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Claire Bowman April 8, 2025

Understanding *Caenorhabditis elegans* Decision-Making in Complex Foraging Environments

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By Claire Bowman

Decisions made by the nematode *Caenorhabditis elegans* when foraging for bacterial food sources have been thoroughly studied in 2D environments using canonical choice assays; however, interactions between *C. elegans* and microbes in structured 3D environments such as soil matrices are currently unclear. Additionally, the scales at which *C. elegans* are able to make foraging decisions in patchy environments are not well understood, and the consequences for microbial ecology are currently unknown. This project seeks to better understand the spatial scales of decision making and worm-microbe interactions by creating patterned bacterial lawns with various strain combinations and performing choice assays using these mixtures. By pairing three bacterial test strains (*Pseudomonas alcaligenes*, *Pseudomonas veronii*, *Salmonella enterica*) with one reference strain (*Pseudomonas citronellolis*) in various assay layouts, we were able to determine that *C. elegans* foraging behavior changes based on the spatial scale of the decision they must make. Additionally, we found that mixing and plating one diluted fluorescent bacterial strain with a separate diluted non-fluorescent bacterial strain at a 1:1 volume ratio produced lattices with small patches of bacteria that can be differentiated using a fluorescent microscope, and that worms behaved differently towards food spots containing two bacterial species than towards food spots with one bacterial species. These results expand our awareness of the decisions made by *C. elegans* in complex foraging environments and serve as foundational data to further investigate the consequences of spatially structured environments on microbial populations.

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1. Introduction

1.1 Microbial community ecology

Microbial communities are vital to our environment. Found abundantly worldwide, these groups are composed of hundreds to thousands of unique microbial species that interact with each other and the environment to exert effects on processes such as nitrogen fixation and food fermentation that keep our society functional[1]. In laboratory environments, desirable microbial communities can be synthesized by manipulating various species' abundances and relevant environmental conditions; however, these communities are often studied using methods that do not accurately reflect the natural environment where microbial communities would develop. For example, propagating bacterial cultures in environments with identical chemical and physical properties in all dimensions (defined as isotropic environments) such as liquid media – a straightforward, commonly utilized laboratory protocol – may prompt different inter- or intra-species interactions than would be observed in anisotropic, structured environments such as agar or soil.

It is well-established that the assembly of microbial communities is constantly influenced by external factors that have significant impacts on which species can grow and what functions they perform, including nutrient availability, energy flow, spatial structure and microbial interactions[2]. Understanding how these factors are related and the exact impacts they have on community composition is crucial for purposes including infectious disease control[3], microbiome health research[4], and the development of synthetic communities[5]. Though literature currently reflects general ideas of the factors governing community assembly, we are yet to completely understand the environmental impacts on community dynamics.

1.2 Spatial effects on microbial communities

Naturally occurring microbial communities can form in environments of vastly different structures, such as the smooth surfaces inside of water pipes where *Legionella* biofilms reside or the mucus-coated large intestine where most of the human gut microbiome is found. The type of structure in which microbial communities form is known to dramatically affect their composition and function. Adding structure to an otherwise isotropic community can increase diversity by allowing competitive species to coexist rather than drive their competitors to extinction, indicating that a community's structural environment directly influences its stability[6]. This can be associated with the fact that spatial constraints reduce the number of cell-to-cell and non-cell-to-cell interactions among bacteria, even when they are present at high abundances[7]. Additionally, spatially constructed communities are associated with stronger rescue effects, where microbial populations that experience loss due to significant environmental disturbances can recover their abundance and functionality[8]; this has been studied in various contexts, including the global ecosystem[9], antibiotic resistance[10], and the human microbiome[11].

Though spatial structure is a crucial aspect of microbial community formation, it is not constant. Environmental changes occur regularly, adjusting the properties of space where microbial communities develop, and organisms external to these communities are capable of manipulating space by changing the physical world. Humans, for example, have dramatically altered the topography of our planet through processes such as urbanization and other forms of development. Interactions between smaller animals and the environment are also responsible for changes in nutrient availability, spatial structure, and the introduction of substrates that can either support or inhibit bacterial growth; as an example, seabird populations on New Zealand islands

facilitate sea-to-land nutrient transport that supports the growth of soil bacterial species, and introducing seabird predators to these islands thus changed their soil microbiome composition[12].

1.3 Effects of Caenorhabditis elegans *decision-making on microbial communities*

One organism that can affect the microbial environment is *Caenorhabditis elegans*, a hermaphroditic nematode that is regularly used as a model system in biological research[13,14]. These microscopic worms are naturally found in soil, where they feed on bacteria and can make decisions about what to consume based on the bacterial density[15] and pathogenicity[16] of available food options, as well as their previous food exposures[17]. According to previous work in the field, worms will choose food sources that can more effectively sustain their growth[18] and can even retain memories of previous food sources - worms fed on specific strains of bacteria for a pre-determined amount of time will preferentially choose the same strain when transferred to new choice assay plates[19]. After choosing and consuming a food source, *C. elegans* can disperse the bacteria further throughout the environment as a result of the worms' movement[20] and excretion from the intestine[21]. The implications of this are evident – depending on which bacterial source *C. elegans* determine to be the most appealing, the structure of microbial communities in which they reside can be dramatically altered. Additionally, previous work in our lab has shown that bacterial evolution can be changed in communities where *C. elegans* are present[22], raising questions about how *C. elegans* behavior affects pathogen evolution and transmission.

Despite our current understanding of factors influencing *C. elegans* decision-making, the effects of spatial structure on *C. elegans* foraging decisions – particularly in environments reflective of their natural habitats such as soil matrices where multiple species of bacteria coexist in very close proximity[23] - is currently unclear. Typical investigation of *C. elegans* foraging choice involves the use of canonical choice assays in which worms are placed in the center of

agar plates with spots of bacteria at a distance typically millimeters away, requiring the worms to identify and travel towards the most appealing source[18,24,25]. Results from these types of experiments have provided insight into various factors used by worms when choosing food sources in these conditions; however, they are not properly representative of the natural environments where *C. elegans* are found. Therefore, further investigation into the effects of microbial spatial structure on *C. elegans* decision-making is warranted to better understand downstream impacts on pathogen spread, microbial community composition, and spatial ecology.

1.4 Project summary

This project investigates the minimum spatial scale of *C. elegans* decision-making and the consequences for spatial ecology of microbial populations. We first performed canonical choice assays to identify pairs of bacteria in which *C. elegans* display an observable, significant preference that can be used as a baseline for further study. These assays were performed at varying spatial scales, with bacterial spots of different sizes (at steps of approximately 6mm, 4mm, 1mm and 150μm in diameter) initially located at different distances from worms (at steps of 2.5cm, 1.5 cm, and 1cm). These bacteria were then used to investigate worm choice on approximately worm-sized patches and in mixed-culture lattices (with small, alternating 'patches' of bacteria that could be differentiated under a microscope) to gain insight into *C. elegans* choices at a smaller scale than we have studied before. The results of this project broaden our understanding of the factors influencing *C. elegans* decision-making, therefore improving our ability to predict and adjust the formation of microbial communities in the laboratory and natural environment.

2. Methods

2.1 Bacterial strains and growth media

Bacterial strains used for choice assays are listed in Table 1. LB medium was prepared by combining and autoclaving 10g tryptone, 5g yeast extract, 5g NaCl, and 1L ddH_2O. Nematode growth medium (NGM) was prepared by combining and autoclaving 3g NaCl, 2.5g peptone, and 1L H_2O; after autoclaving, 25mL 1M KPO_4 buffer, 1mL 1M $MgSO_4$, 1mL 1M $CaCl_2$, and 1mL 5mg/mL cholesterol in EtOH were added. KPO_4 buffer was prepared by combining and autoclaving 108.3g KH_2PO_4, 35.6g K_2HPO_4, and ddH_2O to 1L. M9 worm buffer was prepared by combining and autoclaving 3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, and 1L ddH_2O; after autoclaving, 1mL 1M $MgSO_4$ was added. Agar plates were prepared with a 1.5% agar concentration unless otherwise specified.

#	**Name**	**Modifications**	**Source**
1	*Pseudomonas alcaligenes*	miniTn7-GmR-YFP	Hasan Celiker
2	*Pseudomonas mendocina*	pBTK520-SpR-GFP	Nic Vega
3	*Enterobacter aerogenes*	miniTn7-GmR-GFP	ATCC
4	*Salmonella enterica*	attB::GFP:KmR	ATCC
5	*Pseudomonas veronii*	miniTn7- GmR-YFP	ATCC
6	*Pseudomonas aurantiaca*	miniTn7-GmR-YFP	Hasan Celiker
7	*Pseudomonas aeruginosa*	KmR-YFP	Nic Vega
8	*Pseudomonas putida*	AmpR-GFP	ATCC
9	*Staphylococcus aureus*	pTRKH3-ermGFP	Chris LaRock
10	*Escherichia coli* MG1655	miniTn7-GmR-F1-RFP	CGC

11	*Pseudomonas citronellolis*	miniTn7-TpR-YFP	Hasan Celiker
12	*Ochrobactrum* sp. BS30	pBTK519-BhR-GFP-KmR	*C. elegans* microbiota
13	*Escherichia coli* OP50	miniTn7-GmR-GFP	CGC
14	*Pseudomonas chlororaphis*	miniTn7-GmR-YFP	Hasan Celiker

Table 1. Choice assay bacterial strains. Modified (labeled with pBTK plasmids[26] or miniTn7 insertions[27,28]) strains were used as 'test' strains in preliminary choice assays. CGC = Caenorhabditis Genetics Center. ATCC = American Type Culture Collection. Strains were chosen based on availability, presence of fluorescent modifications, and diversity – though some species had been studied in *C. elegans* before, others' interactions with the nematode were unknown.

2.2 C. elegans *propagation and synchronization*

Experiments were performed using N2 (wild type) *Caenorhabditis elegans* obtained from the Caenorhabditis Genetics Center (CGC). *C. elegans* stocks were maintained at 24.5°C on 10cm NGM + 1.5% agar plates seeded with 100µL of *E. coli* OP50 as a food source. Dauer stocks were chunked onto fresh seeded plates every month to produce breeding stocks, which were then chunked every 3-4 days to maintain active reproduction and feeding.

When necessary for experiments, worm populations were age-synchronized according to standard laboratory protocols[29] by collecting populations from 4 newly starved NGM + 1.5% agar + OP50 plates and rinsing 3 times with ddH_2O, eventually resuspending worms in 1mL ddH_2O. 130µL of bleach and 130µL of 8M NaOH were added to each tube before vigorously vortexing them for 3 minutes to release eggs from adult worms. Once all adult worms were dissolved, the solution was spun down and supernatant was removed to leave a pellet of eggs,

which was then washed four times with 1mL M9 buffer and transferred to a glass culture tube with 9mL M9 buffer. Eggs were allowed to hatch in buffer overnight, and the resulting larvae were washed twice the next day with M9 buffer + 0.1% Triton X-100 before being transferred to NGM + *pos-1* RNAi agar plates to grow without the ability to lay viable eggs for 3 days.

Upon reaching adulthood, worms were placed in 5mL S medium + 2X heat-killed OP50 + 200 µg/mL gentamycin + 50 µg/mL chloramphenicol for 1 hour to purge live *E. coli* from the intestine. These worm populations were then sucrose washed to produce a sample free of dead worms, eggs, or bacteria by first washing populations in 10mL cold M9 buffer at 700xg for 30-60 seconds two times, leaving a pellet of worms, and then adding 5mL cold M9 buffer and 5mL cold 60% sucrose solution. Tubes were mixed by inversion before 1mL of cold M9 buffer was carefully added to the top of each solution without mixing. Tubes were then centrifuged at 1500xg for 3 minutes, causing live adult worms to float between the interface of the M9 buffer and sucrose, where they were collected using a glass serological pipette and transferred to separate tubes containing 8mL M9 buffer. All collected worms were then washed 3 times with 10mL M9 buffer to remove any remaining sucrose before being ready for experimental use.

2.3 Microscopy

Worm quantities on traditional choice assay plates were counted using a Leica KL300 LED microscope. Fluorescence of bacterial strains was confirmed using a Leica DMi8 inverted microscope equipped with a Leica DFC9000 GT sCMOS camera to capture images.

2.4 Traditional choice assays

Bacterial strains to be used in the assay were inoculated from glycerol stock into 200µL LB + antibiotic selection and grown at 25ºC with shaking for 24 hours. After growth, 2µL from each

well was transferred into 198µL NGM + antibiotic selection and grown with the same conditions as before. The next day, 6cm NGM + 1.5% agar plates were inoculated with bacterial spots following the patterns shown below (Figure 1) and incubated at 25ºC for 24 hours. When assay plates were ready, approximately 50 age-synchronized, sucrose washed worms were placed onto the center of each plate, marking the beginning of the assay. The number of worms present on each bacterial spot on the plate, as well as worms that were not located on any bacteria, was counted at multiple timepoints (1h, 2h, 3h, 4h, 6h, 24h). At the 24-hour timepoint, *C. elegans* leaving behavior was analyzed by counting the number of worms located inside and directly outside bacterial spots. Preference was quantified by calculating attraction indices as follows:

$$I_A = \frac{(W_T - W_R)}{n}$$

where I_A = attraction index, W_T = number of worms on test strain spots, W_R = number of worms on reference strain spots, and n = total number of worms on plate. Leaving behavior was quantified by calculating leaving indices at the 24h timepoint as follows:

$$I_L = \frac{(T_E - T_C)}{T}$$

Where I_L = leaving index, T_E = number of worms surrounding test strain spots, T_C = number of worms fully inside test strain spots, and T = total number of worms on or around test strain spots.

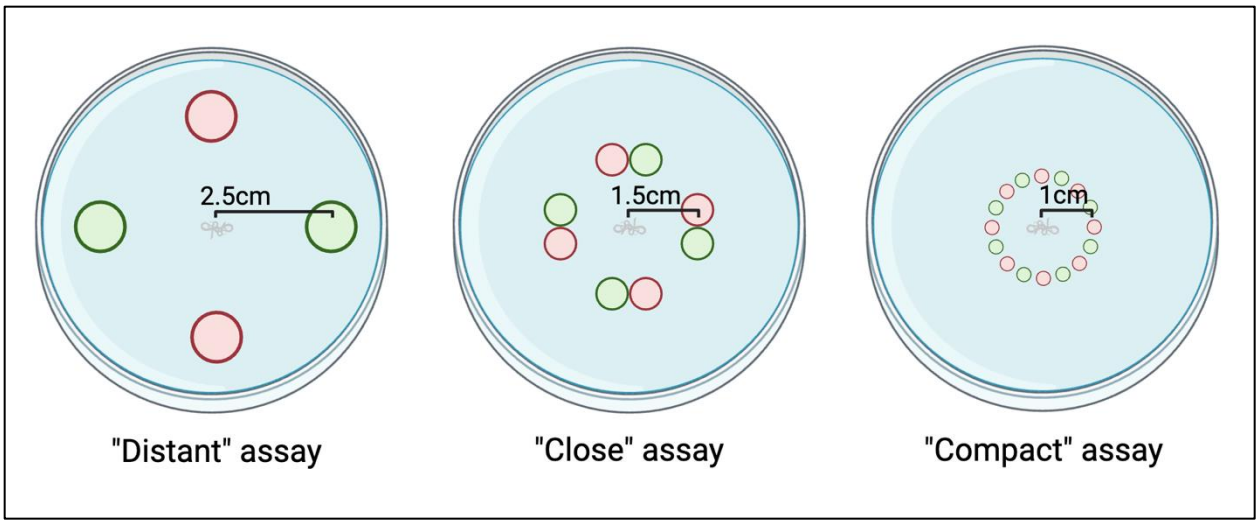

Figure 1. Traditional choice assay design. Layout of experimental plates for "Distant", "Close", and "Compact" choice assays. Plates for distant assays were inoculated with 10µL spots of bacteria. Plates for close assays were inoculated with 5µL spots of bacteria. Plates for compact assays were inoculated with wooden dowels dipped into bacterial cultures and tapped to the surface of the plate. All assays were performed using 6cm NGM agar plates. Red and green spots represent the two strains being tested on each plate. Figure created in BioRender.

2.5 Conjugation

The following strains were grown at 30ºC overnight with shaking: *E. coli* SM10 + pTNS2 in 1mL LB + 100µg/mL ampicillin, *E. coli* HB101 + pRK2013 in 2mL LB + 30µg/mL kanamycin, *E. coli* SM10 pTn7xKS-sfGFP in 2mL LB + 100µg/mL ampicillin, *E. coli* SM10 pTn7xKS-dTomato in 2mL LB + 100µg/mL ampicillin, and unlabeled bacterial species in 1mL LB per species. Mixtures were prepared by combining 0.1 mL of SM10 + pTNS2, HB101 + pRK2013, either SM10 + pTn7xKS-sfGFP or SM10 + pTn7xKS-dTomato, and one of the unlabeled strains with enough LB to reach a final volume of 1mL. Control mixtures without SM10 + pTNS2 were also prepared. Mixtures were centrifuged at room temperature for 2 min at

7,000xg and washed with 1mL of warm (30ºC) LB medium two times before each pellet was resuspended in 30µL of warm LB medium. These 30µL volumes were then pipetted onto pre-warmed (at 30ºC for 30 minutes) 13-mm cellulose acetate filter membranes placed on nonselective LB agar plates and incubated at 30ºC overnight. The next day, sterilized forceps were used to transfer each piece of filter paper to a 1.5mL Eppendorf tube containing 0.2mL of PBS, and each tube was vortexed for 30-60 seconds to resuspend bacterial growth. 0.1mL aliquots of each resuspension were plated onto VBMM + Gm10 + IPTG plates and allowed to grow at 30ºC until exconjugants appeared or one week passed (whichever came first).

2.6 Electroporation

To prepare electrocompetent cells, glycerol stocks of unmodified bacterial strains were used to inoculate flasks containing 12mL LB and grown overnight at 25ºC with shaking. Cultures were evenly divided into microcentrifuge tubes, centrifuged at room temperature at 16,000g for 2 minutes, and supernatant was discarded. Pellets were then washed with 1mL of room temperature 300mM sucrose twice. Four pellets per strain were then resuspended in a combined volume of 200µL of 300mM sucrose, and this mixture was split into 100µL aliquots to be used for electroporation.

For electroporation, one 100µL aliquot of electrocompetent cells was combined with 250ng helper plasmid (pTNS2) and 250ng donor plasmid (pTn7xKS-sfGFP or pTn7xKS-dTomato). Control mixtures were also prepared in which no donor plasmid was added. Cells were mixed by gently stirring with a pipette tip, and each mixture was transferred to an electroporation cuvette stored at -20ºC. Cells were then electroporated at 25uF, 200Ω, 2.5kV, and time constant <5ms, and 1mL LB was immediately added to the cuvette to assist in recovery. Cells were grown at 37ºC with shaking for 1 hour, and 100µL of each mixture was then plated

on an LB + Gm10 plate. The remaining 900µL of each mixture was centrifuged at 16,000xg for 2 min at room temperature and each pellet was resuspended in 200µL LB before being plated on LB + Gm10 plates. Plates were incubated at 37ºC until colonies were identified or until a week had passed.

2.7 Mixture preparation

Bacterial strains to be combined were inoculated from glycerol stock into 200µL LB + antibiotic selection and grown at 25ºC with shaking for 24 hours. After growth, 2µL from each well was transferred into 198µL NGM + antibiotic selection and grown with the same conditions as before. Each culture was then serially diluted in NGM to a concentration of 10^{-6}. Mixtures were created by combining 250µL of a diluted fluorescent strain with 250µL of a diluted non-fluorescent strain, resulting in a total volume of 500µL of mixed culture. When plated on NGM + 1.5% agar plates, these mixtures produce a lattice of fluorescent and non-fluorescent bacteria that can be differentiated visually under a microscope equipped with fluorescent lighting.

2.8 Mixture attraction assays

P. alcaligenes miniTn7-GmR-YFP, *P. veronii* miniTn7-GmR-GFP, and *S. enterica* attB::GFP:KmR were each mixed with unlabeled P. citronellolis as previously described (*2.7: Mixture preparation*). Mixtures were plated as 10uL spots on the top half of a single well in a 24-well cell culture plate previously filled with 1mL NGM agar. Plates were inoculated at 25ºC for 24 hours. Then, ~50 age-synchronized, sucrose washed *C. elegans* were placed on the bottom half of each well and the now seeded 24-well plate was placed on a Leica DMi8 inverted microscope, which captured bright-field and GFP images of each experimental well at 15-minute intervals. These images were then stitched together using Leica software to produce a

visualization of the bacterial mixture in which the fluorescent and non-fluorescent strains could be differentiated from each other and individual worms could be identified. Due to constraints with the speed of the microscope, only 12 wells of the full plate could be imaged per run of the experiment.

3. Results

3.1 C. elegans *strain preferences under traditional conditions*

To identify bacterial strains for use in future experiments, we first performed canonical choice assays to generate preliminary data regarding *C. elegans* decision-making between pairs of bacteria. We characterized the worms' responses to 14 bacterial species when offered *E. coli* OP50, which they were raised on, as an alternative. These experiments provided baseline data for comparison with future results, allowing us to determine the spatial scale at which typical worm decisions are significantly changed, and allowed us to choose a reduced set of bacteria for use in further experiments based on attraction and repulsion data.

Choice assays were performed and analyzed as outlined in Methods (*2.4: Traditional choice assay*). First, a "Distant" choice assay was conducted, in which worms chose between individual test strains (Table 1) and the control (*E. coli* OP50) over a period of 24 hours. The initial distant assay showed that *C. elegans* exhibited distinct food preferences, demonstrated in both their attraction at 2 hours and leaving behaviors at 24 hours (Figure 2). Some bacterial species initially attracted worms but then became repulsive (*P. alcaligenes*, *P. veronii*), while other attractive species did not repel worms after 24h (*E. aerogenes, S. enterica*). Some species were similar to the OP50 control (e.g. *P. citronellolis*).

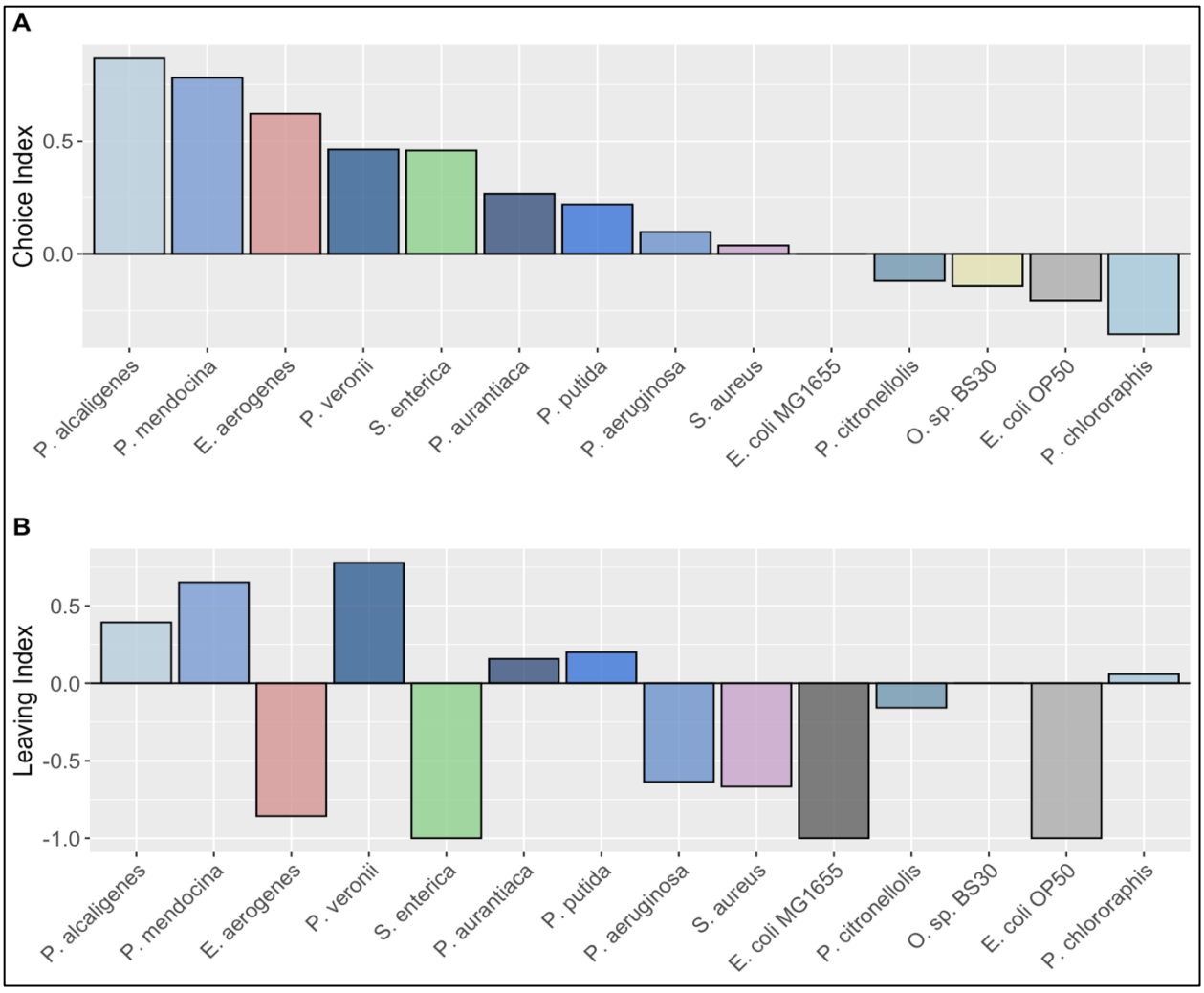

Figure 2. *C. elegans* choice behavior in "Distant" canonical choice assay. All test strains were labeled with fluorescent markers as listed in Table 1. One plate with ~50 worms was analyzed per condition. (A) *C. elegans* attraction to test strains versus unlabeled OP50 control after 2h; higher attraction index values indicate a stronger preference for the test strain, and negative values indicate preference for the control. Attraction index values of 0 imply no preference for either strain. (B) *C. elegans* repulsion to test strains after 24h on test plates; higher leaving index values indicate stronger repulsion, and negative values indicate that worms are more likely to stay on bacterial spots over time. Leaving index values of 0 imply that worms are equally likely

to leave or stay on spots. Color indicates genus of bacteria: *Pseudomonas* in shades of blue, *Salmonella* in green, *Staphylococcus* in purple, *Enterobacter* in red, and *Escherichia* in grey.

Following analysis of these results, specific bacterial strains were selected for further experimentation (*Salmonella enterica*, *Enterobacter aerogenes*, *Pseudomonas citronellolis*, *Pseudomonas alcaligenes*, and *Pseudomonas veronii*). Each strain yielded notable results in the initial distant assay: *P. alcaligenes* was highly attractive but later repulsive, *E. aerogenes* and *S. enterica* were attractive and later not repulsive, *P. veronii* was attractive but later highly repulsive, and *P. citronellolis* was comparably attractive to the control and worms were equally likely to stay or leave on *P. citronellolis* spots after 24 hours. A "Close" choice assay was then performed with each of these strains vs. heat-killed OP50 (HKOP50). HKOP50 was used in this assay instead of live OP50 as before to understand if worms' preferences would be different if they were presented with one live species (the test strain) and one dead species. This assay found that aside from *P. veronii*, all strains were more attractive to worms than HKOP50 (Figure 3). Notably, *P. citronellolis* was chosen by worms at an approximately equal rate to OP50 in the distant assay and did not repel them after 24h; in the close assay, *P. citronellolis* was highly preferable to HKOP50 and still not repellent. Additionally, *P. veronii*, which was initially attractive and later repulsive in the distant assay, was extremely unattractive in the close assay. Because all test strains were either highly attractive or highly unattractive to worms in comparison to HKOP50, we determined it would be best to only use live food sources in future assays to gather a more thorough understanding of worms' preferences.

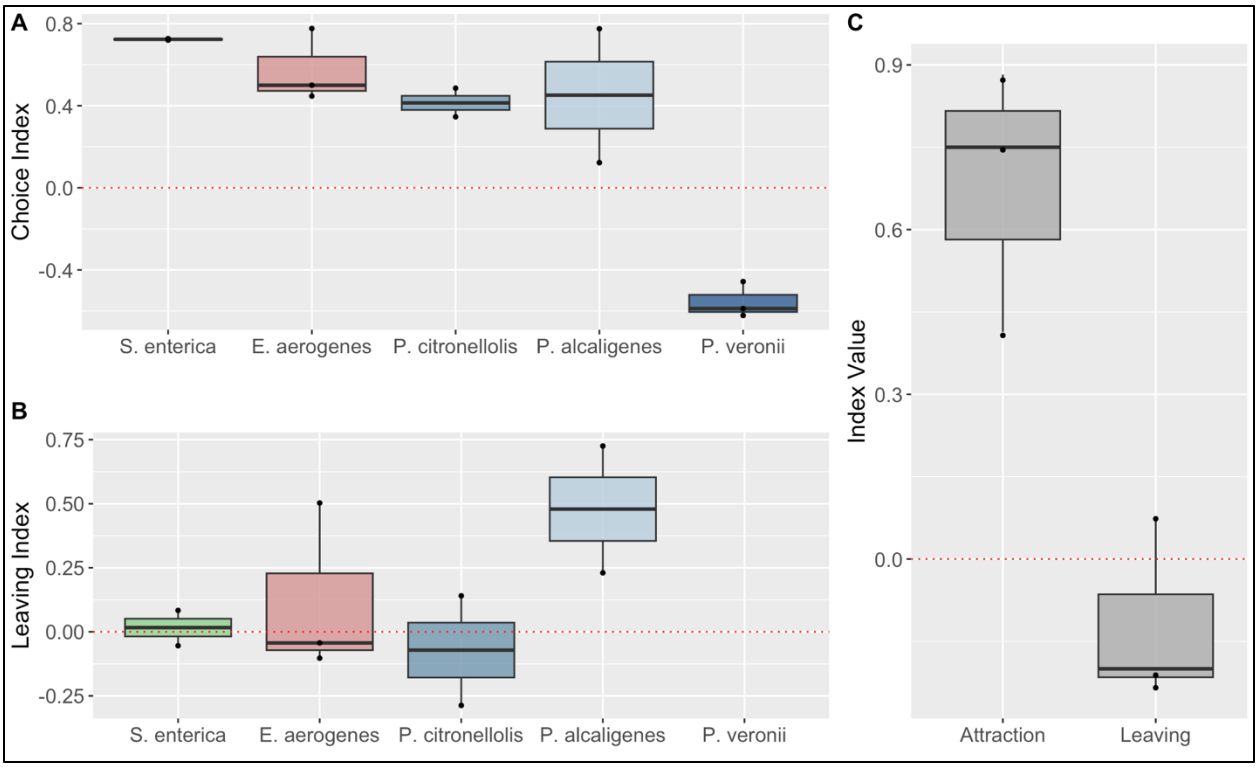

Figure 3. *C. elegans* choice behavior in "Close" canonical choice assay. All test strains were labeled with fluorescent markers as listed in Table 1. Three plates, each with ~50 worms, were analyzed per condition. (A) *C. elegans* attraction to test strains versus heat-killed OP50 control after 2h; higher attraction index values indicate a stronger preference for the test strain, and negative values indicate preference for the control. Attraction index values of 0 imply no preference for either strain. (B) *C. elegans* repulsion to test strains after 24h on test plates; higher leaving index values indicate stronger repulsion, and negative values indicate that worms are more likely to stay on bacterial spots over time. Leaving index values of 0 imply that worms are equally likely to leave or stay on spots. *P. veronii* is excluded from this analysis as there were no worms on or around *P. veronii* spots after 24 hours. (C) *C. elegans* attraction and leaving indices for live fluorescent OP50 in comparison to heat-killed OP50.

To determine if fluorescent or non-fluorescent strains of the same bacterial species could be interchanged reliably in future experiments, we performed a distant assay in which worms were given the choice between single-labeled strain pairs. We expected to see that fluorescence was not a factor in worms' decision-making, and that they would be attracted to and repelled from fluorescent and non-fluorescent strains to the same degree. We used the same strains of interest as before (*S. enterica, E. aerogenes, P. citronellolis, P. alcaligenes,* and *P. veronii*) with the addition of *P. chlororaphis*, a strain that was unattractive to worms in the initial distant assay performed. As expected, this assay showed no significant difference between the attraction or repulsion of each species' fluorescent and non-fluorescent strains (Figure 4), indicating that fluorescence is not a factor in *C. elegans* decision-making. Notably, worms presented with a choice between *P. veronii* miniTn7-GmR-YFP and unlabeled *P. veronii* preferred to avoid both strains; only 17.8% of the 107 worms on these plates were found on bacteria after 2 hours, thus preventing us from calculating choice or leaving indices for this strain.

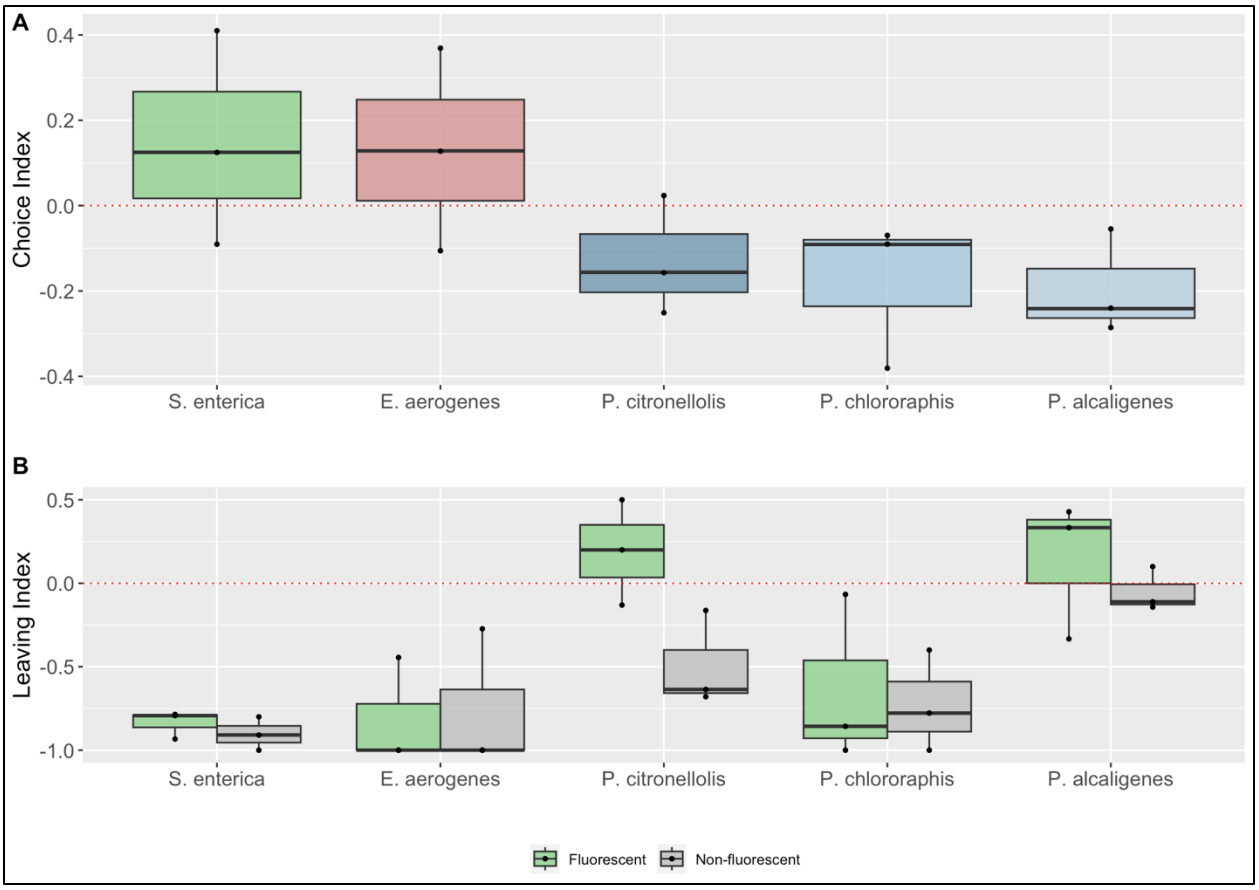

Figure 4. *C. elegans* choice behavior between fluorescent and non-fluorescent strains of the same species. A distant choice assay was performed with fluorescent strains labeled as listed in Table 1 versus unlabeled ancestral strains of the same species. Three plates, each with ~50 worms, were analyzed per condition. *P. veronii* was included in this experiment, but worms avoided these spots so consistently that no analysis could be performed for the species. (A) *C. elegans* attraction to fluorescent versus non-fluorescent strains of the same bacterial species after 2h; higher attraction index values indicate a stronger preference for the fluorescent strain, and negative values indicate preference for the non-fluorescent strain. Attraction index values of 0 imply no preference for either strain. One-sample Wilcoxon signed rank tests of each species found no significant difference between worms' attraction to the fluorescent strain and non-fluorescent strain (*S. enterica* p = 0.5, *E. aerogenes* p = 0.5, *P. citronellolis* p = 0.5, *P.*

chlororaphis p = 0.25, *P. alcaligenes p* = 0.25). (B) *C. elegans* repulsion to fluorescent and non-fluorescent strains after 24h on test plates; higher leaving index values indicate stronger repulsion, and negative values indicate that worms are more likely to stay on bacterial spots over time. Leaving index values of 0 imply that worms are equally likely to leave or stay on spots. Wilcoxon tests found no significant difference between worms' leaving behaviors within fluorescent/non-fluorescent pairs (*S. enterica* p = 0.4, *E. aerogenes* p = 1.0, *P. citronellolis* p = 0.1, *P. chlororaphis* p = 1.0, *P. alcaligenes p* = 0.7).

We then performed a distant assay with single-labeled strains of different bacterial species to gather additional data regarding worms' choices at our largest experimental scale, strengthen the previous assay's findings that fluorescence is not a factor in *C. elegans* decision-making, and begin analyzing potential single-labeled pairs for lattice preparation in the future. In this assay, *P. citronellolis* was used as the reference strain because our previous experiments consistently found that worms were attracted to and repulsed by *P. citronellolis* at similar rates to *E. coli* OP50, and *P. chlororaphis* was removed from testing as its results closely resembled those of *P. citronellolis*. This experiment yielded similar results to our initial assay with *E. coli* OP50 control, with minimal observed differences between fluorescent and non-fluorescent strains (Figure 5). As seen before, both *E. aerogenes* and *S. enterica* were attractive and not repulsive, *P. veronii* was attractive and later very repulsive, and *P. alcaligenes* was highly attractive; however, worms in this experiment did not leave *P. alcaligenes* spots as frequently as observed during the initial assay.

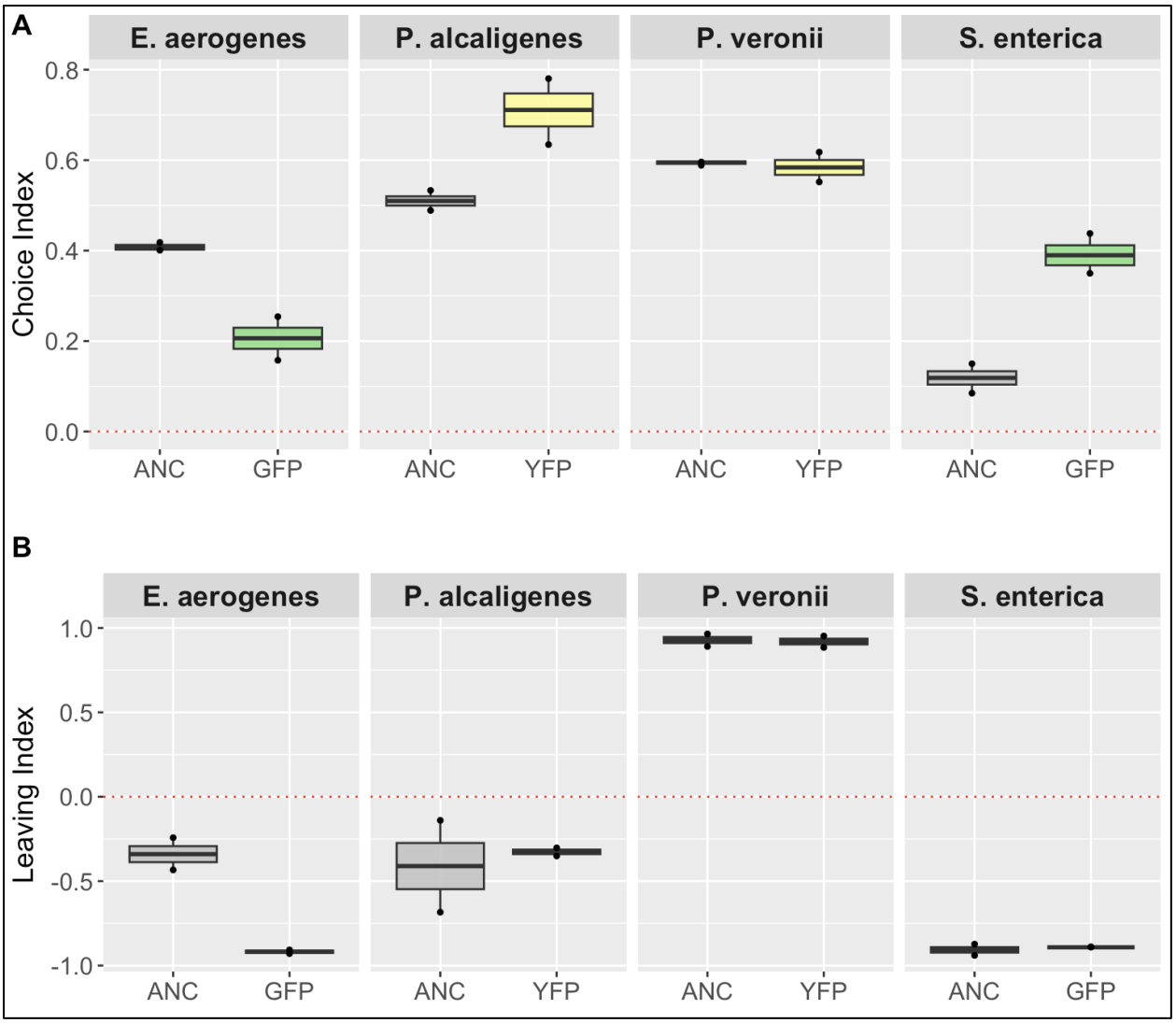

Figure 5. *C. elegans* choice behavior between single-labeled test and reference species.

Attraction at 2h (A) and leaving at 24h (B) of *C. elegans* given a labeled or unlabeled test strain (*E. aerogenes, P. alcaligenes, P. veronii,* or *S. enterica*) in combination with unlabeled or labeled *P. citronellolis* as a reference. Plates were arranged in distant assay format (*2.4: Traditional choice assays*). "ANC" = unlabeled test strain vs. *P. citronellolis* YFP. "GFP/YFP" = labeled test strain vs. unlabeled *P. citronellolis.* Wilcoxon tests found no significant differences between any strain pairs' attraction or leaving indices.

To determine if smaller scales affected the worms' decision-making in comparison to this distant assay, we performed a close assay with a decreased set of strain pairs using either unlabeled *P. alcaligenes, P. veronii,* or *S. enterica* as the test strain and *P. citronellolis* YFP as the reference strain (Figure 6). To streamline experimentation, *E. aerogenes* was removed from this assay (and future assays) due to its similarity to *S. enterica* results. These results reflected our expectations given the previous close assay (Figure 3). Once again, *P. alcaligenes* and *S. enterica* were significantly more attractive to worms than *P. veronii,* and worms were more likely to leave *P. alcaligenes* spots than *S. enterica* spots after 24 hours. Additionally, worms were again so repulsed by *P. veronii* that a leaving index for the strain could not be calculated as there were no worms on or around *P. veronii* spots after 24 hours.

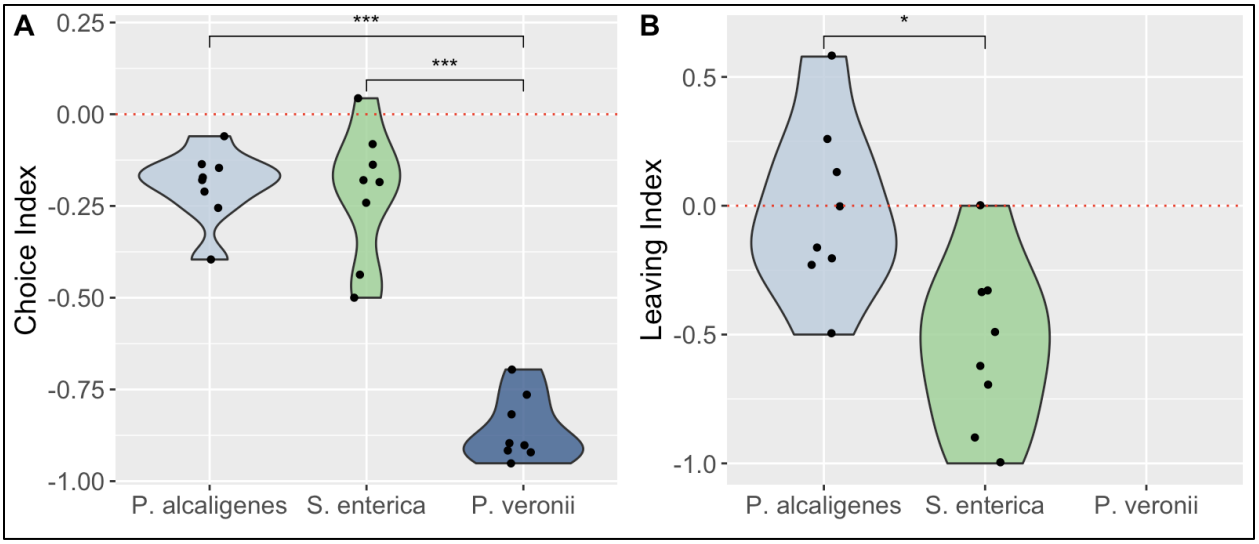

Figure 6. *C. elegans* choice behavior between labeled *P. citronellolis* and unlabeled test strains. Attraction at 2h (A) and leaving at 24h (B) of *C. elegans* given an unlabeled test strain (*P. alcaligenes* YFP, *S. enterica* GFP, or *P. veronii* YFP) in combination with unlabeled *P. citronellolis*. Plates were arranged in close assay format (*2.4: Traditional choice assays*). 8 plates per condition were analyzed, with an average of 46 worms per plate. A leaving index could not

be calculated for *P. veronii* as there were no worms on or around *P. veronii* spots after 24h. Significance levels reflect Wilcoxon test results.

3.2 Lattice preparation

To produce the most easily distinguishable images, we initially planned to create bacterial lattices by combining one GFP/YFP labeled strain with a different RFP labeled strain. Our lab already had glycerol stocks of GFP/YFP labeled strains (Table 1), but none of the species we intended to use were available with RFP tags. Thus, we attempted to insert dTomato plasmids into *P. alcaligenes, P. citronellolis*, and *P. mendocina* with conjugation (*2.5: Conjugation*) and electroporation (*2.6: Electroporation*). No fluorescent *P. citronellolis* or *P. mendocina* colonies were retrieved from either conjugation or electroporation attempt. No fluorescent *P. alcaligenes* colonies were retrieved from the electroporation attempt, but multiple bright red colonies appeared following conjugation. However, attempts to confirm the colonies were *P. alcaligenes* and not another species using PCR yielded inconclusive results. Considering the limited time available for the completion of this project, we made the decision to focus our efforts on performing further experiments regarding *C. elegans* choice behavior and pursue lattice formation with mixtures of one GFP/YFP strain and a different unlabeled strain. Though these mixtures may be slightly more difficult to distinguish, they still allow for accurate identification of strains in patchy environments under fluorescent lighting.

To prepare for future assays, we created and visualized various mixtures of labeled and unlabeled bacterial strains. In each mixture, unlabeled *P. citronellolis* diluted to a concentration of either 10^{-3}, 10^{-4}, or 10^{-5} was combined with a fluorescent test strain (*S. enterica*, *P. veronii,* or *P. alcaligenes*) diluted separately to a concentration of either 10^{-3}, 10^{-4}, or 10^{-5}. After 24h of

growth, the resulting lattices appeared differently under fluorescent lighting depending on which strains were mixed and how diluted the strains were (Figure 7). Dilutions above 10^{-3} did not yield big enough patches for our intended assays, and dilutions below 10^{-5} did not yield enough bacterial density. The ideal mixture for our choice assays was determined to be equal parts of each strain diluted to a concentration of 10^{-3}.

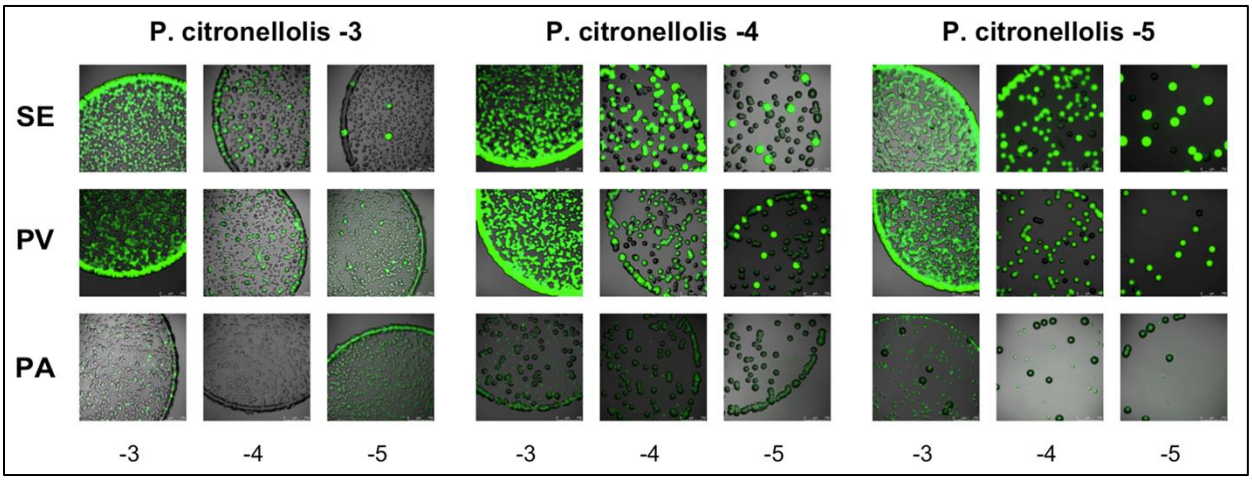

Figure 7. Single-labeled lattice microscopy. *Salmonella enterica* attB::GFP:KmR (SE), *Pseudomonas veronii* miniTn7-GmR-YFP (PV), and *Pseudomonas alcaligenes* miniTn7-GmR-YFP (PA) were combined with unlabeled *P. citronellolis* as outlined in Methods *(2.5: Mixture preparation)*. Different dilutions of test strains (SE, PV, PA: dilution factor on bottom x-axis) were combined with different dilutions of *P. citronellolis* (top x-axis). Images were captured with a Leica DMi8 inverted microscope fitted with GFP lighting.

3.3 C. elegans *strain preferences under complex conditions*

To begin gathering data on smaller scales, we performed a distant assay where worms were given the choice between a single-labeled, two-strain 1:1 mixture and *E. coli* OP50. By putting test strains into mixtures with *P. citronellolis*, some noticeable differences from what we

expected emerged (Figure 8). *P. veronii*, which had previously been extremely repulsive and often unattractive, was extremely appealing to worms when presented as a mixture. *P. alcaligenes* and *S. enterica* remained attractive, but the repulsiveness expected of *P. alcaligenes* was not observed at the 24-hour timepoint.

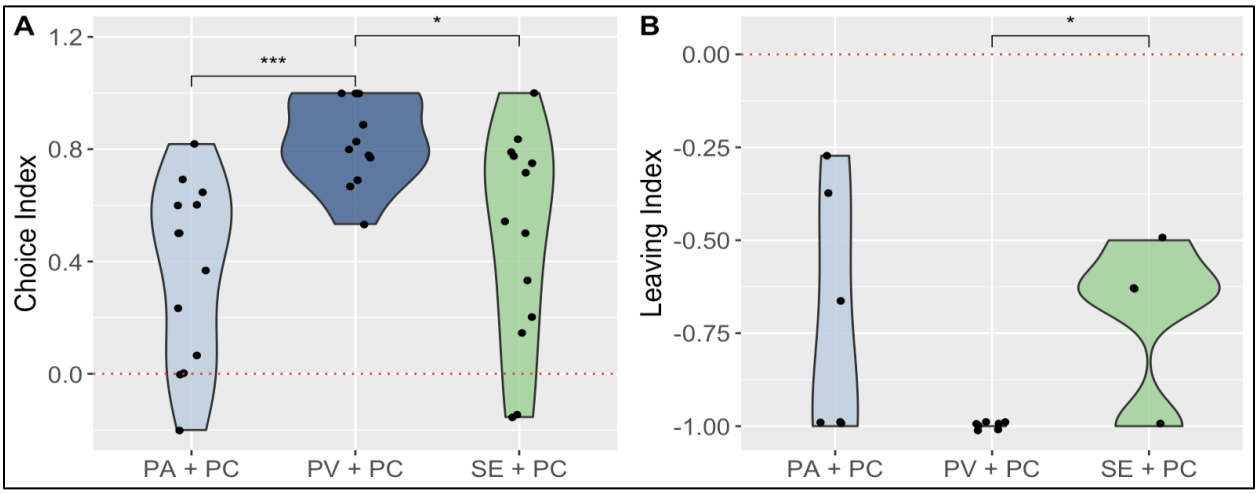

Figure 8. *C. elegans* choice behavior between single-labeled bacterial mixtures and *E. coli* OP50. Attraction at 2h (A) and leaving at 24h (B) of *C. elegans* given the choice between equal densities of a bacterial mixture (PA + PC: *P. alcaligenes* YFP & *P. citronellolis*, PV + PC = *P. veronii* YFP + *P. citronellolis*, SE + PC = *S. enterica* GFP + *P. citronellolis*) and *E. coli* OP50. All strains were diluted to a concentration of 10^{-3}. Plates were arranged in distant assay format (*2.4: Traditional choice assays*). 13 plates per condition were analyzed, with an average of 10 worms per plate. Significance levels reflect Wilcoxon test results.

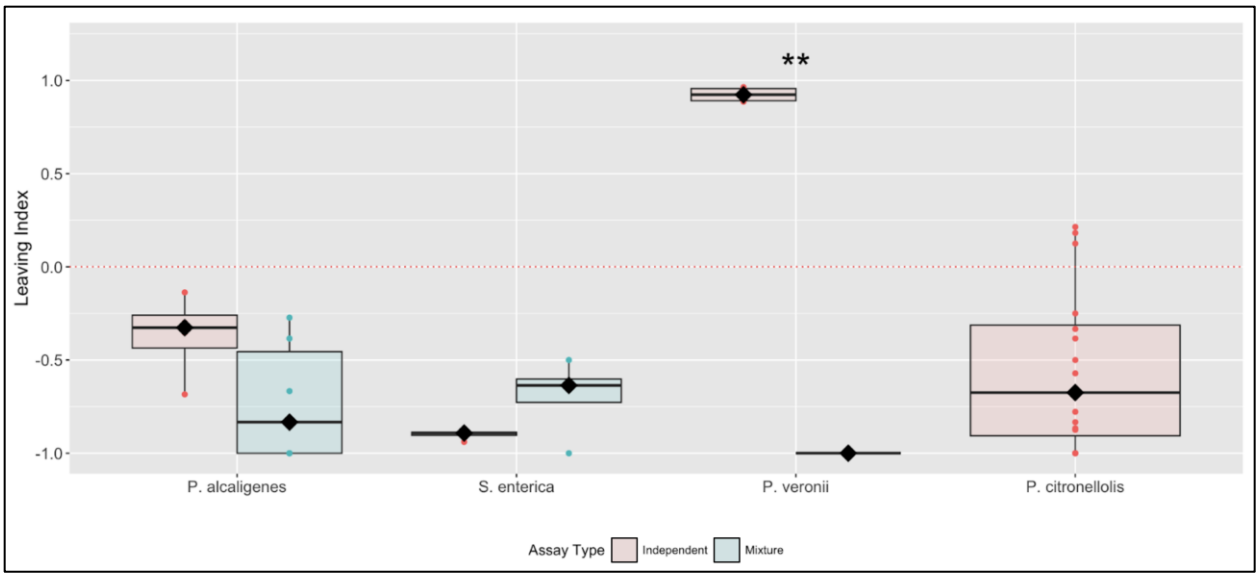

Figure 9. *C. elegans* repulsion to *P. veronii* is significantly different when species is presented in a mixture. *C. elegans* leaving indices to *P. alcaligenes*, *S. enterica*, or *P. veronii* when the species is presented independently (red) or in a 1:1 mixture with *P. citronellolis* (blue) at a distant scale after 24h. *P. citronellolis* leaving indices were determined from a previous distant assay where *P. citronellolis* was used as a control strain. Significance levels reflect Wilcoxon test results.

To understand if a similar pattern could be detected on a larger bacterial scale, we performed a "Compact" assay pairing labeled *P. alcaligenes,* labeled *P. veronii*, or labeled *S. enterica* with unlabeled *P. citronellolis.* The results of this experiment yielded similar results to previous close assays in which *P. alcaligenes* and *S. enterica* were comparably attractive to *P. citronellolis* while *P. veronii* was much less attractive than *P. citronellolis* (Figure 9). Notably, the attractiveness of both *P. veronii* and *S. enterica* was significantly higher in this compact assay than in the previous close assay comparing these strains with *P. citronellolis*, and all strains' attractiveness at this compact scale was significantly lower than their attractiveness at the distant scale (Figure 10). Considering the compact nature of how bacteria were arranged on these

plates, we also measured the number of worms found between two bacterial spots and found that worms appeared to make less decisive choices when offered *P. alcaligenes* or *S. enterica* than when they are offered *P. veronii* (Figure 9); notably, in our other assay where bacterial spots were placed directly next to each other, worms were significantly less likely to be found between two spots of different species (Figure 11).

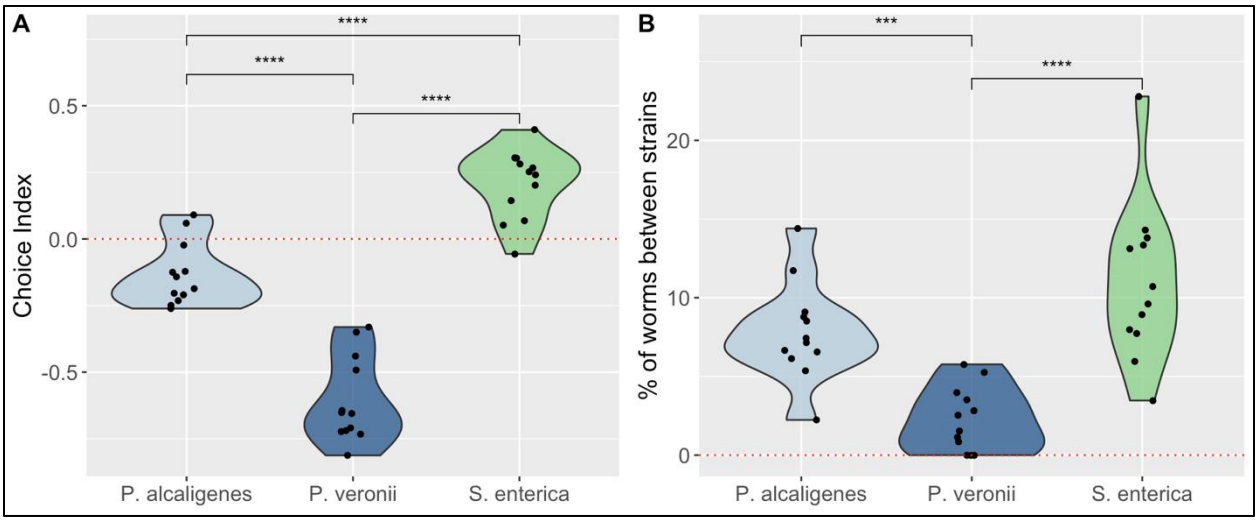

Figure 10. *C. elegans* choice behavior in "Compact" assay setup. (A) Attraction at 1.5h of *C. elegans* to *P. alcaligenes* YFP, *P. veronii Y*FP, and *S. enterica* GFP when paired with unlabeled *P. citronellolis*. (B) Percentage of all worms on plate that were located between two bacterial spots of different species when counted instead of being decisively within one strain's spot or on no bacterial spots. Plates were arranged in compact assay format (*2.4: Traditional choice assays*). 12 plates per condition were analyzed, with a median of 125.5 worms per plate.

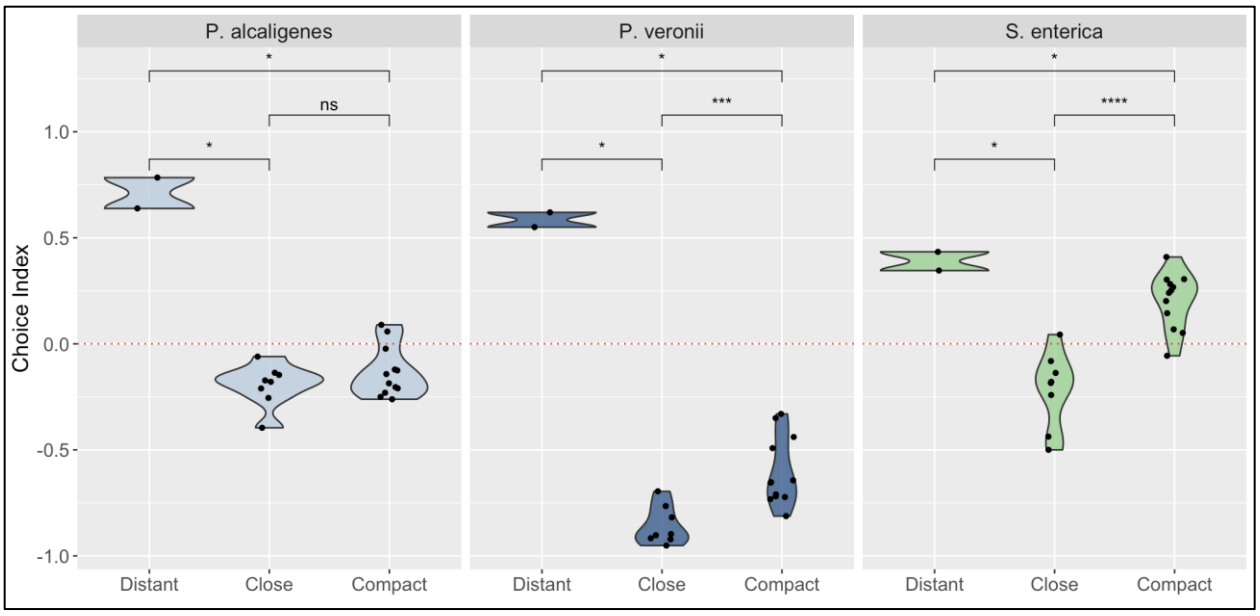

Figure 11. *C. elegans* attraction varies based on spatial scale. Attraction at distant, close, and compact spatial scales to *P. alcaligenes* YFP, *P. veronii* YFP, and *S. enterica* GFP when paired with unlabeled *P. citronellolis* after 2 hours. Significance levels reflect Wilcoxon test results.

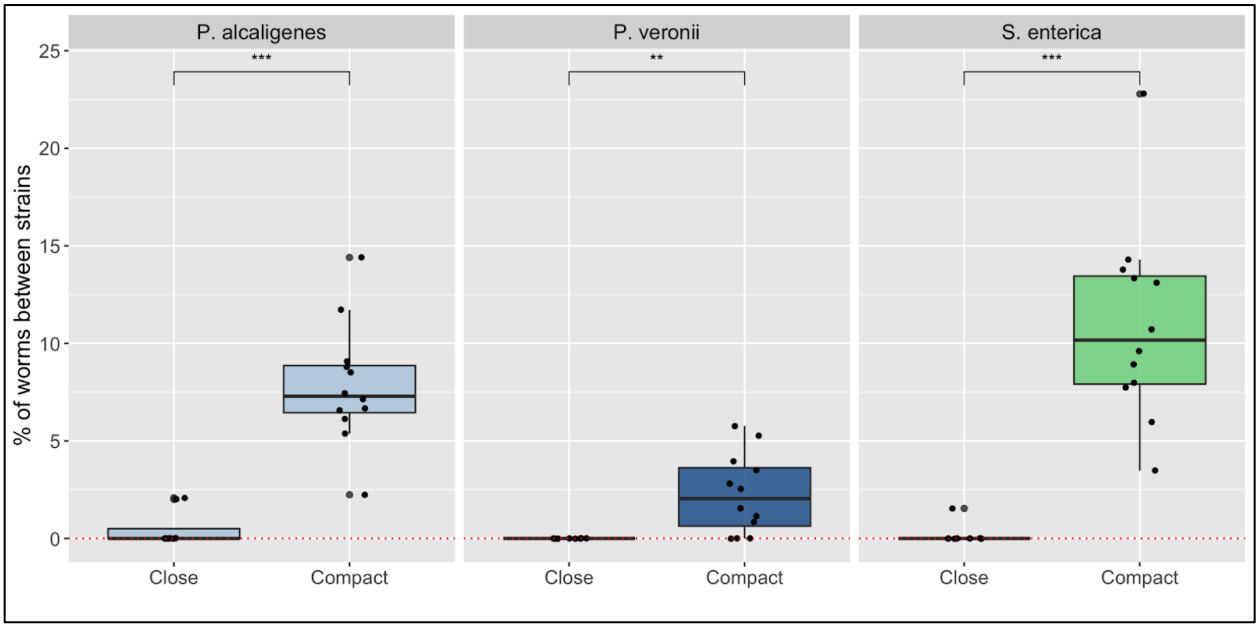

Figure 12. *C. elegans* are found between strains more frequently when in more compact bacterial environments. % of total worms on plate found between test and reference spots at 2h

(close assay) and 1.5h (compact assay). Test spots were either *P. alcaligenes* YFP, *P. veronii* YFP, and *S. enterica* GFP. Reference spots were unlabeled *P. citronellolis.* Significance levels reflect Wilcoxon test results.

4. Discussion

4.1 C. elegans *make distinct feeding and leaving choices based on bacterial species*

Our initial experiments yielded results in agreement with the literature, finding that *C. elegans* make distinct and reproducible choices when provided the choice between different bacterial strains. We found that strains could either be initially preferred (having a positive choice index) or non-preferred (having a negative choice index) when paired with a reference strain, and that they could later become repulsive (having a positive leaving index) or non-repulsive (having a negative leaving index) after 24 hours.

We focused on four bacterial species: *Pseudomonas alcaligenes*, *Pseudomonas citronellolis, Pseudomonas veronii*, and *Salmonella enterica*. Worms showed unique behaviors towards each species. When given the choice between *E. coli* OP50 (our initial reference strain) and *P. citronellolis* in a traditional distant assay layout, worms showed no strong preference for either species, thus prompting us to make *P. citronellolis* our reference strain for future experiments (Figure 2). We also found that worms did not show any significant preference between fluorescently labeled and unlabeled strains of the same species at this scale (Figure 4), opening the door to the use of labeled strains for mixture preparation in future experiments.

C. elegans behavior towards each species was similar in all distant assays we performed. *P. alcaligenes* was initially highly preferable to worms but later repulsed them; *S. enterica*, which was also highly preferable, did not repulse worms at 24h. *P. veronii* was attractive to worms at this scale, but very repulsive. These patterns can be seen when using *E. coli* OP50 as a

control (Figure 2) or when using *P. citronellolis* as a reference (Figure 5), the only exception being that when paired with *P. citronellolis*, *P. alcaligenes* appears to become less repellent.

Similarly, observed behavior was similar across close assays, which decreased the distance between bacterial species. *P. alcaligenes* and *S. enterica* were similarly preferable to each other; both strains were more attractive to worms than heat-killed OP50 (Figure 3) but slightly less attractive than *P. citronellolis* (Figure 6). In both assays, *P. veronii* was extremely unattractive.

4.2 C. elegans *behavior is altered in different spatial environments*

Although the behaviors exhibited by *C. elegans* are consistent when choosing between bacterial species at the same spatial scale, performing assays at different scales appears to prompt different responses to the bacterial species offered (Table 2). In distant assays, where 10µL bacterial spots were positioned with 2.5cm empty space between species (Figure 1), worms were more attracted to *P. alcaligenes, P. veronii,* and *S. enterica* than they were to the reference strains. However, when condensing the scale to the close assay layout where 5µL bacterial spots of alternating strains were positioned directly next to each other, worms treated the test strains differently. *P. alcaligenes* and *S. enterica* were slightly less appealing than *P. citronellolis* at this scale, which did not reflect the results of the less compact assays performed earlier where *P. alcaligenes* and *S. enterica* were more appealing than *P. citronellolis*. Additionally, in the close assay layout, worms treated *P. veronii* spaces much differently; rather than being initially attracted and later repulsed, they were highly avoidant at the 2-hour timepoint. When performing our compact assay – the layout with the smallest patches at which we could still differentiate bacterial species with a dissection scope - *C. elegans* behaviors resembled those seen in the previous (close) assay; once again, worms were similarly attracted to *P. alcaligenes* and *S.*

enterica as they were to the reference strain and chose to avoid *P.* veronii. Notably, in this compact setup, worms were slightly more attracted to each test strain – and significantly so in the case of *P. veronii* and *S. enterica* (Figure 10).

	DISTANT (vs *P. citronellolis*)		**CLOSE** (vs *P. citronellolis*)		**COMPACT** (vs *P. citronellolis*)	
Species	**Attraction**	**Repulsion**	**Attraction**	**Repulsion**	**Attraction**	**Repulsion**
P. alcaligenes	++	-	~	~	~	N/D
P. veronii	++	+++	---	N/A	--	N/D
S. enterica	+	---	-	--	+	N/D

Table 2. *C. elegans* foraging behavior varies based on bacterial strain and spatial scale of choice. Attraction (choice index) and repulsion (leaving index) of *P. alcaligenes*, *P. veronii*, and *S. enterica* as test strains paired against *P. citronellolis*. (+, ++, +++): median choice or leaving index above 0.2, 0.4, or 0.6. (-, --, ---): median choice or leaving index below 0.2, 0.4, or 0.6. (~): median choice or attraction index within ± 0.2 of 0. (N/A): no index able to be calculated. (N/D): data not collected due to experimental constraints.

4.2.1 Pseudomonas veronii *case study*

Throughout our experimentation, we became interested in the worms' behavior towards *P. veronii.* This bacterial strain appeared to have the most notable variations in attraction and repulsion towards worms when presented at different scales. At our largest scale of experimentation (spots 2.5cm apart), *P. veronii* attracted worms initially but later repulsed them. When placing bacterial spots directly next to each other as in the close and compact assays, worms were not attracted to *P. veronii* at all. Though our earliest timepoint measured in these

experiments was 1 hour, it was clear while viewing the plates that worms typically did not enter *P. veronii* spots at any point during this period. When *C. elegans* enter and then leave bacterial spots, they create a trail in the agar following their movement. While counting the number of worms present in each spot at the 1-hour timepoint, we noticed that *P. veronii* spaces with 0 worms appeared untouched, implying that rather than entering and then leaving these spaces, worms were completely avoiding them – an entirely different behavior than seen in the more spaced-out assay, where worms entered the spaces and left them afterwards.

Additionally, we noticed unexpected behaviors when presenting worms the choice between a 1:1 mixture of *P. citronellolis* and *P. veronii* or *E. coli* OP50. In a "distant" assay - our most separated layout, where pure *P. veronii* was seen to attract and later repulse worms – the mixture of *P. citronellolis* and *P. veronii* was attractive to worms at first and *did not* repulse them after 24 hours. In fact, the *P. veronii* mixture was more consistently attractive than the *P. alcaligenes* or *S. enterica* mixtures, and worms were extremely likely to remain on the mixture after 24 hours rather than leave. This leaving behavior contradicts the behavior observed in the same assay layout with pure *P. veronii* instead of the mixture, where worms were highly repulsed from *P. veronii* spots after 24 hours (Figure 9).

The difference in behavior observed when changing scales prompts the question of whether *C. elegans* are making the same decisions more rapidly when their choices are presented closer together. When looking at *P. veronii*, it is evident that worms do not prefer it after a while, regardless of what assay type is being performed. This is especially evident when reviewing the results of our distant assay where we sought to determine if worms preferred fluorescent or non-fluorescent strains of the same bacterial species; when given the choice between labeled and unlabeled *P. veronii,* worms chose to avoid both strains, instead remaining on agar without any

food source. However, during choice assays where worms were given another food source (either *E. coli* OP50 or *P.* citronellolis), they traveled towards *P. veronii* when it is 2.5cm away and stayed there for a while before leaving – but when it is 1.5cm or less away, worms avoided it immediately.

4.3 Study limitations and future directions

The primary limitation for this project was a lack of time. Only eight months (August 2024 – March 2025) were available to perform experiments, thus reducing the amount of data we could collect. Much of this time was spent gathering preliminary data, such as initial choice assays which were used to choose bacterial strains of interest or more in-depth assays with these strains. Because of the time required to grow and age-synchronize *C. elegans*, assays could only be performed approximately every two weeks at best, and experiments did not always go to plan – occasionally, issues with low worm yields or equipment failures would render an experiment's data unusable. Therefore, although we have gathered a large amount of baseline data, there was not enough time available to thoroughly investigate *C. elegans* choice when provided the bacterial mixtures we were able to produce. Further time to iron out the details of some newer protocols (such as *2.8: Mixture attraction assays*) would have allowed for better results; for example, issues with the microscope focus made many images during the mixture attraction assay too blurry to interpret, which could be easily fixed by pouring a more precise volume of agar into each well.

Another effect of this limitation is the lack of RFP bacteria in our mixtures. Though we attempted to insert dTomato into *P. alcaligenes, P. citronellolis* and *P. mendocina* using conjugation and electroporation, neither method was successful. Approximately a month of experimentation was spent trying to successfully perform this insertion, but aside from the

discovery of a highly fluorescent unknown species, there was no success. Because of this, we chose to create mixtures using one labeled (GFP/YFP) and one unlabeled species to preserve time for choice assays. Leica imaging software allows for two channels of an image to be combined – thus, we were able to overlay bright-field images (showing the entire mixture spot) with GFP images (showing only the GFP strain), so distinguishing between strains was possible though not as ideal as a GFP + RFP pair.

Improvements on the methods and experiments performed as part of this project should prioritize (1) improved image quality for experiments measuring *C. elegans* choice within bacterial mixtures, (2) higher consistency of worm quantities placed on choice assay plates between experiments through more experience with synchronization protocols, and (3) the use of GFP/YFP + RFP species pairs in bacterial mixtures rather than GFP/YFP + unlabeled species pairs. Additionally, the results of our experiments raise many questions for further research beyond the initial scope of this project. Though we have generated evidence supporting the hypothesis that *C. elegans* make quicker decisions at smaller spatial scales, we do not yet understand the exact dimensions at which these decisions are altered. We saw that worms behaved differently towards *P. veronii* in 10µL spots (approximately 6mm in diameter) and 5µL spots (approximately 4mm in diameter) containing that species alone than in spots consisting of a 1:1 mixture with another species in which strain patches were approximately 150µm in diameter. However, it is unknown whether the same behavior would be seen in mixtures where patches are any larger or smaller. Therefore, further experimentation to better understand the degrees at which *C. elegans* adjust their behaviors when choosing between bacterial spots is warranted.

Additionally, further research could focus on the causes of the behaviors we observed. It is possible that an interaction between *P. veronii* and *P. citronellolis* makes *P. veronii* less

repellent to *C. elegans* when presented in this mixture, and uncovering the mechanisms behind the worms' behavior when given this choice would be intriguing. Once our understanding of the scales at which *C. elegans* make decisions is better understood, further variables could be introduced into the choices they are offered – for example, increasing the number of bacterial species in each mixture, pairing different mixtures against each other, using human pathogens found in the environment, or combining bacterial species that are known to interact either mutually or competitively. It would also be interesting to perform assays like those performed in this project, but rather than counting worms at predetermined timepoints, utilize video recording technology to gather a continuous picture of when worms make their choices about moving towards – and possibly leaving – food sources.

4.4 Conclusion

The results of this project support our existing understanding that *C. elegans* can identify and consume 'more attractive' bacterial species when presented choices at a distance, as well as deciding to leave after spending time in a food source they determine to be inadequate. In addition, we have found that the scale at which these decisions are presented affects the worms' behaviors with regard to entering and leaving bacterial spots. Analysis of our findings prompts the hypothesis that, when food is presented at a smaller scale, *C. elegans* more quickly identify the properties of each bacterial species and make more immediate decisions in contrast to their typically understood behaviors, where they are more likely to make decisions and then revert them (by entering and later leaving food sources). This project also presents multiple opportunities for further research into the specific scales at which these decisions are altered with examples of experiments that can be performed to better understand the detailed effects of space on *C. elegans* decision-making. In summary, the results of this project present strong

foundational data and many opportunities for expansion into further investigation, thus opening the door for an improved understanding of how spatial scales affect animal behavior – a topic affecting disease spread, microbial community ecology, and the field of biology as a whole.

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