Microbiome Modeling

A Major Qualifying Project submitted to the Faculty of
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In partial fulfillment of requirements for the degree of Bachelor of Science in Bioinformatics and Computational Biology

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Abstract

The intestinal microbiome is a complex microbial ecosystem that affects the health of the host organism. We used both laboratory experiments and computational modeling to study the interactions of beneficial and pathogenic microbes within the intestine of the model organism *C. elegans.* We first observed the movement of fluorescently labeled pathogenic yeast through the worm intestine and measured the transit time. Results showed that the yeast took about 16 minutes on average to travel through the worm, thus altering the performance of an existing agent-based computer simulation. Due to this the model was split into three separate phases of infection, and it was determined that an extra model was to be created to represent the anterior on the intestine. Consistent with our data and the literature, the model predicts fewer pathogenic yeast colonize the worm intestine in the presence of beneficial microbes. Future work to calibrate model output with experimental data will lead to better understanding of microbial interactions and their effects on host phenotype.

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1. Introduction

1.1 Significance

The intestinal microbiome is a complex ecosystem of cells whose interactions are not well understood. The gut is a rich ecosystem that is home to a variety of microbes that interact not only with each other, but also the host. Due to this, there have been possible connections made between the microbial interactions in the gut microbiome and overall human health (Frykman et al. 2015). Thus, it is very important that more research is done into how the microbiome works and what organisms have an effect in order to understand these connections. The microbiome itself is defined as a concentration of microbes such as bacteria within a concentration of biofluid or tissue which can act to promote growth and build resistance to stress-inducing conditions and immune compromission (Zhang, et al., 2017). Researching the microbiome and understanding its influence in the host organism could be later used in the research of other immune and nutritional dysfunctions.

In this project we inspected the intestinal microbiome of the model genetic organism *C.elegans* and examined the effects of pathogenic yeast within the gut. All three organisms used on this project, the worms, yeast, and bacteria, are model organisms. Because there have been many studies and experiments done on these organisms, the knowledge of tools and mutants can be advantageous in an experimental setting in which the understanding of a relatively simple microbiome is being achieved. We used a combination of laboratory experimentation and computational modeling in order to reveal more information about the balance of organisms in the microbiome, the effect on functionality, and observe the host-pathogen interactions on the cellular level. By doing so, we were able to understand more about intestinal microbiomes by observing this interaction between the yeast, bacteria, and the lumen in the intestinal tract of the worm. The main factor for a compromised system was the introduction of *C. albicans*, a yeast species considered pathogenic to the intestinal microbiome of *C. elegans*. This greater understanding of the intestinal microbiome of C. *elegans* will help in the overall process of understanding the microbiome within humans and other more complex organisms.

In the second part of this project, we modified an agent-based modeling program that was started by previous students. Having the model agent-based is useful as the individual microenvironment experienced by cells in the microbiome can be inspected and altered with the goal of understanding how the microbes affect the entire system down to the individual cell to

cell interactions. The overall design of the model gave us a computer-simulated representation of the intestinal microbiome of *C. elegans* so that we could accurately show the results of a change in the balance of that system. The interactions in this model can show how probiotics and pathogenic microbes interact within the intestine to affect each other and the host. The model has been made to simulate these interactions, as well as yeast that colonizes the intestine through biofilm coating, which inevitably results in the overall expansion of the intestine. With the model and the completed laboratory experiments, the results produced by the model allow for a more accurate representation of the biology seen in *C. elegans.* This model iteration will then contribute to the improvement cycle between lab and computational work to give a further understanding of the biological systems.

1.2 Microbiome

1.2.1 C. *elegans* - the host

Caenorhabditis elegans is a species of roundworm that has been growing in popularity for use as a model organism in research settings. Starting in 1965 with Sydney Brenner, scientists have used *C. elegans* as a model organism to study development and behavior in animals (Frézal & Félix, 2015). *C. elegans* has been used as a model organism due to many factors, including their small size of 1.5 millimeters long, which allows for ease of observation in the laboratory. Their rapid life cycle and generation time due to self-fertilization (Altun et al., 2021) is utilized with the ease of cultivation that they have, and they provide the opportunity to look at multiple generations within a single experiment throughout a couple of months as opposed to a couple of years. *C. elegans* has a long-term cryopreservation period increasing the ability to store and transport the worms if needed. Their transparency, low maintenance expense, and ability to reduce gene activity also give rise to more straightforward methods of monitoring during experiments (Frézal & Félix, 2015).

C. elegans has been modified and domesticated to maintain any mutations that may affect the worms' growth, reproductive ability, behavior, pathogen resistance, or their adaptability to the agar environment (Frézal & Félix, 2015). They are an excellent model due to their genetic tractability. Genetic tractability refers to the ability to manipulate genes to create mutants or control gene expression in the organism (Johnston et al., 2019). This has allowed for the use of the organism to observe and monitor host-pathogen interactions. Through experiments, it has

been found that *C. elegans* have obtained mechanisms to interact with different microorganisms through evolution and exposure (Gerbaba, et al., 2017). The manipulation of genetic information has also contributed to the foundation of comparative genomics (Altun et al., 2021). The manipulations done to *C. elegans* were used in the studies of transcription, translation, and other modifications.

The focus of this project is looking at the microbiome in the intestinal tract of *C. elegans*. It is essential to understand that the microbiome of the digestive system of any organism is a significant contributor to the extraction of nutrients, stabilizing and maintaining the immune system by response or protection, and metabolism regulation (Gerbaba et al., 2017). The microbiome of *C. elegans* has been used for microbiome research, which demonstrated that certain bacteria, such as *Acetobacteraceae,* have a positive effect on the worms, including the enhancement of their growth and the resistance to environmental stressors (Zhang. et al., 2017). The balance of microorganisms within the microbiome dramatically affects the health and survival success of the organism. An imbalance or disproportionate amount of even one participant could cause a decline in function and possible death of the host organism.

C. elegans is an ideal species to use for this particular project. The worms are simple and easy to work with when compared to the more complicated biology of a human or other complex organism (Zhang, et al., 2017). Due to their transparency, it is easier to monitor the progress of the yeast throughout their intestinal tract. When fed a fluorescently labeled yeast, it is possible to use a microscope equipped with epifluorescence to very clearly see the progress the yeast has made through the intestine of *C. elegans. C. elegans* also have an intestinal anatomy similar to that of mammals, due to this it is easy to extend the research done in *C. elegans* to larger mammals. Other similarities between mammals and *C. elegans* include the microbial genes that are necessary for virulence in mammals and pathogenicity in the nematodes. Lastly, innate immunity is evolutionarily conserved between nematodes and mammals (Lundquist). These similarities make the *C. elegans* a strong choice in model organism for this project and this line of investigation and research.

1.2.2 *Candida albicans* - the pathogenic microbe

C. *albicans* is a specific species of yeast that can be found within the intestinal microbiome. While it is possible for *C. albicans* to be a normal part of the microbiome, it is also

possible for the yeast to be pathogenic to the intestine. When pathogenic, *C. albicans* are converted to a filamentous form. This morphological switch can be initiated by contact with epithelial walls, or just happen over time. (Zakikhany et al., 2007). This further increases the probability of adhesion, and the formation of the biofilm-producing form of the yeast. Biofilm production involves the secretion of extracellular matrix molecules that results in an adhesive mixture of microbes and molecules. In *C. elegans* infected with *C. albicans*, the yeast accumulates in the intestine, which becomes blocked and leads to the death of the worm.

Some yeast species, such as *C. laurentii* and *C. kuetzingii*, are a source of food for *C. elegans*. There are also probiotic yeast that can inhibit pathogens within *C. elegans*. These probiotic yeast include *Saccharomyces cerevisiae* (KTP) and *Issatchenkia occi-dentalis* (ApC), which can prevent the biofilm formation and adhesion of other yeast strains (Zakikhany et al., 2007).

1.2.3 *B. subtilis* - the beneficial bacteria

Bacteria such as *B. subtilis* have been shown to counteract or inhibit the virulent effect of the yeast (Johnston et al., 2019). The bacteria are suspected of secreting small molecules that prevent the yeast from assuming the filamentous form and compromising the intestinal wall. Filamentation is one of the main factors in the adhesion of the yeast within the intestine.

1.2.4 Modeling

This project will be using an Agent-Based Modeling Simulation to study the interactions between bacteria, yeast, and the *C. elegans* host. Agent-Based Modeling and Simulation (ABM) combines autonomous interacting agents with adaptive learning and decision-making based on the environment to create simulations of real-life situations. ABM has many different applications, such as business, economics, infrastructure, and biology (Macal & North, 2005). The three main aspects of ABM are agents, environment, and conditions. The first aspect, agents, are entities within the simulation that are identifiable, discrete, interact with and recognize traits of other agents, and can make independent choices based on the environment (Macal & North, 2005). These agents can move around and interact with the environment to create a dynamic model (Palsson, 2011). ABMs also have pre-set conditions that can be added to the simulation. These allow for the starting conditions to be changed for the environment or the agents within it, resulting in the ability to simulate and study the different behaviors in the model based on

different starting conditions. Emergent behavior is when coherent, discernable patterns are found based on the interactions of individual agents in the model that then have significance to the researcher. This then helps the researcher draw new conclusions based on the emergent behavior (Chan, Son & Macal, 2010). For example, schooling of fish and flocking of birds can be modeled as emergent behavior that arises from individual fish and birds following simple rules.

The specific platform that will be used to create the simulation in this project is NetLogo. NetLogo is a multi-agent programming language that allows for sophisticated, robust models to be created (Tisue & Wilensky, 2004). This platform is based on the previous ABM platforms, StarLisp, and Logo yet NetLogo has altered aspects to create a more enhanced, easy-to-use version (Tisue & Wilensky, 2004). NetLogo uses a specific type of environment known as individually programmable "patches". These patches can change based on pre-set conditions and interactions with agents (Palsson, 2011). Another tool offered within the NetLogo model is BehaviorSpace, a function that allows the user to input parameters, such as programmed variables, repetitions, and combinations of conditions. BehaviorSpace will then perform systematic tests on the model, following the user-inputted settings. Another advantage of using NetLogo as the simulation platform for this project is that it is written in and built from Java, allowing for consistency across all computers and computing platforms (Tisue & Wilensky, 2004). Finally, NetLogo provides for the easy importing and exporting of data in standard formats, as well as allowing the contents of the graphics window to be saved as an image so data can be easily shared (Tisue & Wilensky, 2004). NetLogo is known to have powerful computational abilities and efficiency (Weston et al., 2015), making it an appropriate choice for the modeling and simulations of this project.

The interaction between lab work and simulation modeling is vital to understand the data fully as well as creating a useful, robust model. Models are built off the information and data found in the lab, therefore when the model is run it allows for new relationships to be discovered between the various components. This, in turn, inspires new experiments within the laboratory setting (Palsson, 2011). If models do not match the scientific data, then the scientist can effectively deduce that something is wrong either in the logic, the model, or the experiment. From there, scientists can then alter aspects of the project to correctly find what needs to be different (Palsson, 2011). Repeatedly going through this cycle of lab work, modeling, and altering will result in the increased confidence that the model is correctly predicting organisms'

behaviors, and the lab work is being done correctly and thoroughly. This process has also been defined as the simulation cycle, a five-step cycle that defines the relationship between modeling and lab work. The first step of the cycle is designing the model based on existing data. The second step is implementing the model. The third step is altering the model based on differences in the model's predictions and the experimental data. The fourth step is further revising the model based on new data or theories. The fifth step is then repeating this cycle (Wilensky & Rand, 2015).

1.3 Analysis of Relevant Past Research

1.3.1 Computational Modeling

In the past, research has been done to study and model the microbiome of various organisms. In one study, a toolbox was created to help model microbial interactions within the microbial communities as well as differences between microbial communities (Baldini et al. 2019). They titled their new toolbox the "Microbial Modeling Toolbox," which is based on the constraint-based reconstruction and analysis (COBRA) toolbox in Matlab. COBRA was limited in that it only allowed for the modeling of single organisms or cells, and the investigators wished to model larger-scale interactions (Baldini et al. 2019). The difference between this study and the goal of our project is their focus on metabolic reactions within the microbiome, whereas we will be focusing on the effect of microbial interactions on biofilm buildup within the intestine.

In another study on modeling the microbiome, investigators took a mathematical approach to analyze and model the interactions between the gut bacteria within the fruit fly (Jones & Carlson, 2018). They concluded that there is a connection between the presence of bacteria in the microbiome and the longevity of the organism, with some bacteria increasing fitness and other bacteria decreasing fitness (Jones & Carlson, 2018). The findings of this study are essential for this project as it will also be looking at bacterial interactions in the microbiome and their effects on the longevity of C. *elegans.* Where the study differs from the needs of this project is in the use of a mathematical model to analyze their data, as opposed to our project which utilizes an agent-based simulation environment, which allows the inclusion of individual cell-cell interactions to better understand the mechanisms increasing longevity.

In another study using a Netlogo model of the microbiome, the research hypothesis was that the gut microbial environment can impact a child's risk of developing autism. The

investigators created a model within NetLogo that looked at three different bacteria: *clostridia, desulfovibrio*, and *bifidobacteria*. The researchers then altered the physiological conditions in the model to study how the different dynamics altered the microbiome and the bacteria's effects on developing autism (Weston et al., 2015). Using the ability of Netlogo to easily test many environmental conditions, the researchers tested the effects of initial bacteria levels, reproduction rates, carbohydrate replenish rates, and the presence or absence of lysozymes (Weston et al., 2015). They concluded the gut microbiome is significant to various aspects of overall health including the development of autism in young children, thus it is important to gain a greater understanding of its functions.

1.3.2 Experimental setup

C. elegans has proved to be a useful host to study infection of the yeast *C. albicans* (Elkabti, et al., 2018); thus, *C. elegans* was used in this study as the model in the laboratory settings, as well as the system that will be modeled in NetLogo. C. *elegans* benefits relevant to the current project include the excellent visibility into its organs as well as having the same intestinal innate immune system that is found in many more complex species (Elkabti et al.,, 2018). The visibility into the organs of *C. elegans* is due to the transparency of the nematode, allowing for live imaging of the host-pathogen interactions within the worm. Fluorescently labeled C. *albicans* can be used to infect *C. elegans* and then tracked using *in vivo* visualization to monitor disease progression. As mentioned earlier, *C. albicans* can be a normal part of the microflora, but in the right circumstances it can transform and become extremely virulent. It is often found to very successfully form biofilm on abiotic surfaces such as catheters, dentures, and other implants (Elkabti et al. 2018). A trait specifically important to virulence in *C. albicans* is the ability to switch from the yeast to the hyphal form, as this allows for the dispersion of the yeast with the hyphal form penetrating deeper into the tissue (Elkabti et al., 2018). *C. albicans* also has traits that allow it to be resistant to drugs, yet it has also been seen that pre-treating the organism with small molecules can decrease its adhesion properties (Elkabti et al., 2018).

In a study of the human microbiome, the intestinal microbiota was found to have an impact on Hirschsprung's Associated Enterocolitis (HAEC), a disorder affecting nerve cells in the colon resulting in irregular bowel movements and impacts on the normal gut flora and microbiome. (Frykman et al. 2015). This study gave a background to how the fungal microbiome

coexists with other bacteria, such that the manipulations of the fungal population within mice would alter the severity of colitis in mice, further suggesting that there could be an influence of health in humans from the mycobiome. A similar study focused on how antimicrobials can pose threats to the gut's flora and disrupt normal functions. Therefore, the study looked at how targeting small molecules can help to stop the disruption of normal gut flora (Dufour & Rao, 2011). Both these studies are applicable to the current project as they give knowledge on how the microbiome works and interacts with the normal flora as well as the impact of small molecules.

Probiotic food-derived yeasts can block the formation of a biofilm by non-*albicans Candida* species (Kunyeit et al., 2019). The organism *C. elegans* was used as a host in the study to determine how the probiotic yeast affects the lifespan of the worm. The worms were infected with *Candida* species alone and their survival times compared with pre-inoculation, co-inoculation, and post-inoculation of probiotic yeast and *Candida.* (Fig. 1; Kunyeit et al., 2019). The increased life span is a result of the probiotic yeasts' ability to block adhesion and inhibit biofilm formation of non-*albicans Candida*. This is especially relevant to this project as survival curves will be consulted in the creation of an accurate model.

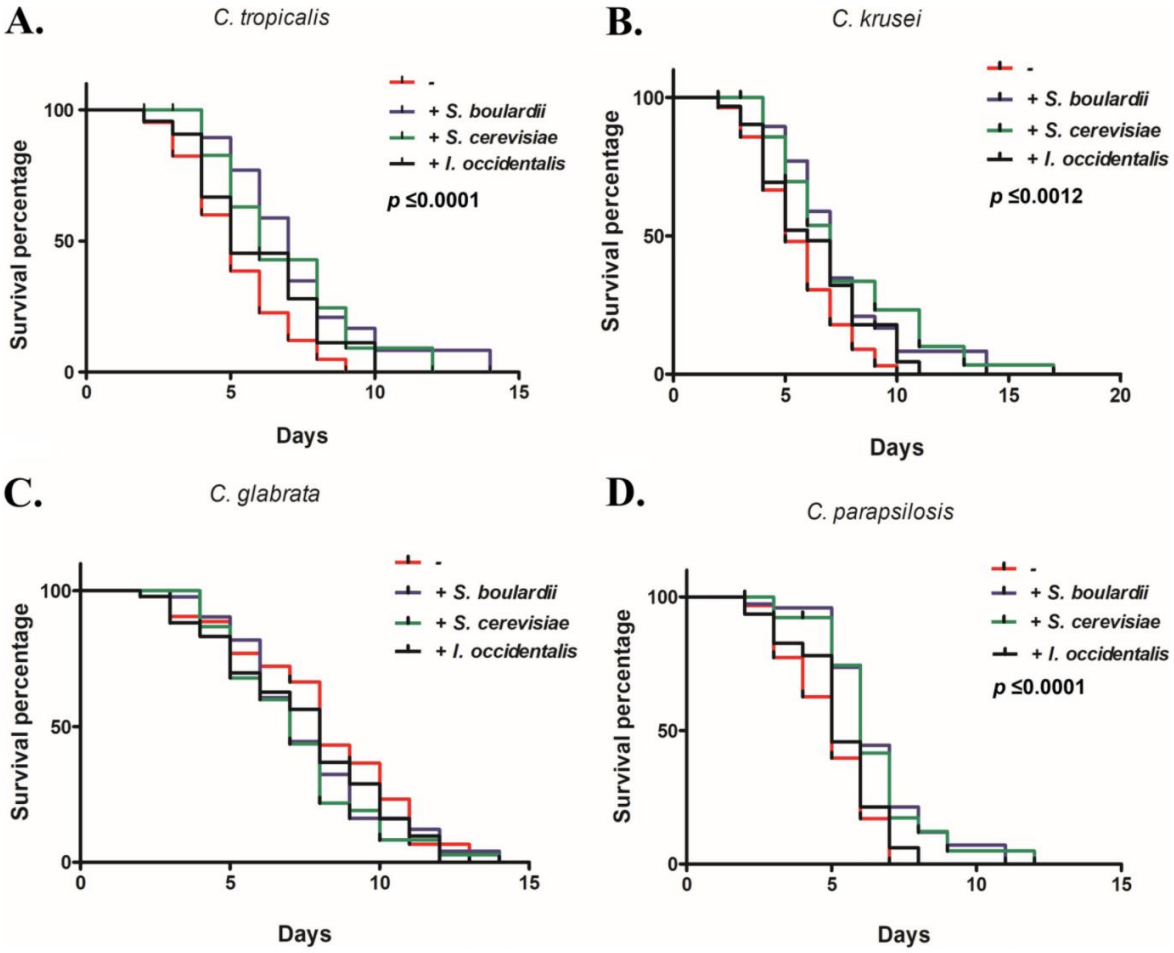


Figure 1. ***C. elegans*** **survival curves with probiotic treatment of non-*albicans Candida* infection.** The red line in all graphs indicates worms treated with only non-*albicans* Candida. The rest of the lines within the graphs shows the survival curves of worms exposed to probiotic yeasts. P values < 0.05 indicate significant differences in life-span. The species *Candida tropicalis, Candida krusei, Candida glabrata, Candida parapsilosis, and Candida auris* were the pathogenic strains of *Candida* used within this study. The probiotic strains were *S. boulardii, S. cerevisiae, and I. occidentalis*. (taken from Kunyeit et al., 2019).

Bacillus subtilis can also modulate the effects of *Candida* infection on C. *elegans* (Rao et al., unpublished results). Organisms that consumed *B. subtilis* along with *C. albicans* tended to live longer, thus supporting the hypothesis that *B. subtilis* can impact the adverse effects of C. *albicans* (Fig. 2). *In vitro* results show that *B. subtilis* secretes a small molecule that changes the phenotype of the yeast, thus affecting its adhesion properties (Rao et al., unpublished data). Thus, the effects of *B. subtilis* on pathogenic yeast infecting *C. elegans* are similar to those observed with probiotic yeast. This is important to note as these organisms diverged in evolution

billions of years ago, yet a similar outcome is observed, pointing to the idea that the mechanisms that assist in this process are highly conserved.

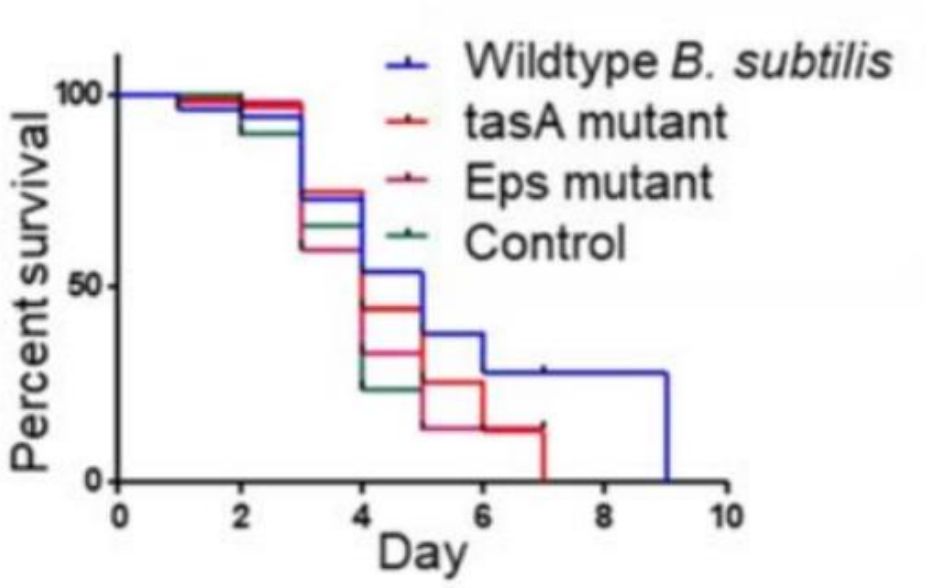


Figure 2. ***C. elegans*** **survival curves after consuming wildtype or mutant strains of** ***B. subtilis***. Control worms (green) were exposed only to *C. albicans*. Worms exposed to wildtype *B. subtilis* (blue lines), but not to non-adherent B. subtilis mutants (red, pink), had increased lifespans. (Rao et al., unpublished results).

3.3. Correspondence between laboratory and computational models

This project built on previous models created using NetLogo to simulate actions within the microbiome. In previous iterations, the model was created to show the interactions between C. *albicans,* B. *subtilis,* and the host C. *elegans.* It is also important to consider the expansion of the lumen seen within the intestine due to biofilm build-up (Fig. 3 Panel D, Rao and Ryder, unpublished results). This is important to consider when creating an accurate model as the expansion has an effect on travel time and survivability.

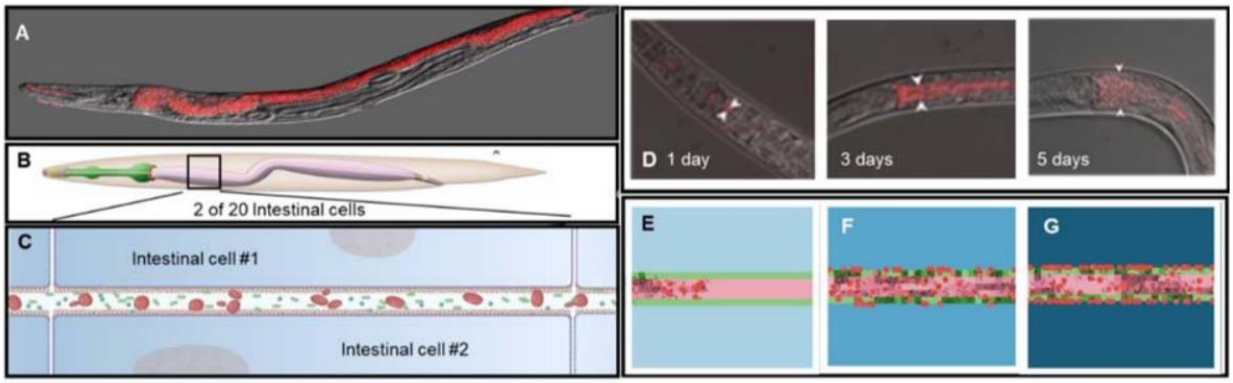


Figure 3. **Comparison between *C. elegans* infection and simulation**. Panels A and D depict images of the intestine of C. *elegans* when yeast (red) is present. Note expansion of the intestine over time of infection (arrowheads, panel D). Panel B shows a schematic of the worm (pharynx in green). Panel C shows one cell length of the worm's intestine. In C the blue represents the intestinal epithelial cells, while the white represents the lumen, and the red ovals and green lines show the yeast and bacteria respectively. Panels E-Gshow a model that was created within NetLogo to visualize yeast moving through a section of the intestine over time., The upper and lower blue areas of the model represent intestinal cells #1 and #2 from panel C; darker blue indicates more pathogenic yeast are adhering to the intestine. (*C. elegans* images from Jain, 2012)

1.4 Project Goals

The objective of this project was to improve the understanding of the interactions of bacteria and yeast within the intestinal microbiome of *C. elegans* through time-course experiments and the creation of accurate NetLogo models. These were intended for the prediction and simulation of how microbes in the intestinal tract can affect organisms over time and potentially contribute to the early death of the organism. Within the laboratory, the goal was to use *in vivo* observation of fluorescent yeast, C. *albicans* within the gut of C.*elegans* to monitor yeast behavior and record an accurate travel time of the yeast through the intestine. This data was then combined with past data to improve the computer model and the rules it is built from. The interconnected use of laboratory and computer approaches worked in tandem to modify and improve the effectiveness and accuracy of the overall program simulation. The project resulted in a more accurate version of a program that can simulate the effect of *C. albican*s and *B. subtilis* on the intestinal tract of *C. elegans.*

2. Methods

2.1 Laboratory

2.1.1 Examination of anesthetized worms

Slide spacers were created by placing tape on two glass slides surrounding another slide. 2% agarose was melted in a boiling water bath. A drop of agarose was pipetted to the central slide using a Pasteur pipette, and a second slide was placed perpendicularly to form an agarose pad (Fig. 4). The top slide was then removed, and 3 ul of 10mM tetramisole was pipetted to the pad. Worms were picked to the spot of anesthetic and covered with a coverslip. Worms were examined using a high-powered dissecting microscope equipped with epifluorescence (Zeiss Discovery microscope).

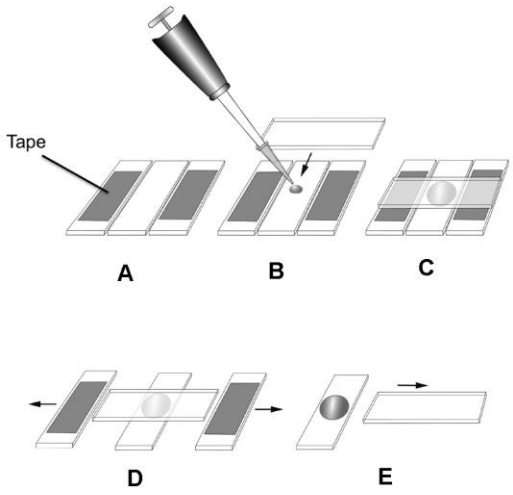


Figure 4. **The process of slide prepping for viewing of C. *Elegans*.**

2.1.2 NemaGel as Recoverable Anesthetic

NemaGel was prepared by keeping it in an ice bath in order to get it into a liquid state. A ring of NemaGel was squeezed out of the bottle on the slide. Then 5 microliters of M9 were pipetted into the space left in the middle of the ring of NemaGel. Next, 2 worms were picked from the yeast plate and placed into the M9 bubble on the slide. A drop of NemaGel was then applied to a coverslip and placed such that the drop of NemaGel aligned with the center of the

ring. The coverslip was then pressed down to ensure every area containing the worms was covered.

2.1.3 Yeast culture

48 hours before worm infection experiments, well-separated colonies on streaked plates of *Candida albicans* expressing mCherry, a wildtype strain from the wheeler lab that has an RFP tag on a histone gene, were picked to 5 ml YPD and cultured overnight. The next day, 24 hours before the experiment was run, the yeast was taken out of the roller drum and spun down in a bench-top centrifuge and 20mililiters were pipetted onto an NGM plate. This allowed enough time for the yeast to dry on the agar plate, without the plates starting to overgrow.

2.1.4 *C. elegans* infection 'pulse chase' experiments

Worms were maintained on agar plates seeded with OP50 bacteria. On the day of the pulse-chase experiment, 10 to 20 L4 or young adult worms were picked from the OP50 plate and were transferred to an agar plate without food. The worms were left to starve for two hours before being removed and transferred to a yeast plate. Starvation was needed because the worms would not eat the yeast under normal conditions. The worms were again left to eat on the yeast plate for between two and two and a half hours. After this time, the worms were picked off the plate onto individual plates seeded with OP50 bacteria.

Once the worms were transferred to the OP50 plates, they had to be carried quickly to the Discovery microscope to capture pictures of the fluorescent yeast within them (Fig. 5). Pictures of each worm were taken approximately every 3 to 5 minutes for about 30 to 40 minutes, or until the yeast appeared to have exited the worm, or had not moved in a 40 minute time interval. The resulting pictures can be found on the Final Worm Data slide show (Appendix 1).

Figure 5. ***C. elegans* captured with ingested fluorescent yeast.** An example image of a worm with fluorescent yeast taken with a fluorescent microscope.

2.2 Computer Modeling

To create the current working model our team picked up from a model previously created by another student for this same research. This model already depicted two cells of the *C.elegans* intestine as well as how yeast and bacteria interacted throughout the microbiome. The model interface is shown in Figure 6. The initial biological "rules" that created this model centered around four main points:

1. The movement of pathogenic yeast and beneficial microbes down the lumen at a particular rate and their ability to slip past each other within a certain probability (for yeast this is influenced by the amount of yeast biofilm present).
2. The ability for the beneficial microbe to secrete a small molecule (SM) at a particular rate and for the molecule to degrade at a particular rate.
3. The probabilities of the yeast and beneficial microbe adhering and dislodging from the lumen. These numbers are influenced by the concentrations of beneficial microbe small molecules (SM) and appropriate biofilms.
    a. Beneficial microbe SM decreases the chance for yeast adherence
    b. Yeast biofilm increases the chance of adherence for the yeast within a certain radius and does not have an influence on the other agent type
4. If yeast or beneficial microbes are stuck for a set amount of time they become stuck permanently as biofilm and start secreting their respective biofilm.

In order to properly visualize the microbiome, various colors were used to depict different states of microbes and cells (Fig. 6). Specific sliders to note within the created NetLogo simulation are yeast-deadhesion-probability, yeast-adhesion-probability, yeast-intake-probability, and beneficial-microbe-intake-probability as these had the greatest impact when determining the results. Specifically, the intake probabilities affect how likely it is for the worm to ingest a microbe. The adhesion and deadhesion probabilities dictate the likelihood that the yeast will change from unstuck to stuck states and vice versa. These states control the ability of the agents to be stuck within the worm intestine.

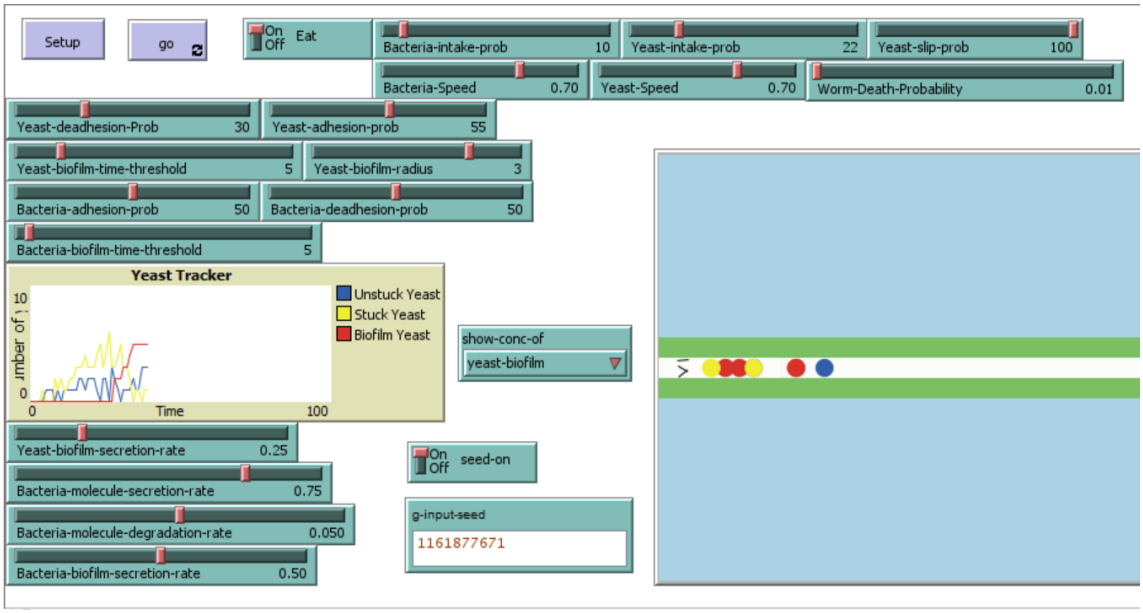


Figure 6. **Netlogo microbiome model interface showing parameter settings.** The blue circle shows a free yeast cell, yellow is a stuck yeast, and red is a yeast cell producing biofilm. Lastly, the white, green, and blue patches represent the intestinal lumen space, mucous layer, and epithelial cells respectively.

In order to make needed model changes, the NetLogo documentation was heavily used, as well as source code from sample models in NetLogos free “Models Library”. Using these resources allowed for searching for necessary commands to build aspects of the model. One particular model used for building the structure of the worm anterior to the intestine was “Autumn” which depicted a tree in different conditions. The diagonal branching shape gave the inspiration to make the shape of the cup. The next step to add was the expansion of the pharynx to this model. This was done by inspecting the number of the yeast of the bottom-most patch of

the pharynx and ‘pushing’ the mucous patch up and adding a lumen patch, depending on the number of yeast present.

3. Results

In order to improve the predictability of our microbiome model, we first needed to gather information from the laboratory on how yeast travels along the worm intestine during infections of *C. elegans* by *C. albicans*. To begin, we developed a proper procedure to determine the travel time of yeast, such that the worms were starved and then live monitored under the microscope. After performing this procedure, we discovered that the yeast took a median time of 12.5 minutes to travel through the worm. We also noted specific behaviors of the yeast; a knocking movement pattern within the intestine, the likelihood of getting stuck, and excretion patterns. Due to these findings, we decided that various aspects of the model needed to be changed including the time scale, adding the expansion of the intestine due to yeast colonization, altering from bacteria to beneficial microbes, changing yeast movement, and adapting a secondary model to represent the anterior portion of the worm intestine. After calculating the time scale, we then determined that the length needed for accurate data was too cumbersome for NetLogo, thus the models were split into three separate infection phases and comparative data was generated to ensure that the beneficial microbes had a positive effect on preventing lumen expansion.

3.1 Laboratory

3.1.1 Developing *C. elegans* infection and 'pulse-chase' protocol

The first aspect of this project step was to develop a successful procedure for tracking the yeast's movement through the worm. In the first attempt at tracking the yeast, the goal was to anesthetize the worms at specific time points after ingestion of fluorescently labeled yeast in order to examine the position of the yeast along the intestine. We picked worms to slides, anesthetized with tetramisole, and examined using epifluorescence. New worms were picked for each time point. We followed this process in hopes that consistent progress of yeast through the intestine would be observed over time. However, the worms did not ingest the yeast in a consistent enough pattern to draw conclusions by examining groups of separate worms at different time points.

Our next step was to attempt to follow individual worms over time by recovering the worms from slides and picking the worms back to the yeast plate. This procedure also proved to be unsuccessful, as the anesthetic was too strong, even extremely diluted; the worms took hours

to recover and often died. This procedure also made it very difficult to pinpoint exactly when the worm was fully recovered and eating the yeast again.

Due to this problem, we used another anesthetic, NemaGel, in a second attempt to recover individual worms. NemaGel is an anesthetic used specifically for anesthetizing worms and then recovering the worms back to a fully active state. While the anesthetic aspect of the NemaGel worked well for our purposes, when examining the worms under the microscope it created a 3D substrate; the worms were not in a consistent focal plane. Due to this problem, we were unable to document the progress of the yeast (Fig. 8).

As part of the goal was to examine the yeast behavior within the worm while disrupting worm behavior as little as possible, we decided to observe the infected worms with no anesthetic after picking them off the yeast plate and onto a bacterial lawn. This protocol was successful and resulted in the generation of all data described below (see Methods for detailed protocol).

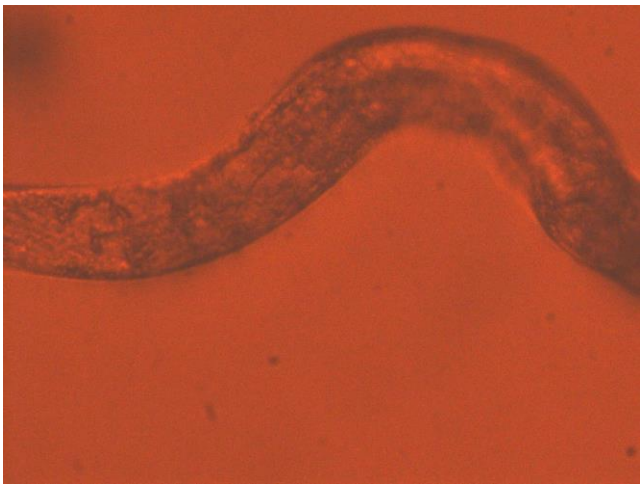
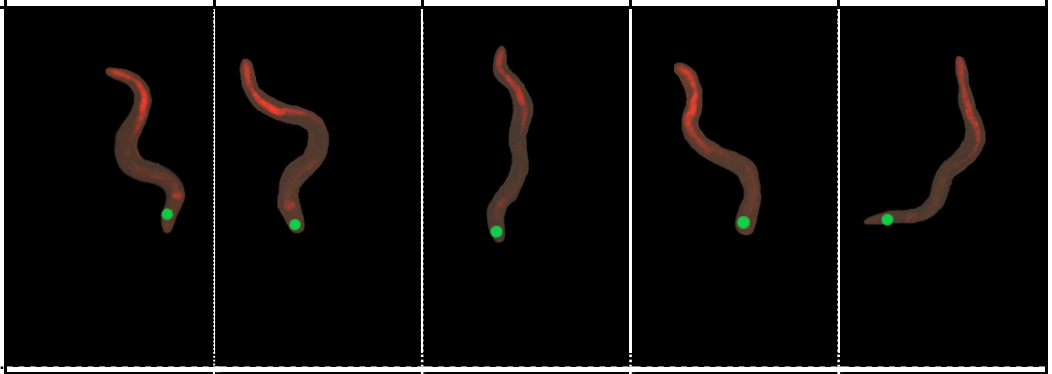
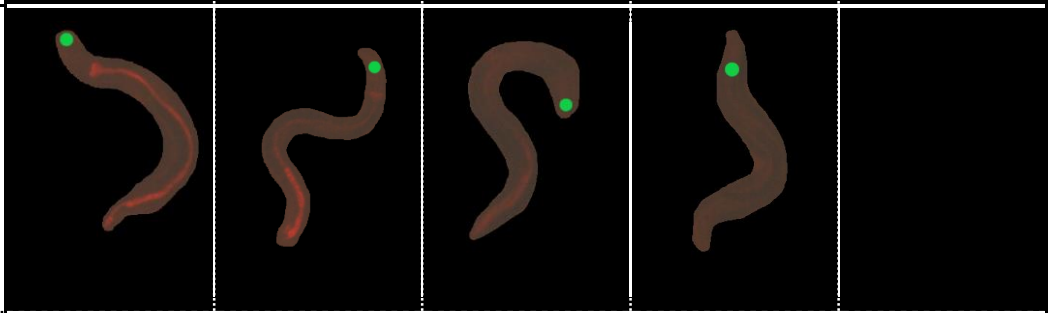
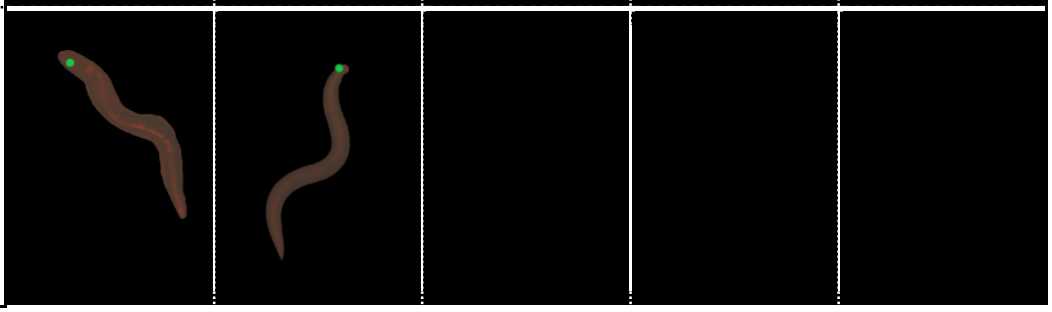


Figure 8. **Worm anesthetized using NemaGel.** The head of the worm is in focus while the body of the worm is out of focus.

3.1.2 Observations of yeast in *C. elegans* intestine

While collecting the time course data we were also able to observe other properties of the yeast within the intestine of the worm. We observed that the yeast tended to get stuck in a specific pattern (Fig. 9). It would first accumulate for a short period of time at the anterior of the intestine, just posterior to the pharynx, as well as the posterior end of the worm (Fig. 9A). In most cases, after a few minutes of the worm actively moving and feeding on a bacterial lawn, this yeast would then become dislodged and gradually clear out of the worm (Fig. 9C). In other cases, the worm would become full of yeast in the posterior of the worm and after watching these

worms for an extended period of time we deemed that the yeast may have become permanently stuck within the worm (Fig. 9A). Another pattern we observed was the way *C. elegans* would excrete the yeast; this was done in small, continuous clumps (Fig. 9D). The final pattern that we observed was worms in which the yeast freely moved directly through the worms. In these cases the yeast would clear out of the worm in a matter of minutes, leaving no stuck yeast left inside (Fig. 9B).

Category	0-3min	3-8min	8-15min	15-20min	20+
A **Stuck**					
					
B Free Flowing					
					

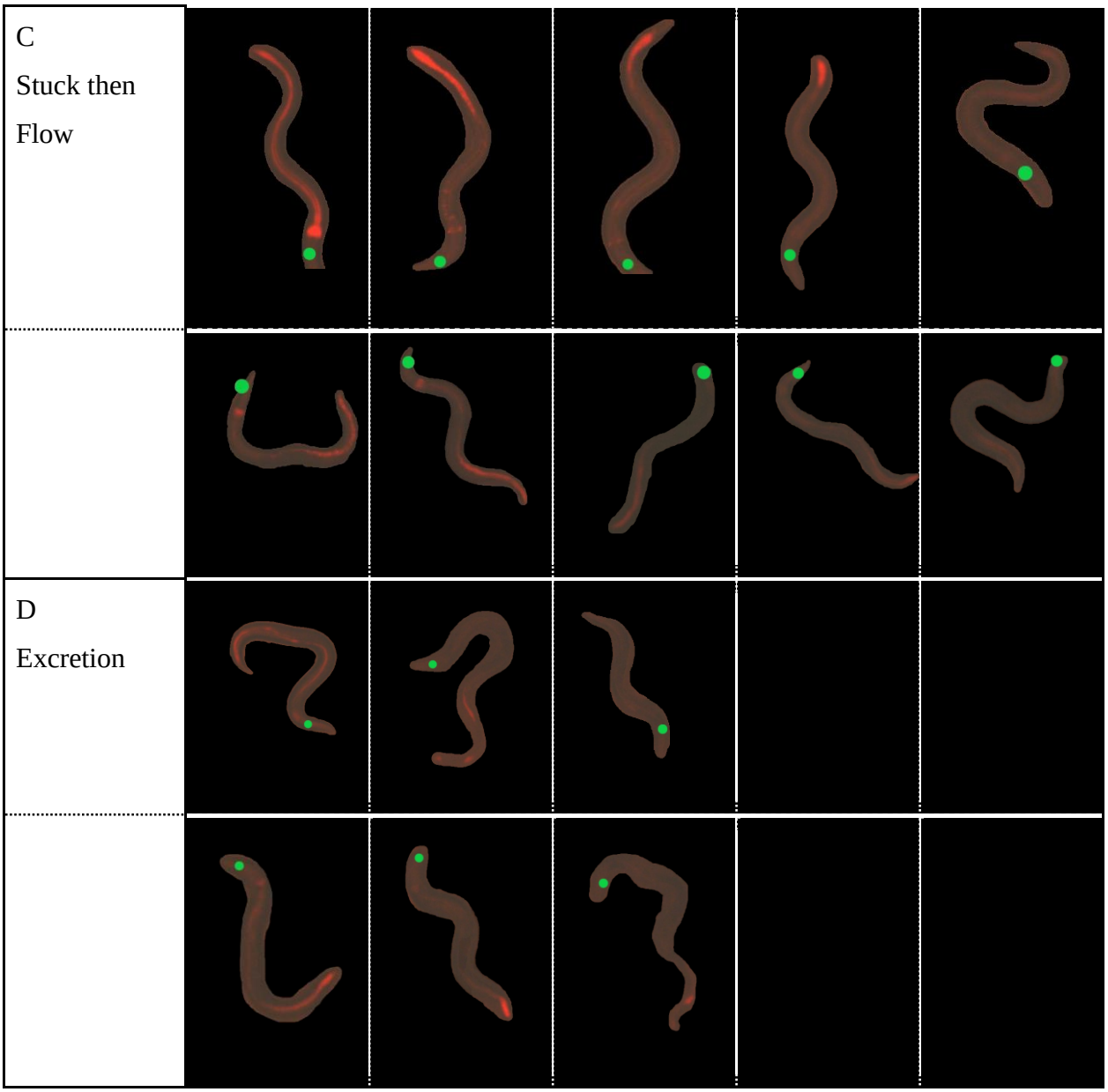


Figure 9. **Micrographs showing fluorescently labeled yeast traveling through *C. elegans* intestine over time**. Two examples of each main observation category are shown. A corresponds to worms in which the yeast was fully stuck. B is a worm in which the yeast flowed freely through the worm. C is when the yeast was initially stuck, yet eventually cleared. D shows examples in which the worm's excretions can be viewed. Green dots show the position of the pharynx, just anterior to the intestine. Spots that are empty indicate that there were no pictures taken at these time points.

3.1.3 Analysis of yeast travel time in *C. elegans*

During this experiment, we found that the average travel time of the yeast was 16.2 minutes while the median was 12.5 minutes. In most of the inspected worms, we found that the yeast flowed freely fast through the worm, while in about 10% of the worms the yeast became stuck inside of the intestine. This result showed that in this time scale the yeast can stick inside the worm, but typically it transits the intestine fairly rapidly.

Data Set	Number of Worms	Mean Travel Time	Median
1	10	20.2	15
2	20	14.15	10.5
Combined	30	16.2	12.5

Figure 10. **Table of Important Statistics from Pulse-Chase Experiments.** Calculated mean and median for the travel time for the yeast within the worm given each data set and the combined data sets.

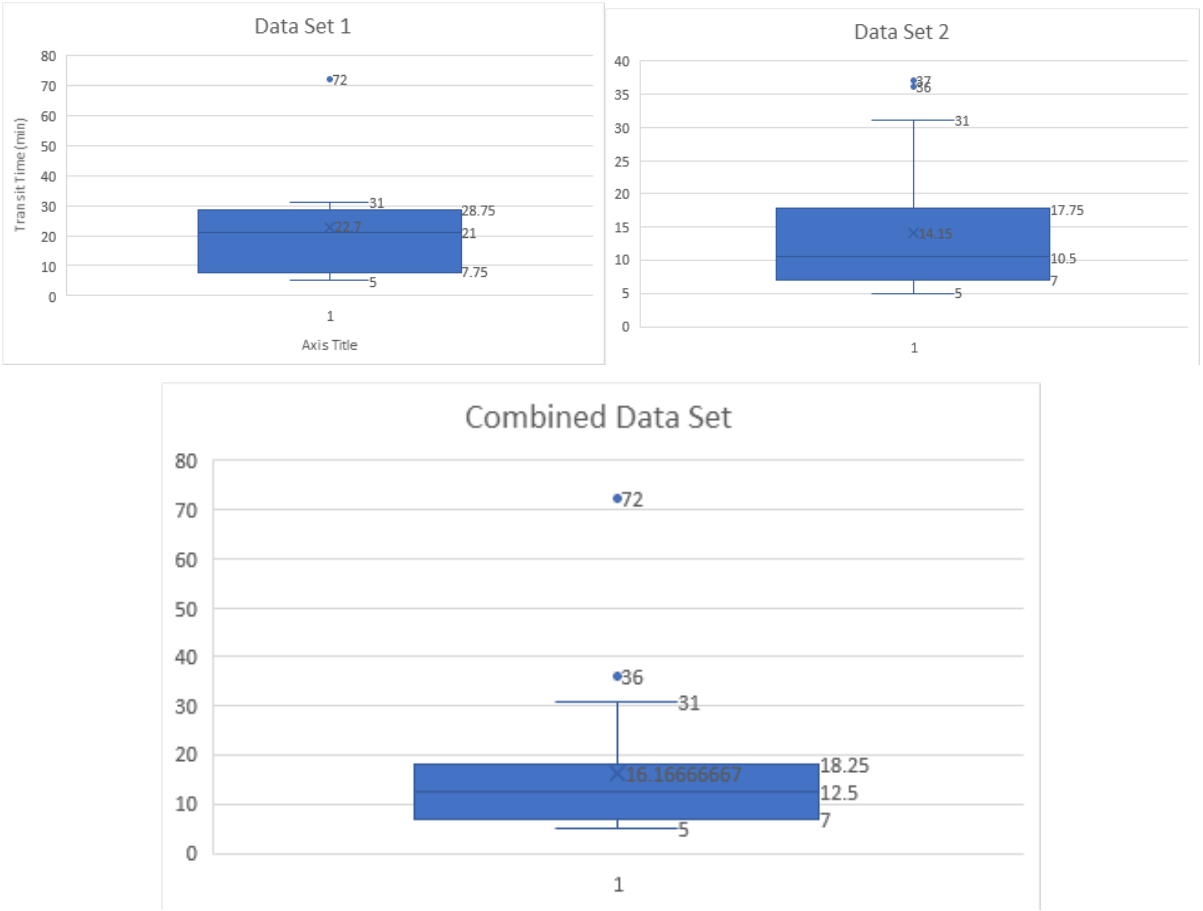


Figure 11. **Box and Whisker of Worm Travel Times.** The three figures above show the box and whisker plots that are generated from the raw data of yeast travel time within the worm.

3.2 Modeling

The initial model represented an over-simplification of the biology of the system. We added several new rules to increase the model's accuracy:

1. Yeast and beneficial microbes enter a biofilm state when others of their kind are also able to progress into a biofilm within a certain radius
2. A set amount of time must pass with yeast or beneficial microbe in the "stuck" state and near other stuck agents for their respective agents before becoming stuck permanently as biofilm, thus secreting their respective biofilm.

After examining the data found from our experiments within the lab, we made several additional modifications to model rules. We modified the rules for yeast movement so that yeast 'push' each other along the intestine rather than slipping by:

3. When there is a stuck yeast directly in front of the moving yeast the stuck yeast will become unstuck at a certain probability

To represent the expansion of the worm intestine that is seen when there is a buildup of yeast over time, we added the rule:

4. If more than two stuck/biofilm yeast are located in the lumen, patches shift in a specific pattern to mimic the expansion of the intestine observed over time in infection experiments in the laboratory.

The full set of specific rules can be found in Appendix 4.

Various changes were made to the model in order to ensure biological accuracy. In a general change we altered the bacteria, so they became beneficial microbes that looked and moved more like the previously designed pathogenic yeast, yet still, secrete the small molecules that decrease the adhesion of pathogenic yeast. We also determined that it would be beneficial to design a new, separate model that represents the anterior intestine as we noticed that in many of the worms, we inspected yeast would first get stuck in this area. Lastly, we also decided that the timing of the model needed to be altered in order to match the survival curves and the yeast travel times more accurately.

In order to calculate the relative travel time of yeast within Netlogo, the first step was to look at the movement of the NetLogo agent within the simulation. A single stuck yeast was followed and timed from when the yeast first appeared in the model to when it reached the end of the simulation and was no longer seen. It was found that the yeast took about six seconds to travel from start to finish with the model running at "normal speed", the equivalent measurement in ticks found it to be about 90 ticks for the yeast to travel the length of the simulation interface (15 ticks/second). The simulation interface represents 1/20th of the entire worm (the length of an intestinal cell), so it can be assumed that if the yeast was to travel the entire length of the worm in NetLogo it would take 120 seconds or 2 minutes (1800 ticks). Since our data show that ingested yeast typically takes about 20 minutes to travel through the intestine, the model with its current settings is running at about 10 times the speed of real life. The next step was to calculate the number of ticks that would be expected until the death of the worm. Referencing the past survival curve data, it was found that on average it would take a maximum of 10 days for a worm to die and using the 10x speed for the model this would be 1 day. In one day there are 86400 seconds; at the rate of 15 ticks/second, it would take the worm on the order of 1,296,000 ticks to die.

While this would be a successful representation of biology, it was found that the run time was too long in order to generate multiple data sets necessary for analyzing data. Due to this issue, the planning of the model was slightly altered to accommodate a large amount of data processing. The solution found was to split the model into three sub-models or phases in which the first phase is before any yeast biofilm and expansion is present, the second includes the beginning of yeast adhesion and intestinal expansion, and the third includes biofilm formation and worm death (Fig. 12).

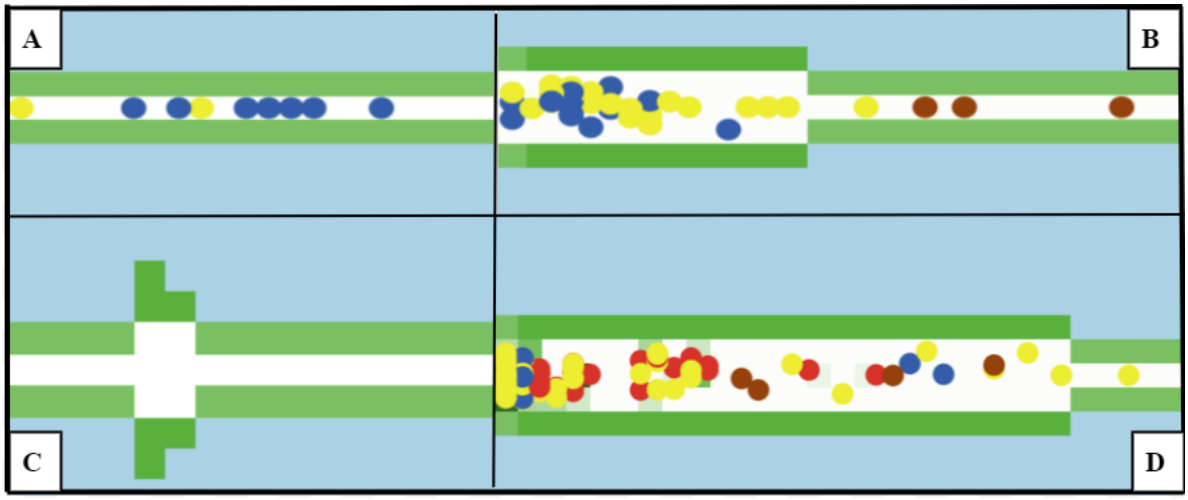


Figure 12. **Microbiome models.** In this figure, each panel is a screenshot from one of the main models/sub-models that were created. In panels A, B, and D the model interface represents the middle of the intestine. These are all the same base models with different presets to represent the three separate phases. A is the first phase with no expansion and little to no stuck yeast. B is the second phase with some expansion and some stuck yeast, but no biofilm. D is the third and final phase which contains biofilm and greater expansion. Lastly, C shows the environment for the anterior intestine model, with the settings for pathogenic yeast and beneficial microbes being the same as for the middle intestine model. The blue circle shows a free yeast cell, yellow is a stuck yeast, and red is a yeast cell producing biofilm. Lastly, the white, green, and blue patches represent the intestinal lumen space, mucous layer, and epithelial cells respectively. The brown circles depict the beneficial microbes, while the darkening patch colors are used to visualize the levels of small molecules on each lumen cell. Model setting parameters are included in Appendix 3.

From here points of comparison were used to ensure that the presence of the beneficial microbes impeded the pathogenic yeast's ability to stick, and thus decreased the expansion of the intestine. The main point of comparison used was measuring the total percent of yeast considered stuck after running the phase 2 middle intestine model for 2000 ticks. This was done by running the simulation with a gradually increasing probability of beneficial microbe intake. The first data set was created using a pathogenic yeast intake probability of 50 and a beneficial microbe intake probability of 0. The next set of data was created using the same yeast intake probability of 50, while the beneficial microbe intake probability was increased to 5. For the third data set, this pattern was followed with the yeast intake probability set to 50 and the beneficial microbe probability set to 10. Data sets were created following this pattern until the beneficial microbe intake probability reached 50.

The increased amount of beneficial microbe's present decreased the amount of pathogenic yeast left in the worm, as well as decreased the percentage of this yeast that was in the stuck state once the simulation had run the desired 2000 ticks. (Fig. 13) The presence of the beneficial microbes also made it significantly more difficult for the intestine in the model to expand, accurately representing the presence of the beneficial microbes in the intestine. When no beneficial microbes were present the expansion happened relatively quickly, whereas with the beneficial microbes present the expansion happened at a much slower pace, and sometimes never reached full expansion (Fig. 14).

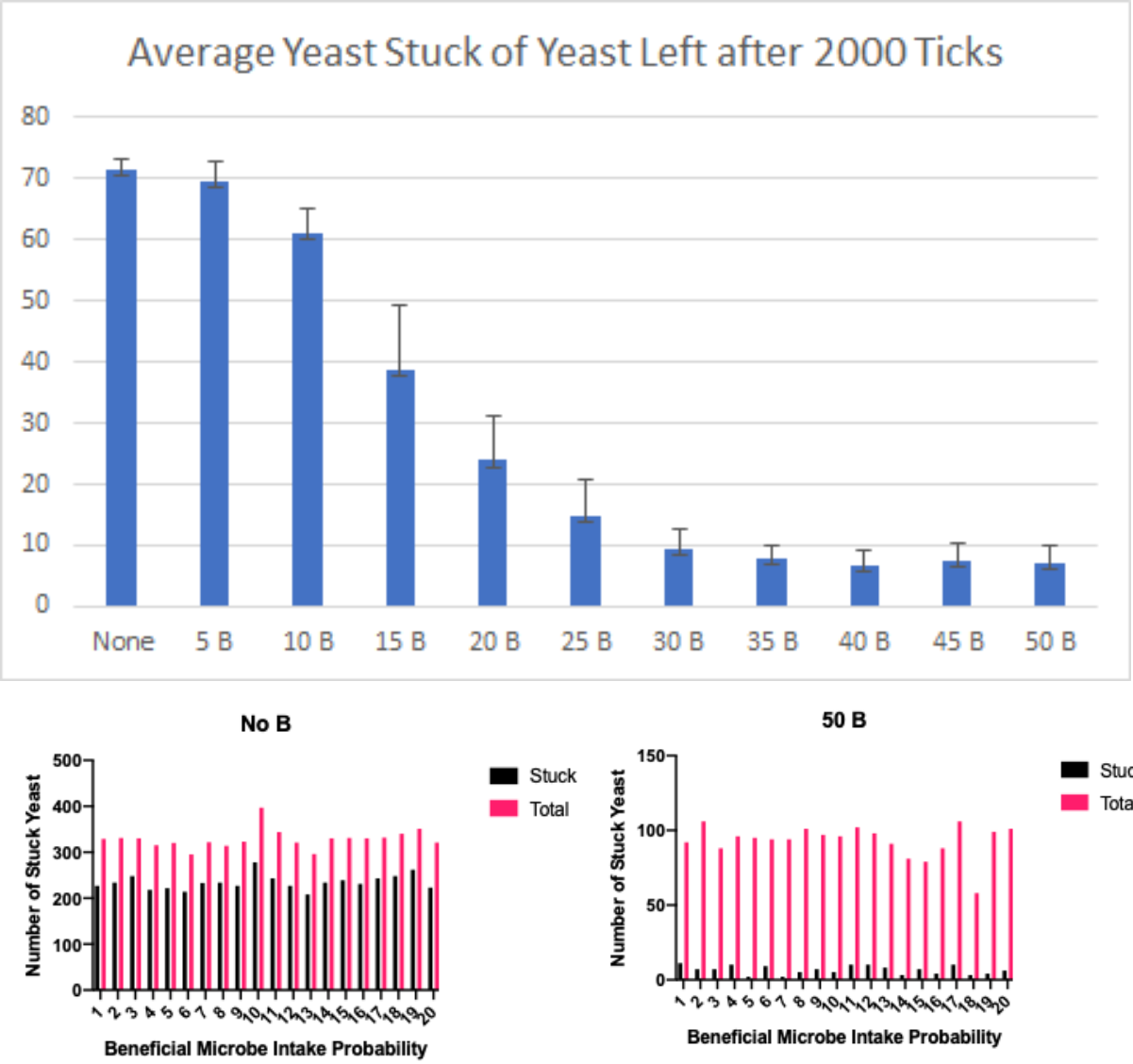


Figure 14. **Comparison of total and "stuck" yeast remaining in the simulation for different beneficial microbe intake probabilities.** Panel A: the percent of pathogenic yeast left in the simulation after 2000 ticks that were categorized as "stuck". (Mean, SD). Panel B, C show the exact number of yeast stuck and total yeast given no

beneficial microbe (No B) and high beneficial microbe (50 B) for each of 20 trials.. The full list of data can be found in attached files.

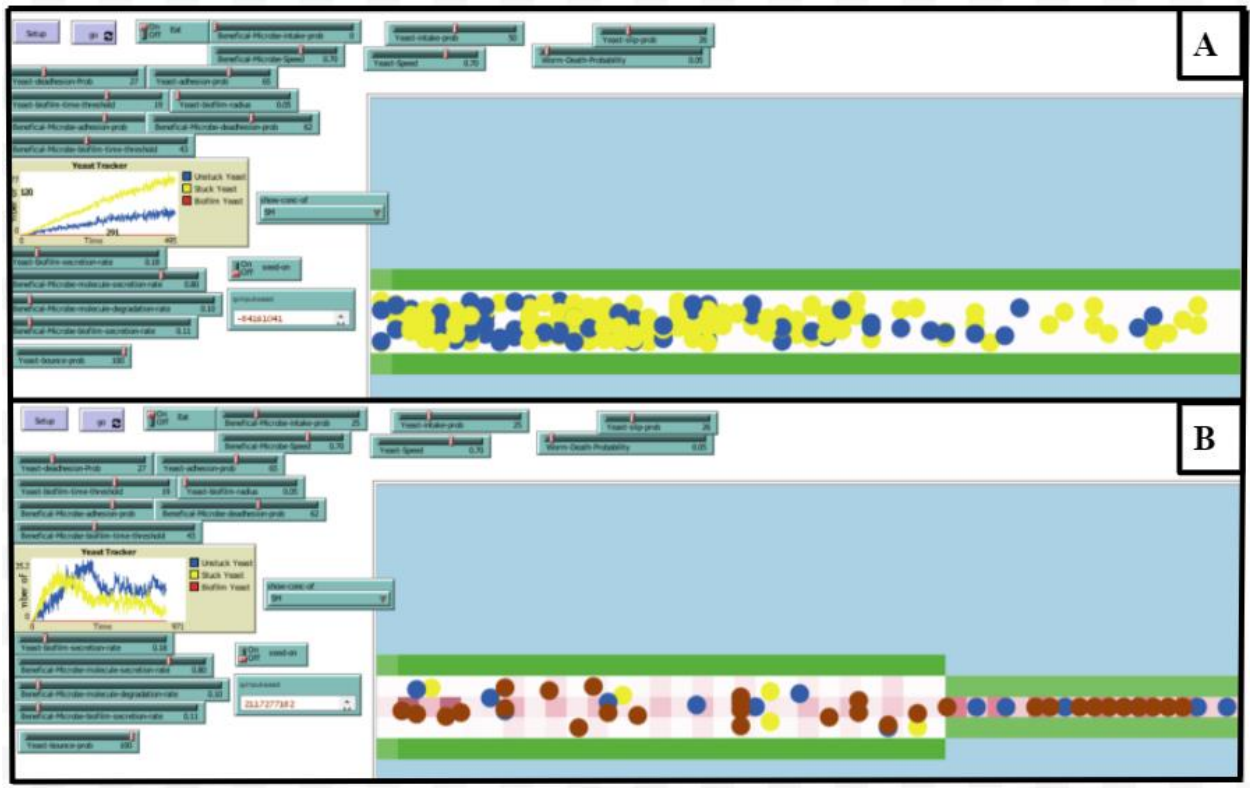


Figure 14. **Comparison of expansion with the phase two model when beneficial microbes are present versus when they are not.** In panel A there are no beneficial microbes and thus there was quick expansion and there are many stuck yeast cells. In panel B the beneficial microbe is present and actively secretes small molecules, as can be seen by the red patches, thus resulting in less expansion and fewer stuck yeast within this variation.

The second model that was created focused on the anterior intestine of the worm. The lab data showed that yeast seemed to adhere more to the anterior of the intestine just posterior to the pharynx than to the middle of the worm. We determined that this portion of the intestine is anatomically distinct (Altun et al., 2021); thus, we determined it important to create a model that focused specifically on this region (Fig. 15). As seen in the figure the diagonal-like portion represents the anterior of the intestine of *C. elegans*. This model is a base environment for future iterations, thus has not yet reached a stage to collect substantial quantitative data.

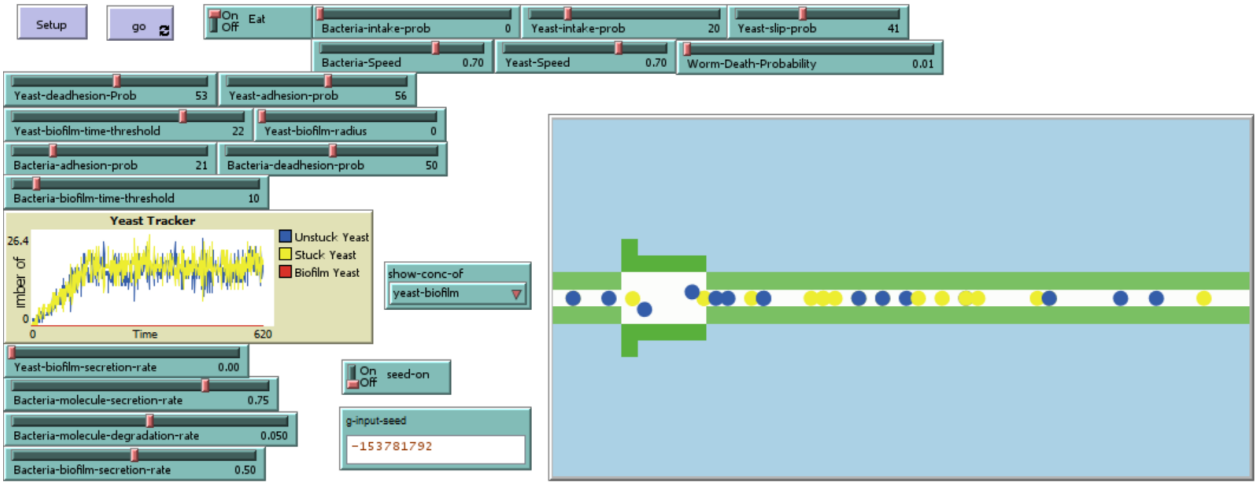


Figure 15. **A secondary model created to depict the anterior of the intestine.** The model was created to simulate the anterior intestine portion of the model. The pathogenic yeast, beneficial microbe, and expansion properties are all very similar to the middle intestine model, yet it begins with the “pouch” to indicate the morphology of the anterior of the intestine.

4. Discussion

The results from the laboratory experiments gave a significant perspective for seeing the general speed of the yeast's movement through the worm. The speed of the yeast was determined by the amount of time it took for the yeast to travel through the worm. Getting an accurate measurement for this will greatly improve the accuracy of the model and better simulate what happens within a specific time frame. The results of the laboratory experiments also made it possible to create a model that closely resembles and demonstrates the patterns of flow among the three states — stuck, unstuck, biofilm — yeast within the worm.

Because the experiments were done such that the worm was checked under the microscope at set time intervals, we were unable to record the exact times of the yeast's passage through the worms. More specifically, we were also unable to consistently monitor when the worm ingested and excreted the yeast as well as the amount. There were challenges in determining if the yeast was permanently adhering to the intestinal wall. Future work could investigate whether leaving worms on the yeast plate to eat for an extended time would result in more yeast adhering within the intestine. Another issue in observing the yeast was that fluorescence is used to mark the yeast and make it visible under the UV light would fade with the increased period of exposure to the UV.

There are many aspects to the laboratory investigations that can be explored further. One of the more difficult challenges was finding the right timing for the overall passage of yeast through the worm. A focal point might be finding ways of making this more accurate by finding a way to monitor when the worm begins ingesting yeast and following the yeast through the worm the entire time. During our experiments, we did not use *B. subtilis* at all. Future groups should work with pre-infection or co-infections of the worm with *B. subtilis*. This would be useful in determining if the bacteria had a direct effect on the ability of the yeast to colonize the worm. If the *B. subtilis* bacteria were given a fluorescent tag, their movement could be tracked through the worm along with the yeast.

Another observation worthy of further investigation is the movement of yeast within the intestine. While watching infected worms under the microscope, we occasionally observed a "swishing" of the yeast back and forth through a large portion of the intestine of the worm. It

was not clear whether this movement resulted from the worm moving or if there are other factors contributing to it. This movement could affect the adhesion and biofilm formation of the yeast within the worm and it could change our understanding of the mechanisms of sticking and the interactions between the bacteria and the yeast while they are in the intestine.

Running an experiment to find the time frame of biofilm production and monitor it through the process in the worm would also help to better model the effects within the simulation. Knowing how long the yeast is stuck before the biofilm builds up and spread would be important to the model accuracy and could impact the scaling and calculations of the timing within it. Making this model as accurate as possible is significant because it will allow for the predictive analysis of the microbiome of *C. elegans* intestinal tract. This predictive analysis will be used as a visualization for the expansion with a representative scaling of the time period in which it happens.

Future work can further improve the model by better incorporating the phases of infection of the yeast within the worm, including the phases as infection, expansion, and death of the worm. The model could also be modified in order to incorporate the entire worm from the mouth to the tail. The method of intestinal expansion used currently has the issue of the small molecule secretion stopping the expansion when it it set too high; it prevents yeast sticking at the expansion point due to its build up on a particular patch. Once this problem is addressed, there will be a more accurate prediction of how long it takes for the full expansion of the intestine of the worm. In using this method that combines laboratory experiments and computer modeling, the results could eventually be scaled up and be applied to the intestinal microbiome of humans and other organisms.

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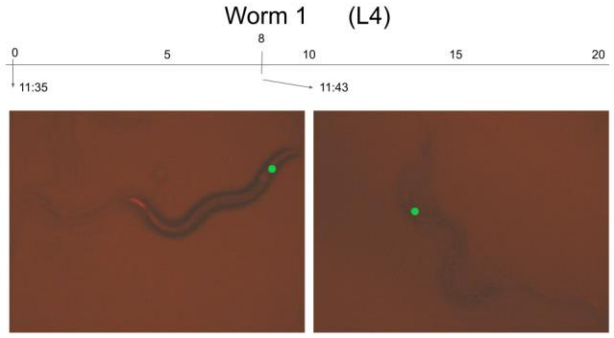
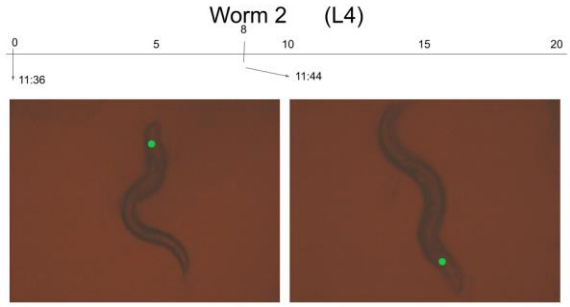
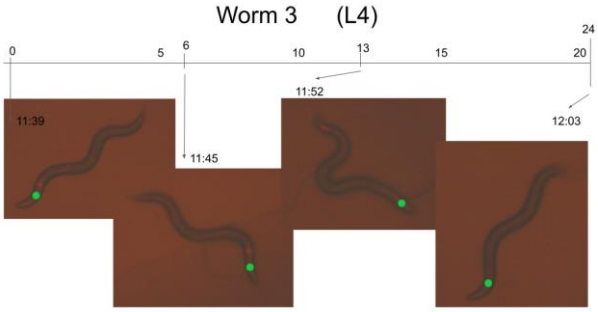
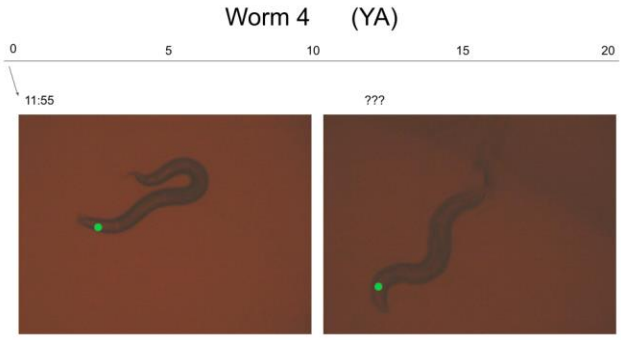
6. Appendices

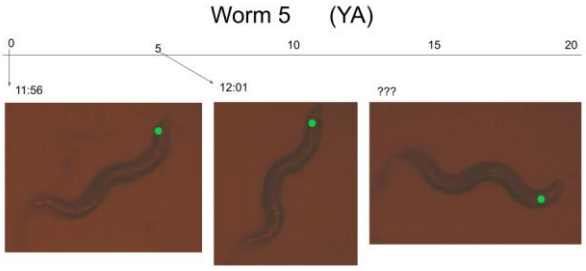
Appendix 1 -- Final Data and Pictures Collected of all worms

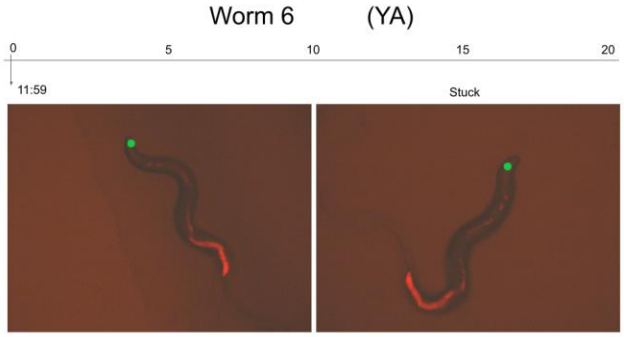


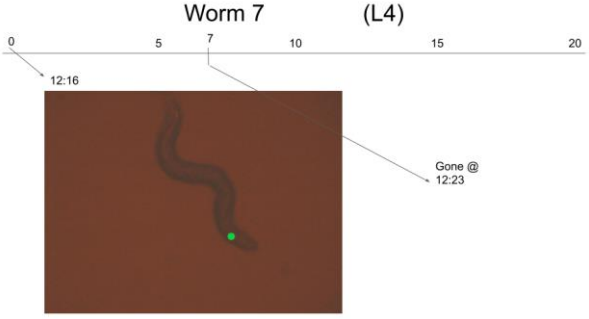


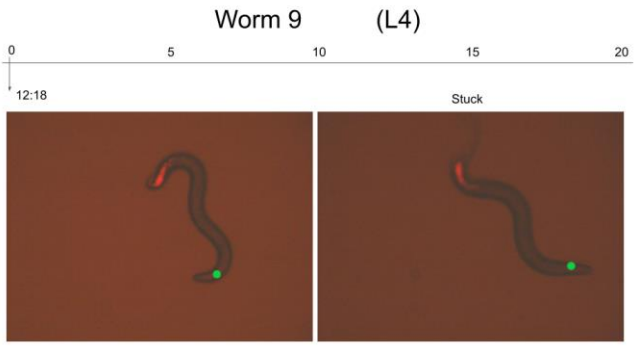


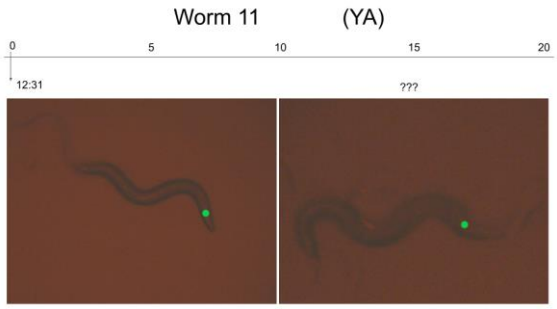









Worm 9 (L4)
0
5
10
15
20
12:18
Stuck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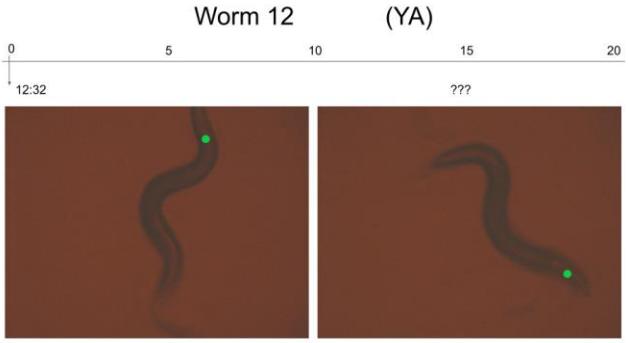
0
5
10
15
20
12:31
???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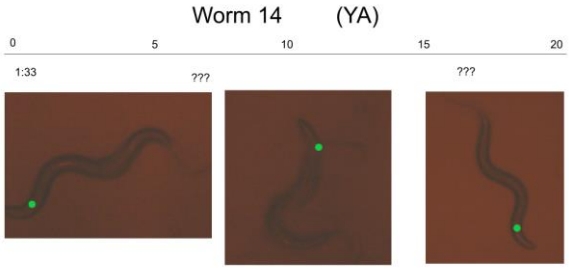
0
5
10
15
20
12:32
???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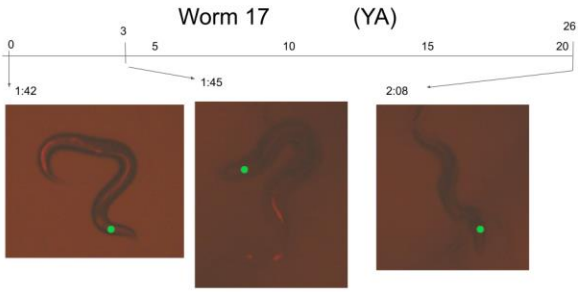
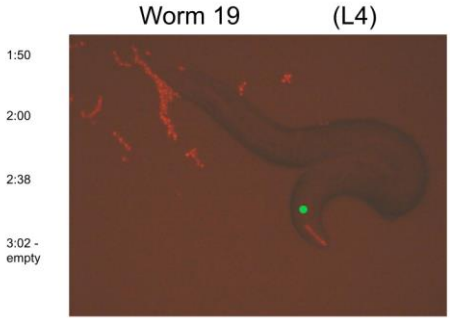
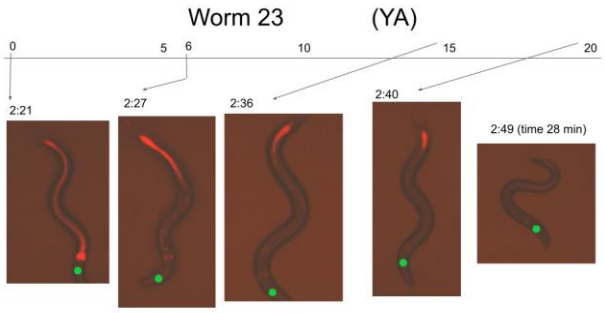
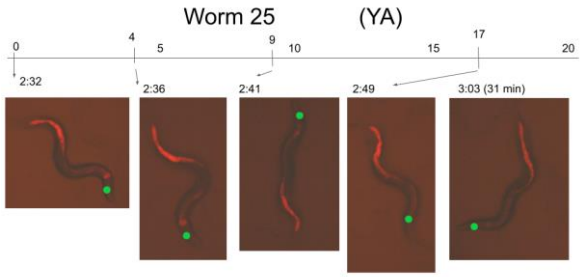
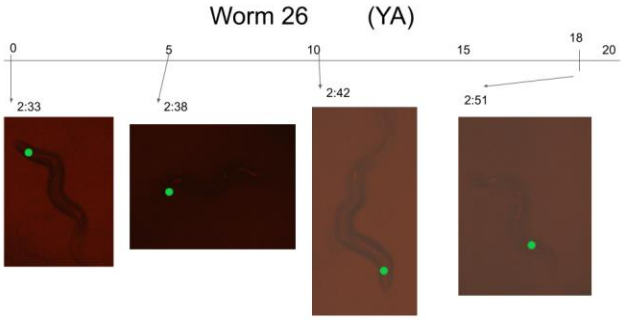
0
5
10
15
20
1:33
???
???

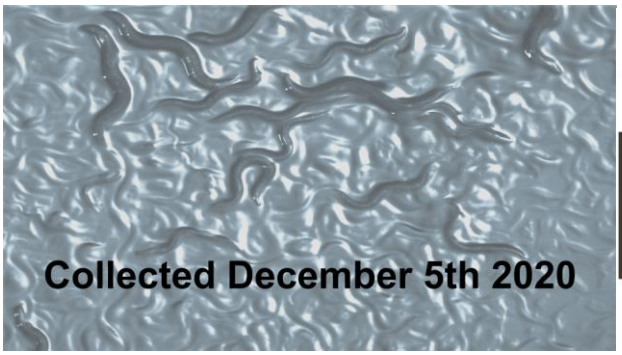

Worm 17 (YA)
0
3
5
10
15
20
26
1:42
1:45
2:08

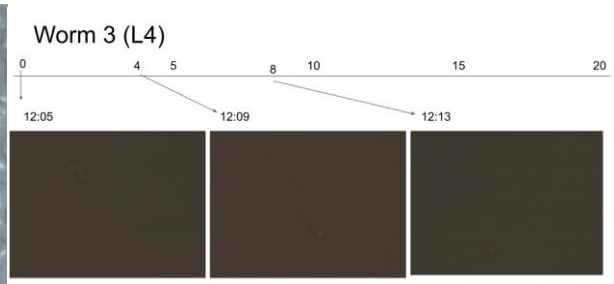

Worm 19 (L4)
1:50
2:00
2:38
3:02 -
empty

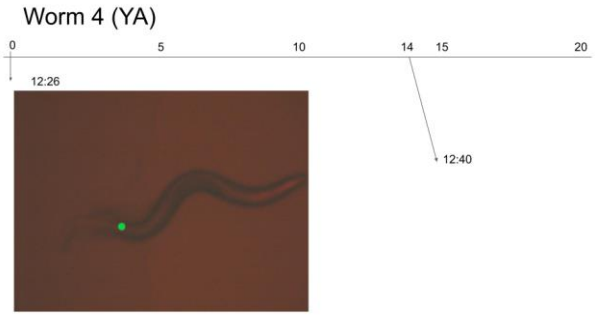

Worm 23 (YA)
0
5
6
10
15
20
2:21
2:27
2:36
2:40
2:49 (time 28 min)

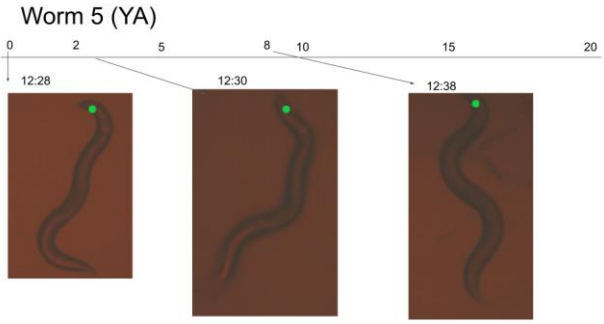

Worm 25 (YA)
0
4
5
9
10
15
17
20
2:32
2:36
2:41
2:49
3:03 (31 min)

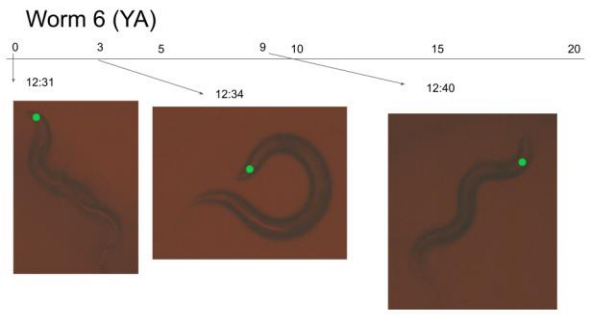

Worm 26 (YA)
0
5
10
15
18
20
2:33
2:38
2:42
2:51

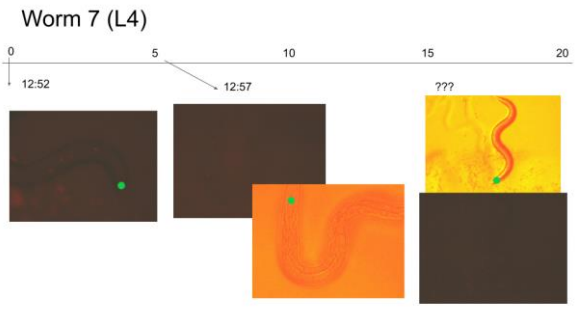

Collected December 5th 2020

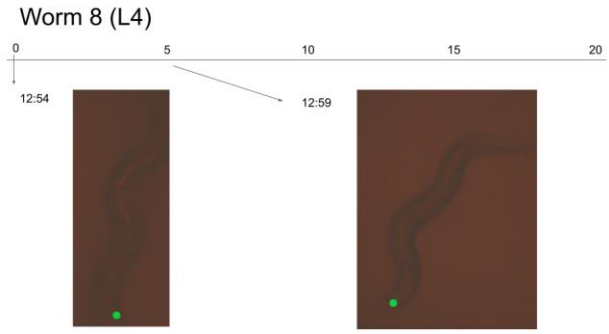

Worm 3 (L4)
0
4
5
8
10
15
20
12:05
12:09
12:13

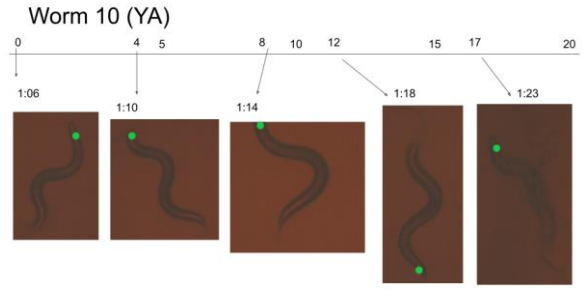

Worm 4 (YA)
0
5
10
14
15
20
12:26
12:40

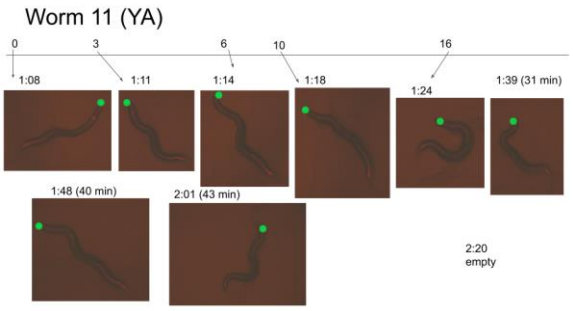

Worm 5 (YA)
0
2
5
8
10
15
20
12:28
12:30
12:38

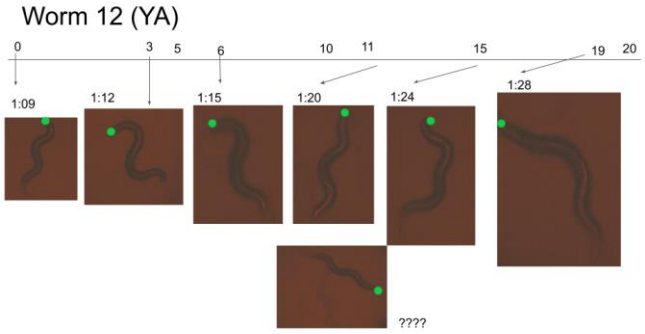

Worm 6 (YA)
0
3
5
9
10
15
20
12:31
12:34
12:40

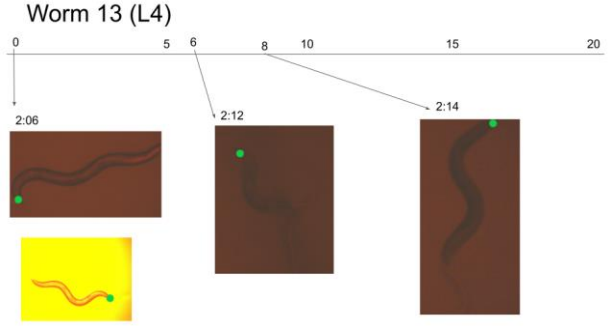

Worm 7 (L4)
0
5
10
15
20
12:52
12:57
???

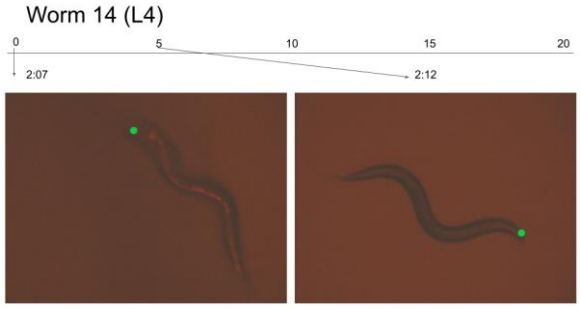

Worm 8 (L4)
0
5
10
15
20
12:54
12:59

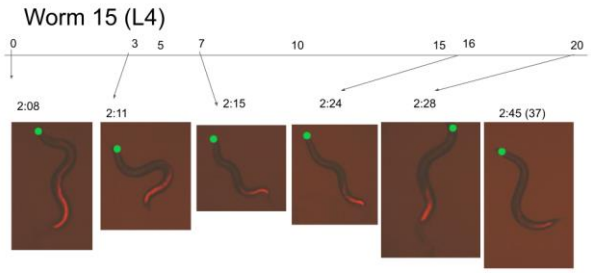

Worm 10 (YA)
0
4
5
8
10
12
15
17
20
1:06
1:10
1:14
1:18
1:23

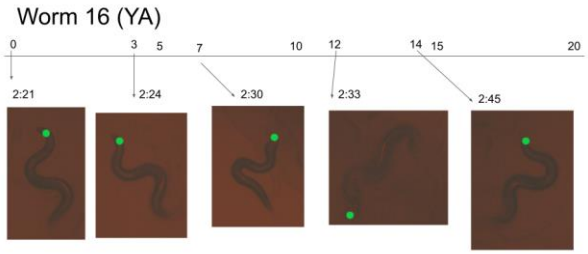

Worm 11 (YA)
0
3
6
10
16
1:08
1:11
1:14
1:18
1:24
1:39 (31 min)
1:48 (40 min)
2:01 (43 min)
2:20
empty

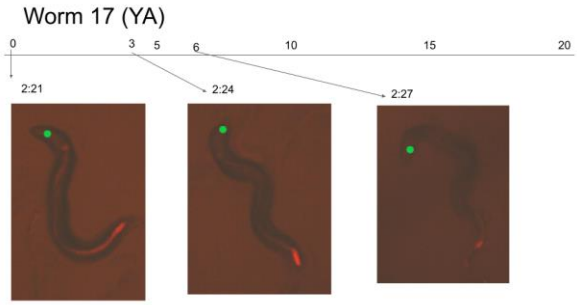

Worm 12 (YA)
0
3
5
6
10
11
15
19
20
1:09
1:12
1:15
1:20
1:24
1:28
????

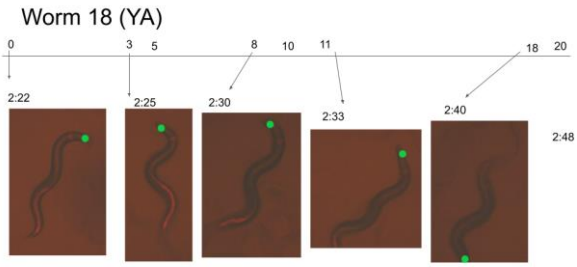

Worm 13 (L4)
0
5
6
8
10
15
20
2:06
2:12
2:14

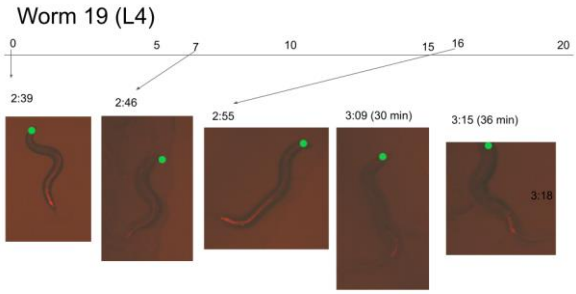

Worm 14 (L4)
0
5
10
15
20
2:07
2:12

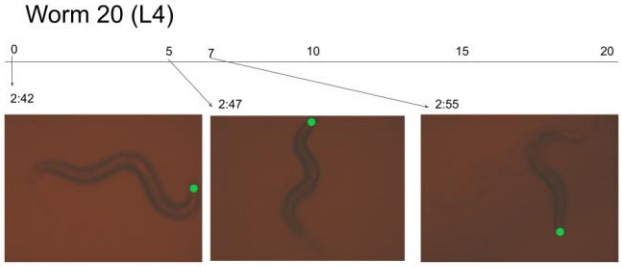

Worm 15 (L4)
0
3
5
7
10
15
16
20
2:08
2:11
2:15
2:24
2:28
2:45 (37)

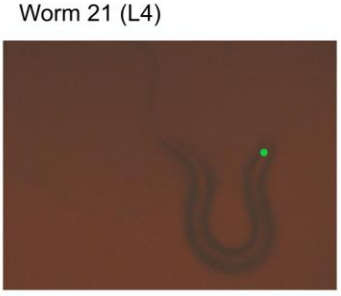

Worm 16 (YA)
0
3
5
7
10
12
14
15
20
2:21
2:24
2:30
2:33
2:45

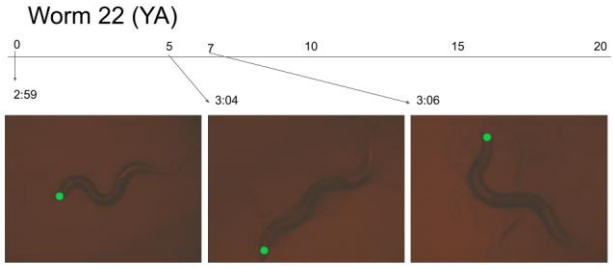

Worm 17 (YA)
0
3
5
6
10
15
20
2:21
2:24
2:27

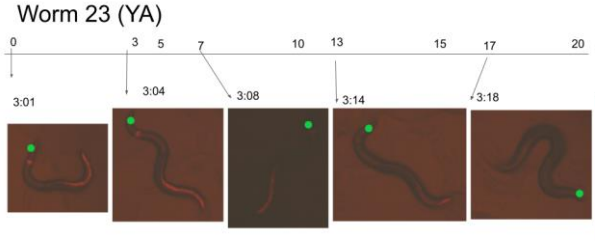

Worm 18 (YA)
0
3
5
8
10
11
18
20
2:22
2:25
2:30
2:33
2:40
2:48

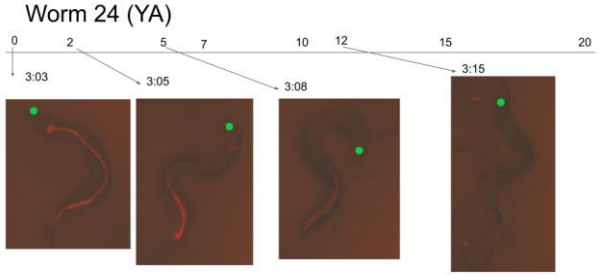

Worm 19 (L4)
0
5
7
10
15
16
20
2:39
2:46
2:55
3:09 (30 min)
3:15 (36 min)
3:18


Worm 20 (L4)
0
5
7
10
15
20
2:42
2:47
2:55


Worm 21 (L4)
2:44
RIP


Worm 22 (YA)
0
5
7
10
15
20
2:59
3:04
3:06


Worm 23 (YA)
0
3
5
7
10
13
15
17
20
3:01
3:04
3:08
3:14
3:18


Worm 24 (YA)
0
2
5
7
10
12
15
20
3:03
3:05
3:08
3:15

Appendix 2 -- Full Set of Rules Pre-Edits

Model rules-- 7/16/20

1. Yeast and bacteria move down the lumen at a particular rate. Yeast stop when another yeast cell is in front of them, but bacteria can slip by.
2. Bacteria secrete a small molecule (SM) at a particular rate and the molecule degrades at a particular rate. (SM is degraded by patches)
3. Both yeast and bacteria have a probability of sticking and a separate probability of unsticking. These numbers are influenced by the concentrations of bacterial small molecules and appropriate biofilms.
    a. Bacterial SM increase the chance for bacterial adherence and decrease the chance for yeast adherence
    b. Biofilms increase the chance of adherence for their respective agent type and currently do not have an influence on the other agent type
4. If yeast or bacteria are stuck for a set amount of time they become stuck permanently as biofilm and start secreting their respective biofilm.

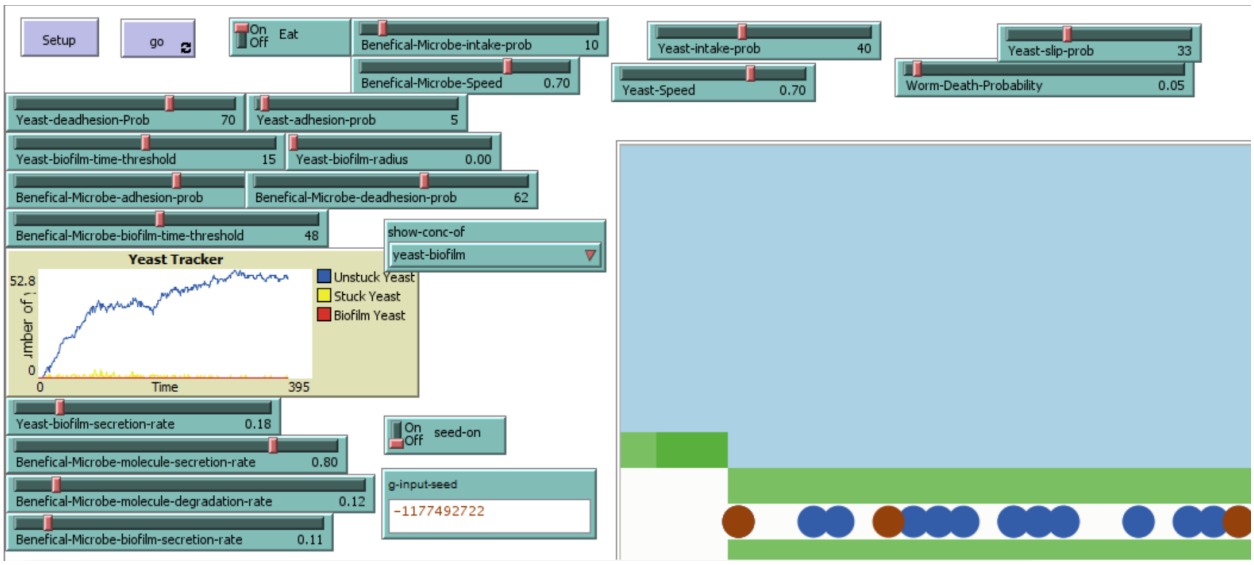
Model Rules Update (8/10/20-- version 2.5.1)

Above rules apply except:

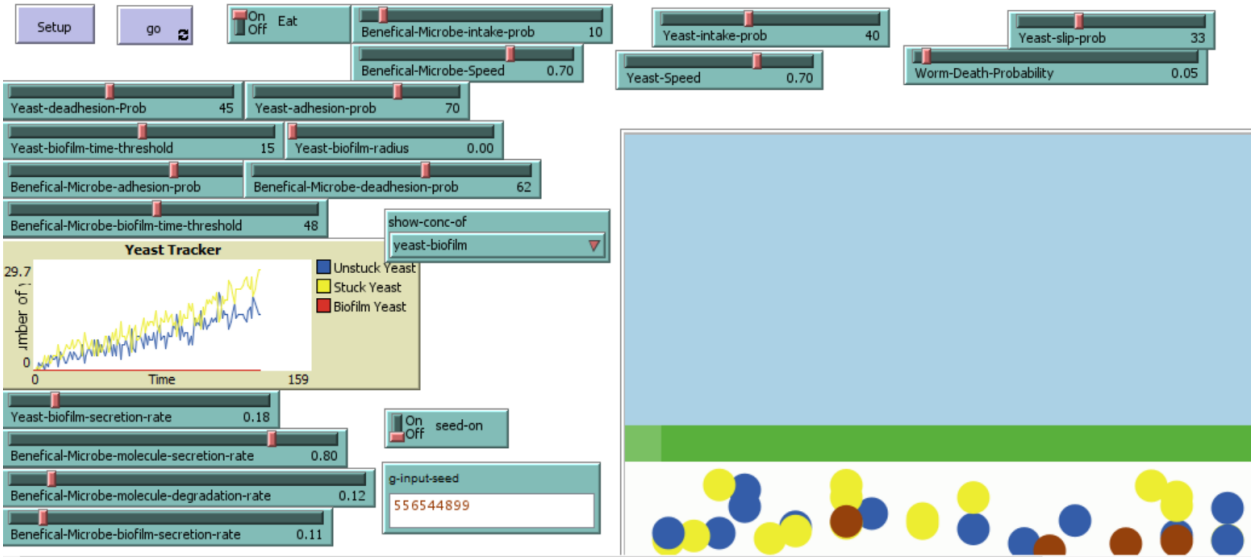
1. Bacterial SM no longer increases bacterial adherence
2. Yeast and bacteria enter biofilm when others of their kind are also able to progress into a biofilm within a certain radius
3. Bacteria now limited to moving within the lumen
4. Yeast may slip by one another as a certain probability, which is influenced by the amount of yeast biofilm present
5. Amount of bacteria entering the worm may now be increased substantially in comparison to the amount of yeast

Appendix 3 -- Model Presets

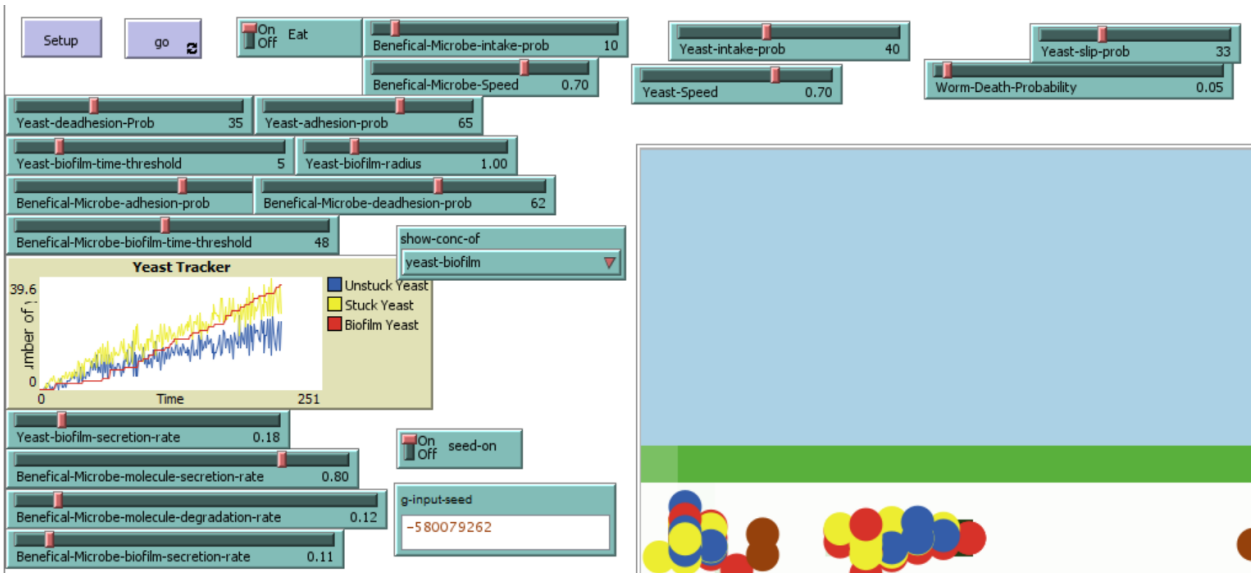
Middle Intestine Model - Phase 1



Middle Intestine Model - Phase 2



Middle Intestine Model - Phase 3

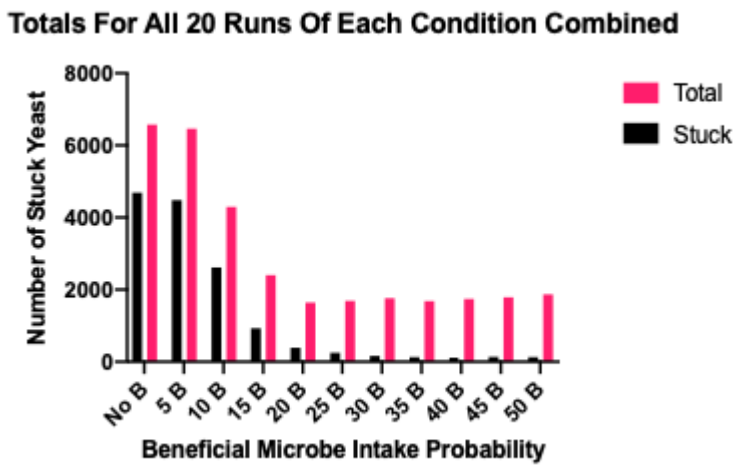

Setup
go
On
Off
Eat
Benefical-Microbe-intake-prob 10
Benefical-Microbe-Speed 0.70
Yeast-intake-prob 40
Yeast-Speed 0.70
Yeast-slip-prob 33
Worm-Death-Probability 0.05
Yeast-deadhesion-Prob 35
Yeast-adhesion-prob 65
Yeast-biofilm-time-threshold 5
Yeast-biofilm-radius 1.00
Benefical-Microbe-adhesion-prob
Benefical-Microbe-deadhesion-prob 62
Benefical-Microbe-biofilm-time-threshold 48
show-conc-of
yeast-biofilm
Yeast Tracker
39.6
0
Time
0
251
Unstuck Yeast
Stuck Yeast
Biofilm Yeast
Yeast-biofilm-secretion-rate 0.18
On
Off
seed-on
Benefical-Microbe-molecule-secretion-rate 0.80
Benefical-Microbe-molecule-degradation-rate 0.12
g-input-seed
-580079262
Benefical-Microbe-biofilm-secretion-rate 0.11

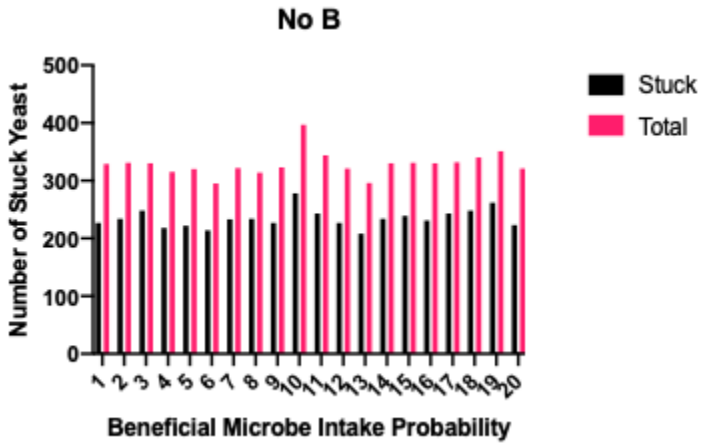
Appendix 4 -- Current Rules

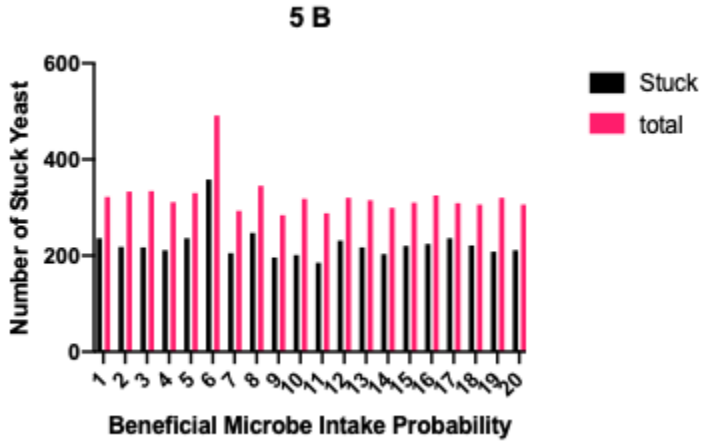
Current model rules-- 9/30/20

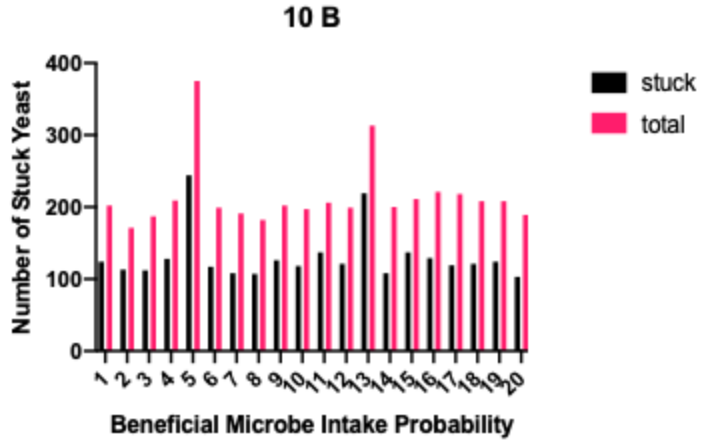
1. Yeast and bacteria move down the lumen at a particular rate, but bacteria and yeast can slip by within a certain probability (for yeast this is influenced by the amount of yeast biofilm present).
2. Bacteria secrete a small molecule (SM) at a particular rate and the molecule degrades at a particular rate. (SM is degraded by patches)
3. Both yeast and bacteria have a probability of sticking and a separate probability of unsticking. These numbers are influenced by the concentrations of bacterial small molecules and appropriate biofilms.
    a. Bacterial SM decreases the chance for yeast adherence
    b. Biofilms increase the chance of adherence for their respective agent type and currently do not have an influence on the other agent type within a certain radius of their respective agent; yeast only secrete biofilm if they are within range of yeast, and been there for a bit
4. Yeast and bacteria enter a biofilm state when others of their kind are also able to progress into a biofilm within a certain radius
5. A set amount of time must pass with yeast or bacteria in the "stuck" state and near other stuck agents for their respective agents before becoming stuck permanently as biofilm, thus secreting their respective biofilm.
6. We also talked today about adding in probiotic yeast to this model, so that we could potentially generate data for the proposal related to Rao lab published work.using probiotic yeast instead of probiotic bacteria.
7. If more than 2 stuck/biofilm yeast is located on the lumen patch shift in a specific pattern. Or shift by half on either side
8. The bacteria is now probiotic yeast
    a. Acts more like the previously coded yeast in shape, size, movement. It still has the same positive effects on the biofilm

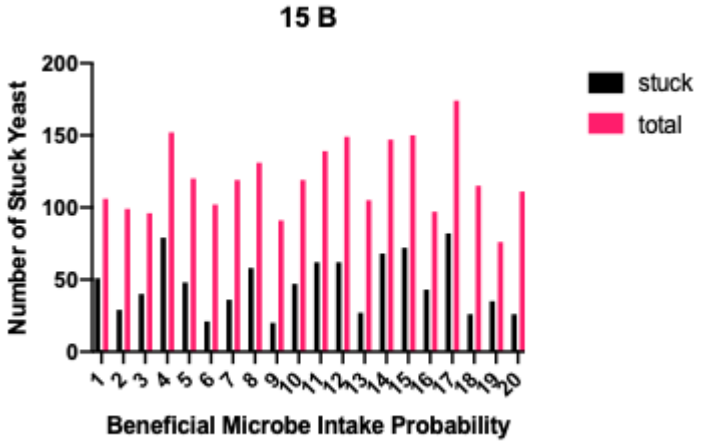
Appendix 5 -- Data Graphs

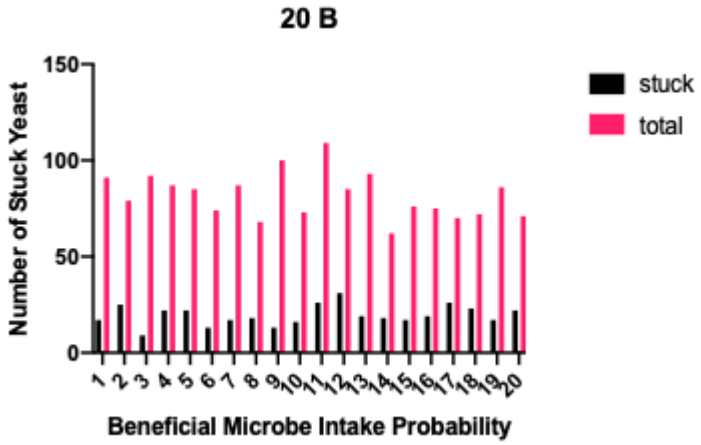


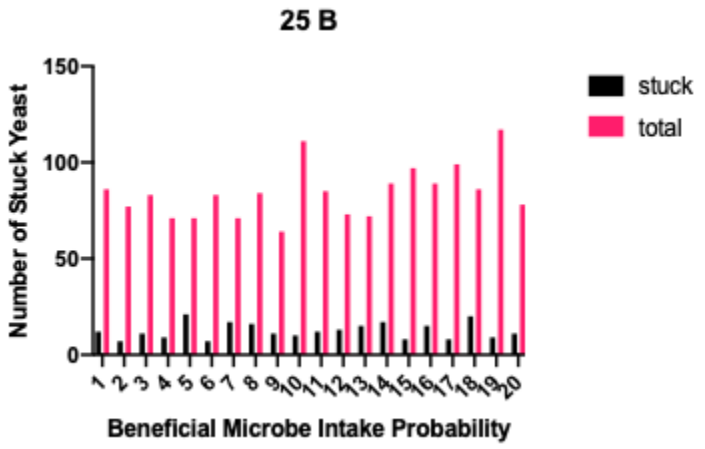


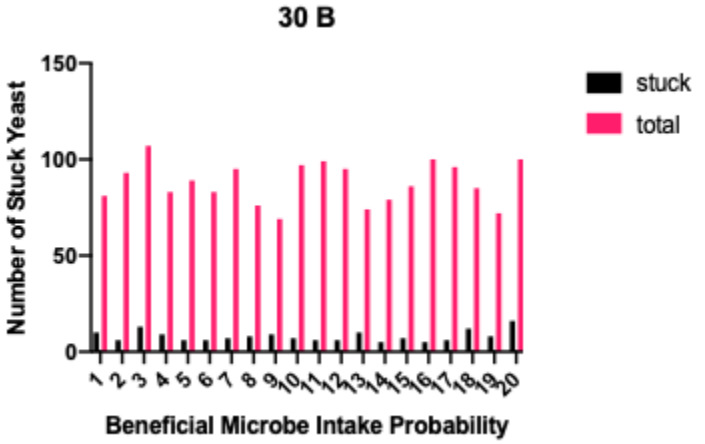


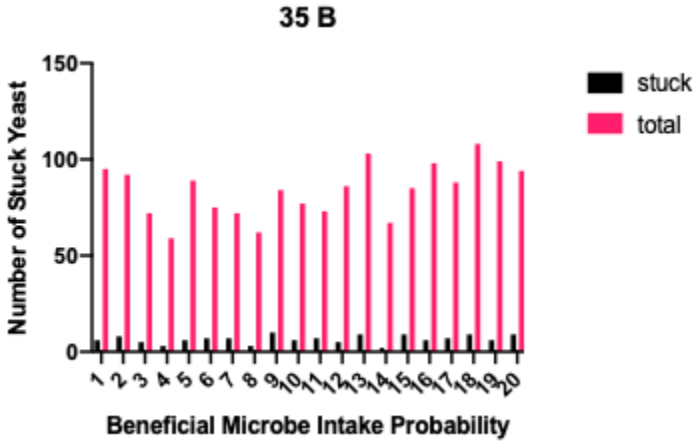

10 B
Number of Stuck Yeast
400
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
stuck
total

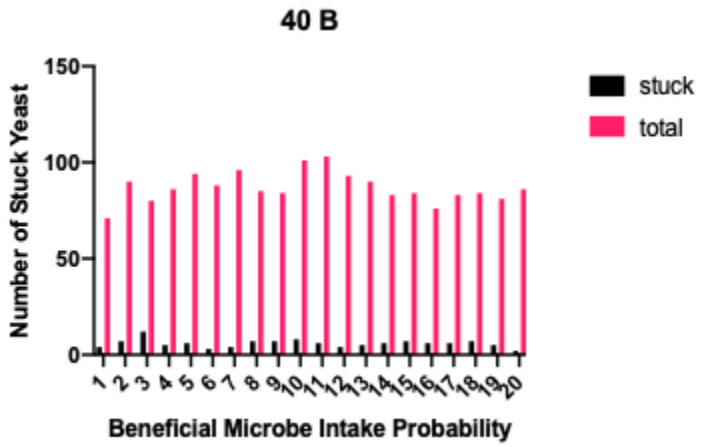

15 B
Number of Stuck Yeast
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
stuck
total


20 B
Number of Stuck Yeast
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
stuck
total


25 B
Number of Stuck Yeast
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
stuck
total


30 B
Number of Stuck Yeast
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
stuck
total


35 B
Number of Stuck Yeast
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
stuck
total


40 B
Number of Stuck Yeast
150
100
50
0
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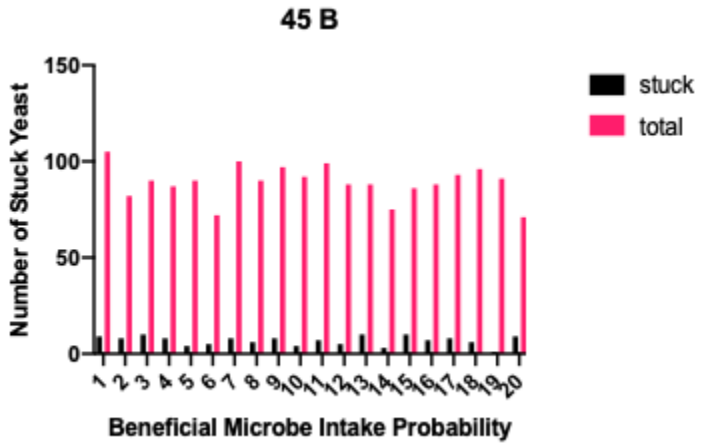
Beneficial Microbe Intake Probability
stuck
total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Number of Stuck Yeast
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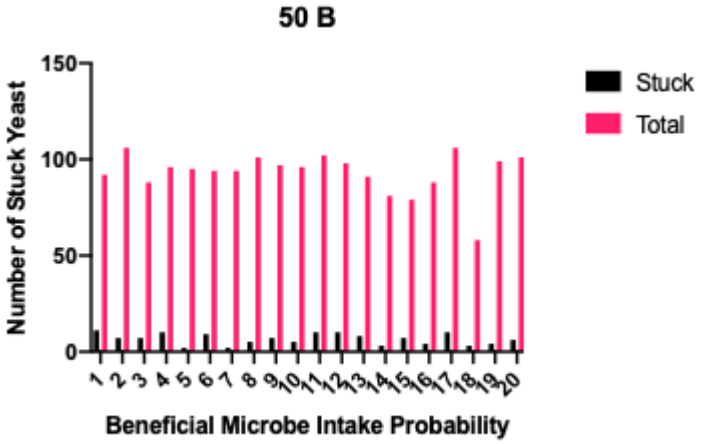
Beneficial Microbe Intake Probability
stuck
total


50 B
Number of Stuck Yeast
150
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Beneficial Microbe Intake Probability
Stuck
Total