light illumination, and then moved to the corresponding photograph taken with either blue or green light illumination. This ensured that the entirety of the worm was covered by the polygon. The software then calculated the area, lowest fluorescence intensity in a pixel, highest fluorescence intensity in a pixel,

integrated fluorescence density, and mean fluorescence intensity. In the positive control group, the same measurements were taken of a random sample of the background of each photograph. In that group, the mean fluorescence density of the background was subtracted from that of the worm, and could then be compared over time. In the negative control group and experimental group, the teal mean fluorescence density of the worm (without subtracting the background) was divided by the orange mean fluorescence density to obtain a ratio. That ratio could then be compared between the negative control and experimental groups, and over time. All data was analyzed using t-tests between the first day of each group and the other three days.

Results:

As shown in Figure 1, the average teal to orange ratio of mean fluorescence densities in the negative control group varied very little over the first three days, but was followed by a decrease on the last day of the experiment (though not statistically significant). The average teal to orange ratio was 1.0572 on the first day, 0.9362 on the second day, 1.1441 on the third day, and 0.5539 on the fourth day. T-tests conducted between the first day and each other day of the experiment revealed a p-value of 0.103 between the first and second days, a p-value of 0.396 between the first and third days, and a p-value of 0.054 between the first and fourth days.

As shown in Figure 2, the teal mean fluorescence density varied very little over the course of the experiment. The teal mean fluorescence density was 0.8779 on the first day, 0.8153 on the second day, 0.9756 on the third day, and 0.8965 on the fourth day. T-tests conducted between the first day and each other day of the experiment revealed a p-value of 0.911 between the first and second days, a p-value of 0.040 between the first and third days, and a p-value of 0.019 between the first and fourth days.

As shown in Figure 3, the average teal to orange ratio of mean fluorescence densities in the experimental group increased significantly on the second day, followed by a significant decrease on the third day, followed by a moderate increase on the fourth day. The average teal to orange ratio was 1.0065 on the first day, 1.3388 on the second day, 0.7152 on the third day, and 1.0161 on the fourth day. T-tests conducted between the first day and each other day revealed a p-value of 0.010 between the first and second days, a p-value of 0.811 between the first and third days, and a p-value of 0.323 between the first and fourth days.

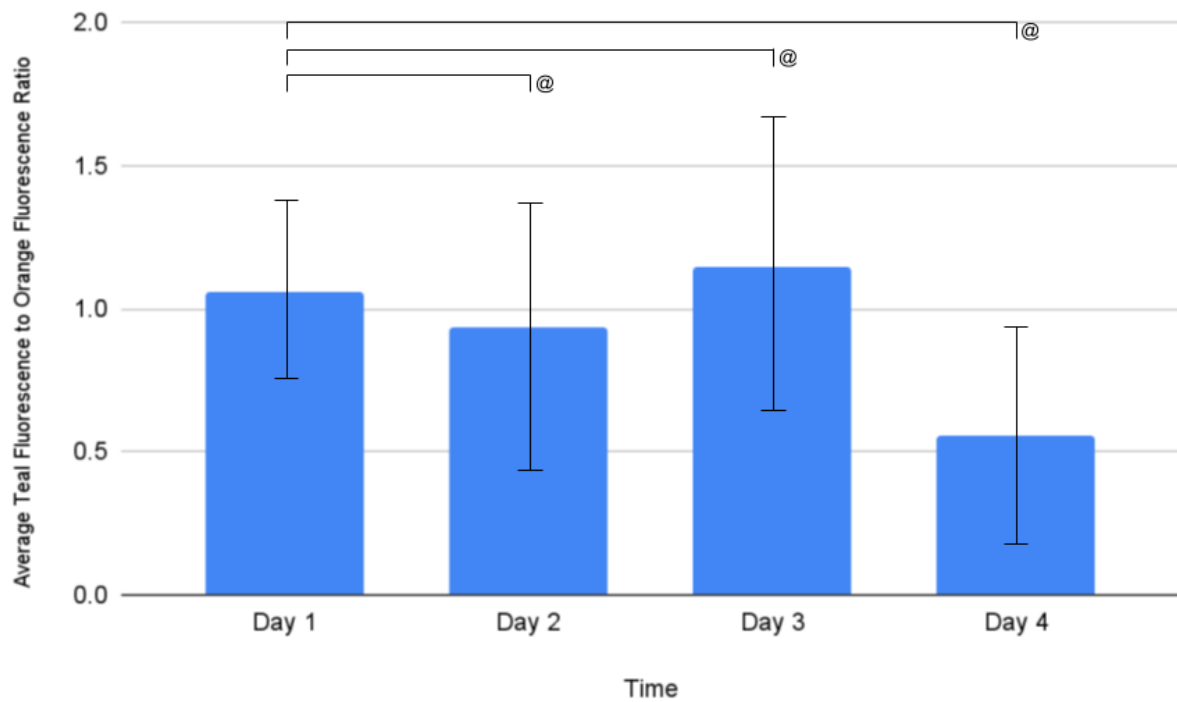


Figure 1: A comparison of the ratio between teal and orange mean fluorescence density across the negative control group. Bars show average across at least 15 worms per day with the error bars showing standard deviation. “@” indicates $P > 0.05$, “β” indicates $P < 0.05$, and “ββ” indicates $P < 0.01$.

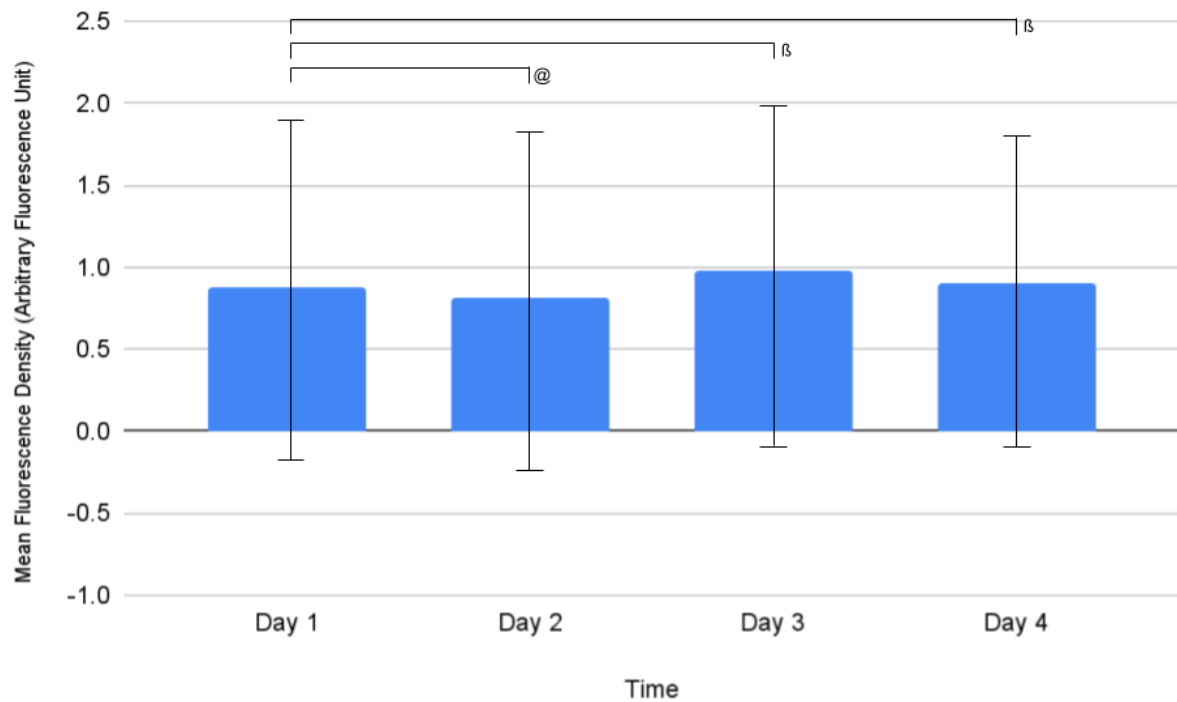


Figure 2: A comparison of the mean teal fluorescence density across the positive control group. Mean fluorescence density in the background was subtracted from that of the worm. Bars show average across at least 15 worms per day with the error bars showing standard deviation. “@” indicates $P > 0.05$, “β” indicates $P < 0.05$, and “ββ” indicates $P < 0.01$.

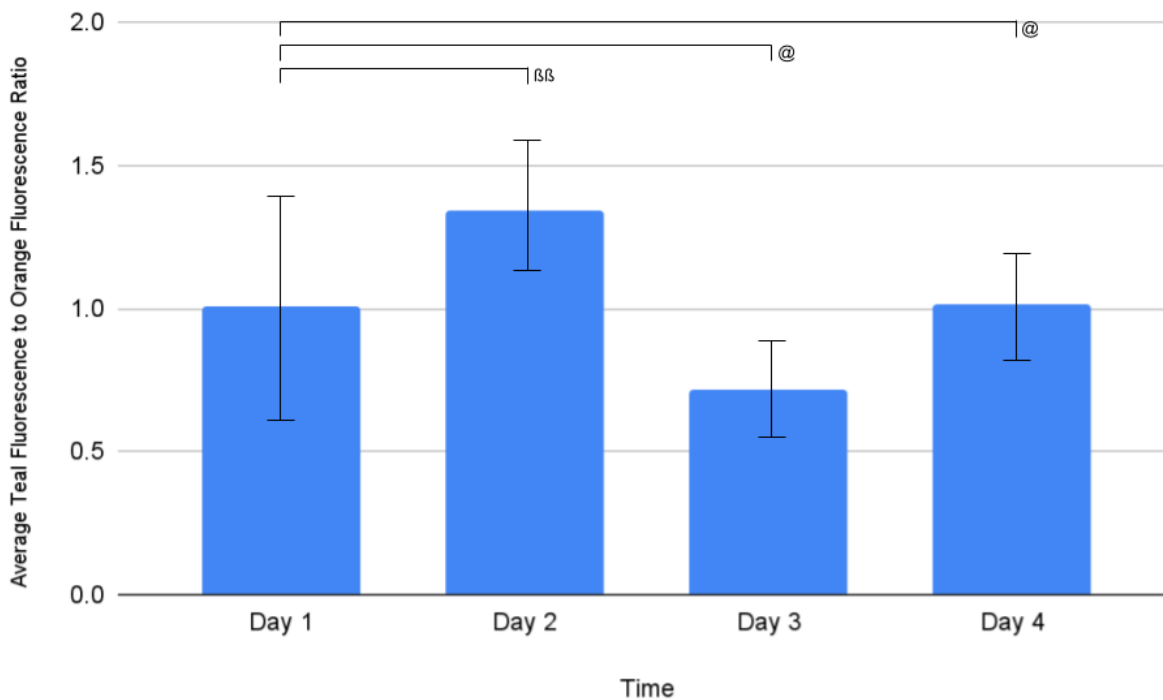


Figure 3: A comparison of the ratio between teal and orange mean fluorescence density across the experimental group. Bars show average across at least 15 worms per day with the error bars showing standard deviation. “@” indicates $P > 0.05$, “β” indicates $P < 0.05$, and “ββ” indicates $P < 0.01$.

Discussion:

For the negative control group and experimental group, the change in the teal to orange mean fluorescence density ratio was minimal. As seen in Figures 1 and 3, the standard deviation on each day overlapped greatly, and the t-tests revealed high p-values, suggesting that there was no significant difference over time. In the experimental group, the t-test between the first and second days suggested a significant difference due to finding a p-value less than 0.01. However, this is deceptive, as there was still a great deal of variation within the Day 2 group, and that p-value could easily be interpreted as a false positive.

For the positive control group, the mean fluorescence density varied little over time. Here, too, the lower p-values (two of which, the Day 1-Day 3 p-value and the Day 1-Day 4 p-value, were below 0.05) seem to be deceptive. The immensely wide variation of mean fluorescence density on each day, and the standard deviation overlap overrule the p-values and clearly posit that there was no significant difference over time in that group.

The immensely wide standard deviations within the positive control group can likely be attributed to an imprecise section of the method, namely, the measuring of background mean fluorescence density. While it was assumed that the background of the photo would have more or less an equal distribution of

fluorescence, this was not the case, and the arbitrary method of moving the worm-shaped polygon to any part of the plate not containing a worm involved a great deal of random error. This was avoided in the positive control group and experimental group, as using a ratio between two different measures of the same worm negated the environmental differences between plates. In the future, similar experiments should use the ratio as the measured data, and reject the subtraction method for its uncertainty.

As all groups seem to have little to no significant difference over time, the hypothesis is rejected and it can be concluded that the microbiome is a somewhat permanent construct, and can resist manipulation from outside sources for long periods of time. This is significant, as it might suggest that treatment for gastrointestinal diseases through microbiome manipulation, such as in a fecal transplant, may not need to be done as frequently.

In the future, this experiment should be conducted over a longer period of time, in order to further gauge the permanence of the microbiome. Additionally, the same experiment could be conducted utilizing a different second organism. In other words, the orange fluorescent bacteria in this experiment could be replaced by something more distinct from the teal fluorescent bacteria, such as a member of a different species of bacteria. Finally, a future experiment in this vein might add stress to the *C. elegans*'s environment (such as temperature stress or starvation) to see how this affects microbiome transience.

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