The Role of the Map Kinase Pathway in *C. elegans* Immune Response Against *C. albicans*

A Major Qualifying Project Report submitted to the Faculty of Worcester Polytechnic Institute in the partial fulfillment of the Degree of Bachelor of Science.

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Abstract

The term “butterflies in the stomach,” stems from the complex bidirectional communication between the gut and brain. *C. elegans* worms are a great model to understand this pathway with their similar digestive pathway and their innate immune system conserved in humans. This research focuses on the link between innate immunity and the learned behavior of pathogen avoidance. Through the measurement of gene expression by qPCR after exposure periods to fungal infections (*C. albicans*) the research will show the correlation between the immune system and the gut-brain pathway.

Acknowledgments

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Table of Contents

Introduction

1.1 Gut Health and Mental Health: The Gut-Brain Axis

The Gut-Brain Axis, an exciting area of study in biology, explores the complex, bidirectional communication between the gut and brain. This work focuses on the gut microbiota, which has the ability to influence the psychological aspects of the brain. The gut is home to the enteric nervous system which governs the "brain of the gut." The gut contains a network of autonomic neurons that connect the central nervous system to the gastrointestinal tract. It is known that the neurons of the brain travel a complex, dense pathway, but it is also similar to the neuronal components of the gut (Bargman, 2005). The neurons of the gut receive direct input and transmit the information from that input to the remainder of the autonomic nervous system (Gautron, 2013). Communication between the gut microbiota and the central nervous system relies heavily on short-chain fatty acids (SCFAs), secondary bile acids (2BAs), and tryptophan metabolites known as microbial-derived intermediates. (Osadchiy, 2019).

Several sources contribute to the research that neurotransmitters play a large role in communication between the gut and the brain. Specific neurotransmitters: serotonin, norepinephrine, epinephrine, GABA, and dopamine, play a large role in the relationship between the gut and brain and disease (O'Donnell, 2020). When these neurotransmitters are deficient in the gut, it directly correlates to brain activity specifically functions in mood, sleep, and behavior regulation (Gautron, 2013). Mental health-related disorders alter the levels of these neurotransmitters and when they are in lower amounts or in abundance in the body, this affects the gut-brain axis as a fair amount of these neurotransmitters lie directly in the gut. Dysregulation in any of these neurotransmitters plays a large role in health and activity. (Mittal, 2017)

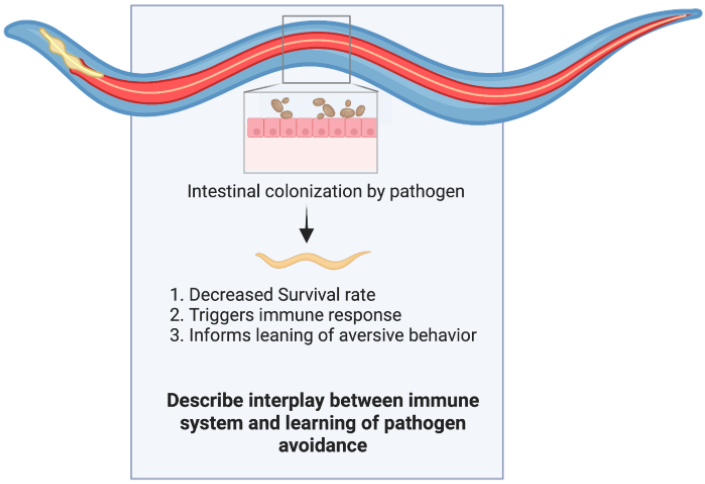
According to the World Health Organization (WHO), the consumption of probiotics contributes to an increase in mood, immune system support, improvement in skin health, and enhances resistance to allergens. Microorganisms in the gut contribute to this, creating the gut microbiota. The gastrointestinal (GI) tract is home to various microorganisms that contribute to overall human health. Studies in recent years have shown that the microbiota directly works in correspondence with the brain affecting aspects of brain function, behavior, emotions, and brain systems (Mohajeri, 2018).

1.2 *Caenorhabditis elegans* Model Organism

Caenorhabditis elegans or commonly called *C. elegans* are nematodes that have become a popular choice of model organisms in the field of biology. They are easy to maintain, generate large amounts of offspring over a short period of time, and are one of the first genomes of a multicellular organism to be sequenced. (YourGenome, 2021) These worms are very small, roughly 1mm in size, with a life span of about 2.5 weeks, producing numerous offspring throughout their lifetime. *C. elegans* worms have a simple system and even similar life stages. They go through 4 larvae stages, L1-L4, into adulthood, and then death, making them a fantastic model as results are seen in a short period of time (Meneely, 2019).

Starting from its mouth to the anus, the digestive system of the nematode is essentially a tube that runs the length of the animal. *C. elegans* are transparent making it easy to visualize the contents of their alimentary canal. Furthermore, the experimental tools available in this model make is easy to study infections of the intestine. (Elkabti, 2018). Facets of the innate immune system of the worm are conserved in humans making it a relevant model to study the host response to pathogens that colonize the digestive system (WormAtlas, 2019). The digestive system of *C. elegans* also mimics mammalian gut morphology as it contains microvilli which are finger-like membrane protrusions. Microvilli are important for nutrient absorption which makes this animal an incredible study model as it is similar to the mammalian gut (WormAtlas, 2019).

C. elegans are a functional model for researchers because of their simple nervous system. *C. elegans* have roughly 350 neurons which makes them an excellent model to study, considering the average human has 86 billion neurons (Meneely, 2019). Most of these neurons have a simple structure with only one or two processes that exit the cell body. (Zhang, 2020). The unique component of these neurons is that their axons both give and receive synapses, which makes their nerve conduction extremely passive with no sodium-potassium-dependent action potential (WormAtlas, 2019). They have a similar nervous system set up to humans and have similar neurotransmitters including the discussed serotonin, norepinephrine, epinephrine, and dopamine (Meneely, 2019). *C. elegans* do not have sight and perception so they rely on their olfactory system to communicate. They have many chemosensory neurons through G-coupled-Protein receptors for signaling and communication (Corsi et al., 2015).



<u>Figure 1:</u> Image of the C. elegans worm and how a pathogen affects the intestinal integrity of the worm with a link to innate immunity and pathogen avoidance.

Another important component of *C. elegans* is how their immune system is conserved in humans and the adaptability of their immune system (Figure 1). It has been seen that this animal has been chosen as a model to learn more about innate immunity, but also how it defends itself against infection (Gravato-Nobre, 2005). There are many links between the genes of *C. elegans* and the pathways associated with different processes. These molecular mechanisms are common and linked to immunity (Fabian, 2021).

1.3 Molecular Components of *C. elegans*

The goal of this research is to observe the expression of genes at the beginning of infection and then after a time period with distinct checkpoints. These genes will be correlated with the living evidence of *C. elegans* on the lawn to see if there is solely a link between the gut-brain axis, or if manipulation of genes plays a role. The genes tested include *PMK1, NPR1, FLP18, FLP21,* and *DAF7.* Some of these pathways can be seen in Figure 2.

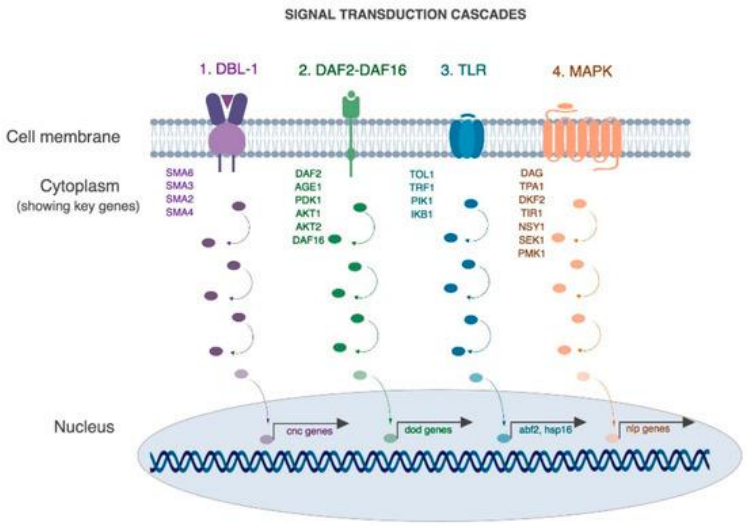


Figure 2: Found in the Elkabti, 2018 paper discussing the molecular pathways that play a central role in mounting immune response in C. elegans. This includes the DBL-1 pathway, the insulin signaling pathway, the Toll pathway, and the MAPK pathway (Elkabti, 2018).

1. **PMK1 (Mitogen-Activated Protein Kinase 1)** is a signaling pathway that regulates the expression of genes in response to pathogenic challenges in the nematode worm Caenorhabditis elegans (Shivers, 2008). PMK1 pathway activation can increase susceptibility to pathogens, but it is also essential for defense against intestinal pathogens. The PMK1 pathway is thought to be an independent pathway that contributes to the induced immune response in C. elegans. By looking at this pathway and any mutations in this pathway, the induced immune response can be observed.

2. **NPR1 (Neuronal PAS Domain-containing Protein 1)** is a transcriptional co-activator that plays a role in the regulation of behavioral differences in C. elegans. *NPR1* is linked to aero taxis behavior, which is the response of nematodes to changes in the concentration of oxygen and carbon dioxide. NPR1 is also associated with a neuronal globulin domain protein that affects the behavioral response to changes in the concentration of CO2 and O2 (Sterken, 2015).

3. **FLP18 (FMRFamide-Like Peptide 18)** is a gene that encodes six distinct FMRFamide-like peptides that share a common C-terminal, which is linked to a loop on NPR1. FLP18 is linked to a decrease in the odor response after starvation, which suggests a role in the regulation of sensory activity. FLP18 is also involved in activating NPR1 signaling, which decreases neuronal and circuit activities (Li, 2014) .

4. **FLP21 (FMRFamide-Like Peptide 21)** is a gene that is directly related to chemotaxis and is involved in transmitting stress signals through its receptor NPR1. A lack of the FLP21 gene results in aggregation behavior, which shows a link between FLP21 and NPR1. Mutations in the FLP21 gene result in increased thermal thresholds of heat avoidance, suggesting that activation of this gene decreases the thermal threshold (Li, 2014).

5. **DAF7 (Dauer Formation 7)** is a gene that is directly related to the regulation of serotonin and the storage of fat in C. elegans. DAF7 is involved in the regulation of glucose metabolism and is thought to mediate a significant portion of the signaling pathway in C. elegans (Wu, 2022). DAF7 also has a role in regulating serotonin input in chemosensory neurons, which are involved in the perception of chemical signals (Zheng, 2011).

1.4 Fungal Pathogens: *Candida albicans*

Candida albicans (C. albicans or F15*)* is a yeast found in the gastrointestinal tract and is responsible for a variety of infections that can range in lethality (Poupet, 2019). It is the most common fungal pathogens found in human health, accounting for thousands of infections and mortalities. It has recently been seen that this fungus is becoming resistant to the leading antifungal agents on the market. New research has proposed new ideas for the approach to treating infections of *C. albicans* (Poupet, 2019). This research will focus on exposing *C. elegans* to these fungal pathogens over several hours of infection periods to see if there is any alteration at the molecular level.

1.5 Caenorhabditis elegans as gut-brain axis Model with C. albicans

It is important to note the flexibility of *C. elegans* as they can be used as a model to understand more about *C. albicans.* Because the intestine of *C. elegans* is similar to the intestinal cells of a human, they are used as a model to understand more about the GBA (Murphey, 2019). The traditional food for worm growth is the Escherichia coli strain, OP50, as it is nutritious for the worms and not harmful. Research that exposed worms to *C. albicans* as a food source, led to infection in the *C. elegans* worm, distention of the gut, and premature worm death (Pukkila-Worley, 2011).

It is also known that the gun-brain axis contributes to overall health because of the benefits this pathway has on the immune system. *C. elegans* worms are known. The immune system of these worms does not function by phagocytic activity, but rather through the mounting of epithelial cells. (Albany, 2019). This is similar to the microvilli found in mammals, which makes them an impactful model for studying the immune system and gut microbiota. Due to the transparent nature of the worms, the entire length of a fungal pathogen going through the worm can be observed and analyzed, contributing to the knowledge of fungal pathogens through the human digestive system (Pukkila-Worley, 2011).

1.6 Goals and Objectives

The goal of this research is to describe the connection between the immune system and the learned behavior of pathogen avoidance. As worms are the perfect model to understand innate immunity with immunity conserved in humans, by exposing them to pathogens, the expression of key genes involved in the immune response pathway during this infection can be measured.

Methodology

2.1 Lab Conditions and Worm Conditions

The N2, or wild-type strain of *C. elegans* worms is used for this project in gene exploration. These worms have been grown and maintained throughout the lab, coming from a frozen stock of worms that are thawed and maintained. The worms are grown on Nematode Growth Medium (NGM) agar plates that are seeded with *Escherichia coli* (*E. coli)* OP50. OP50 is grown in LB broth overnight at 37°C on a shaker and then stored overnight at 4°C.

2.2 Egg Preparation

Worms were maintained at the L4 stage on NGM agar plates, seeded with OP50 at 20°C until they had aged for three to four days, and there was a substantial presence of adult worms and eggs on the plate (Figure 3). Once this was achieved, the worms and eggs were washed off the plates with M9 buffer (14mL). The buffer and worm mixture was left in a tube stand until a pellet formed and there were no worms seen in the buffer. The supernatant was discarded and resuspended in M9 buffer (14mL), and the process was repeated two more times until the worms and eggs were washed. After the third wash, the supernatant was aspirated, and the worms were immediately resuspended in a bleaching solution (5mL). The suspension was gently mixed by inverting the tube for roughly two minutes until the adult worms had disintegrated and only eggs were left. This was confirmed under a dissection microscope. M9 was then added to the remainder of the solution (up to 14mL) and centrifuged for five minutes at 2,500 rpm. The supernatant was then aspirated, and the pellet was resuspended in M9 buffer. This process was repeated three times. Finally, after the final supernatant was aspirated, the pellet was resuspended in 200µL of M9 buffer and plated respectfully on several NGM agar plates, seeded with OP50 (Issi, 2017).

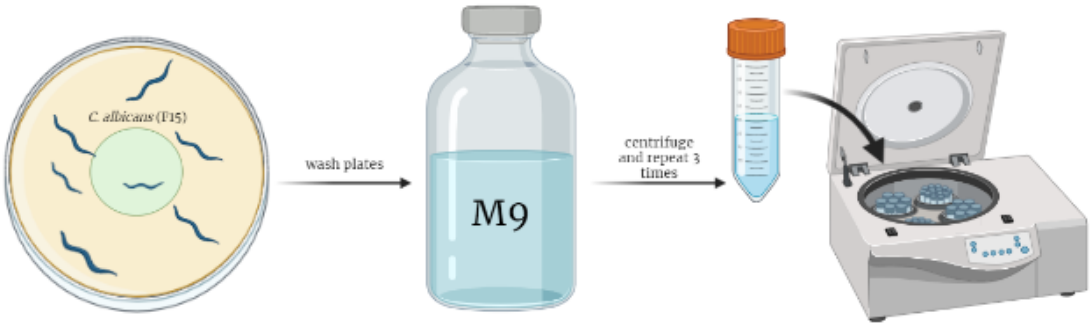
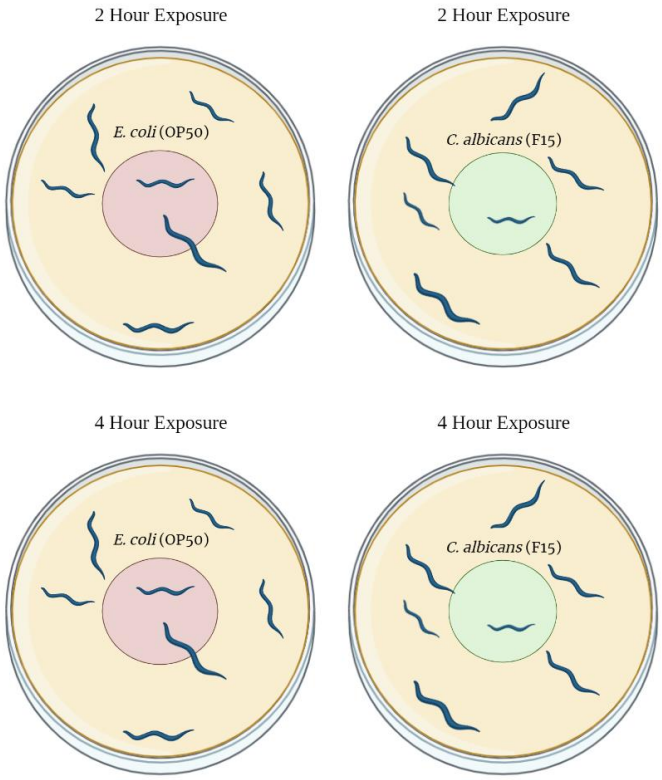


Figure 3: The process involved in making plates during an egg preparation for the infection. Worms are washed off plates with M9 and spun down to just eggs.

2.3 *C. albicans* Infection

To understand the effect *C. albicans* has on the worms as a fungal pathogen, the worms were infected with the standard laboratory strain of *C. albicans* Sc5314. After the egg preparation, the worms were left to grow in OP50-seeded agar plates for two days, or until they had reached the L4 stage of the life cycle. Several plates were made, labeled as two hours or four hours, and seeded with F15 or OP50. Once at the phase, the worms were transferred to respective plates with either a two-hour or four-hour infection (Figure 4). Typically, triplicates were done for each infection period to have enough worms to run the next part of the experiment. The worms were left on their plates for the respective hours and washed off with M9 solution. The worms were washed well so there were no remnants of strain *Sc5314* by resuspending the pellet in different volumes of M9 multiple times. After the supernatant was removed, roughly 500ul of M9 was left in the tube with the worms and transferred to an RNAse tube. In the fume hood, Trizol (1mL), an RNA extraction agent, was added to the tubes and the tubes were stored in the -80-degree freezer until ready for the RNA extraction (Issi, 2017).



<u>Figure 4:</u> This figure demonstrates the two time periods of exposure during the infection period tested on the worms in two different conditions. These plates were done in triplicates for analysis with substantial amounts of worms for each mutant.

2.4 RNA Extraction

To begin the RNA extraction protocol, the tubes were flash-freeze in liquid nitrogen and thawed on a heat block of 37C. This step was repeated four additional times and vortexed in between. The tube was then vortexed for 30 seconds and rested for 30 seconds and repeated four additional times.

Next, for the RNA isolation from the Trizol Sample, chloroform (200uL) was added to the sample under the fume hood. The sample was shaken vigorously for 15 seconds and incubated at room temperature for three minutes. The sample was then centrifuged at room temperature at 10,000 rpm for 5 minutes. The upper phase of the tube was then transferred to an RNase-free tube. The volume of the upper volume was noted before continuing to the next step. 100% Ethanol was added (1.25 volumes) and pipetted into a filter cartridge. The mixture was then centrifuged for 15 seconds to pass through the filter and the flow through was discarded. The Wash Solution 1 was then added to the filter cartridge (700ul) and centrifuged again for 15 seconds. The filter was then placed in the same tube and the 2/3 Wash Solution was used (50ul) and drawn through the filter. This process was repeated twice. The filter and tube were spun once more time for 1 minute to remove any excess liquid. The filter was transferred to a new tube and 95 C nuclease-free water was added to

the filter (20ul) and spun. The eluate which contained the RNA was then measured via Nanodrop to measure the concentration with Qubit RNA. The final concentration was then stored at -80C (Rochester University, 2023).

2.5 cDNA Preparation

To conduct a cDNA analysis of the samples, a reverse transcription (RT) master mix was prepared based on the nanodrop findings and RNA concentration levels of each sample. This RT master mix was placed on ice as the tubes were prepared. To prepare the tubes, the RT master mix was pipetted into each PCR tube (10ul). A volume (10ul) of the RNA sample was pipetted into the tube as well and the tubes were sealed and centrifuged to spin down all contents and eliminate air bubbles. The plate was then loaded into a thermal cycler and the reaction was run at different temperatures over the course of two hours (Applied BioSystems, 2006)

2.6 qPCR Analysis

The last step to gain a full analysis of the samples was analysis through qPCR (Figure 5). To begin, plates were made to have correct volumes of cDNA and primers. These calculations were done by diluting the cDNA and mixing it with primers. The master mix was prepared as well and placed in the qPCR tube.

Figure 5: Gene expression analysis was done via cDNA samples by analysis through qPCR.

A volume (2ul) of cDNA sample was placed in the tube and 8ul of master mixed was placed as well. Several tubes were set up with housekeeping genes as controls and comparisons. The housekeeping gene used for this experiment was *CDC-42*. This gene was used in comparison as it is always expressed in the cell. To set up the qPCR machine, the plate layout was inserted into the software, the dye SYBR was used, and no quencher was involved, the target was labeled as the correct target gene and the qPCR ran for two hours (Nguyen, 2020). The values were then taken

and a series of averages of the Delta Delta value were taken, and the standard deviation was done. These values were then graphed, and the data was represented.

Results

To fully analyze the expression of genes in *C. elegans* immune response to fungal pathogen, several statistical analyses were performed to obtain values of comparison. It is important to note that in each plate run for the qPCR, housekeeping genes were used to always have a comparison. Housekeeping genes always express despite environmental differences, including a normal environment versus an environment with a fungal pathogen.

It also must be noted that several genes were discussed in terms of expression, however, in this research, *PMK1*, *NPR1*, and *DAF7* were analyzed by qPCR to understand more about expression in wild-type worms. Relative gene expression in *PMK1* was much greater after four hours of exposure to a pathogen compared to two hours. The substantial difference in gene expression can be noted (Figure 6) demonstrating that in longer periods of fungal pathogen infection exposure, there is greater relative gene expression.

By taking the averages of the Cq values, which are the overall expression of the gene, it can be seen in Table 1 and Table 2 that the housekeeping gene averages were taken in all exposure.

cdc-42			AVERAGE		PMK		AVERAGE		^CT	^Ct Gen - ^ C	2^-CT
wt1	23.63963	17.29236	20.465995		24.95126	18.87104	21.91115		1.2245325		
wt2	20.11926	21.69522	20.90724		23.57718		23.57718		2.8905625		
			20.6866175						2.0575475		
F15 1		17.98132	17.98132		18.92574	17.72948	18.32761		1.65676	-0.4007875	1.32022837
F15 2	17.15277	13.56799	15.36038		18.80914	14.74156	16.77535		0.1045	-1.9530475	3.87191559
			16.67085						0.88063		1.80431534

cdc-42			AVERAGE		NPR1		AVERAGE		^CT	^Ct Gen - ^ C	2^-CT
wt1	23.63963	17.29236	20.465995		21.46537	15.47329	18.46933		-2.2172875		
wt2	20.11926	21.69522	20.90724		17.87321	21.64554	19.759375		-0.9272425		
			20.6866175						-1.572265		
F15 1		17.98132	17.98132		17.64657	21.65527	19.65092		2.98007	5.1973575	0.02725458
F15 2	17.15277	13.56799	15.36038			19.49962	19.49962		2.82877	3.7560125	0.07401633
			16.67085						2.90442		0.03306555

cdc-42			AVERAGE		DAF7		AVERAGE		^CT	^Ct Gen - ^ C	2^-CT
wt1	23.63963	17.29236	20.465995		13.95523	17.98132	15.968275		-4.7183425		
wt2	20.11926	21.69522	20.90724		15.30869	20.80593	18.05731		-2.6293075		
			20.6866175						-3.673825		
F15 1		17.98132	17.98132		26.4667838	26.885588	26.6761859		10.0053359	14.7236784	3.696E-05
F15 2	17.15277	13.56799	15.36038		25.9905379	25.9563346	25.9734363		9.30258625	11.9318938	0.00025594
			16.67085						9.65396106		0.00015484

*Table 1: The averages of gene expression for each gene at **2 hours** were taken and compared to the housekeeping gene, cdc-42. The CT value was used for each, and the standard deviation was taken and graphed.*

cdc-42			AVERAGE		NPR1		AVERAGE		^CT	^Ct Gen -	2^-CT
wt1		22.34415	22.34415		25.17807		25.17807		3.554225		
wt2	22.75729	19.04979	20.90354		20.62039	23.70163	22.16101		0.537165		
			21.62385						2.045695		
F15 1		24.8712	24.8712			24.98402	24.98402		0.311837	-3.24239	9.46359
F15 2	23.48473	25.4616	24.47317		18.19294	25.95864	22.07579		-2.59639	-3.13356	8.775963
			24.67218						-1.14228		0.486225

cdc-42			AVERAGE		DAF7		AVERAGE		^CT	^Ct Gen -	2^-CT
wt1		22.34415	22.34415		23.29217	26.93514	25.11366		3.48981		
wt2	22.75729	19.04979	20.90354		25.52864		25.52864		3.904795		
			21.62385						3.697303		
F15 1		24.8712	24.8712		18.48341		18.48341		-6.18877	-9.67858	819.49
F15 2	23.48473	25.4616	24.47317		16.86524	23.84766	20.35645		-4.31573	-8.22053	298.2808
			24.67218						-5.25225		368.5505

cdc-42			AVERAGE		PMK1		AVERAGE		^CT	^Ct Gen -	2^-CT
wt1		22.34415	22.34415		24.10625	19.13478	21.62052		-0.00333		
wt2	22.75729	19.04979	20.90354		24.84172	25.37855	25.11014		3.48629		
			21.62385						1.74148		
F15 1		24.8712	24.8712		22.18722	21.34879	21.76801		-2.90418	-2.90085	7.46865
F15 2	23.48473	25.4616	24.47317			23.89651	23.89651		-0.77567	-4.26196	19.18574
			24.67218						-1.83993		8.285234

*Table 2: The averages of gene expression for each gene at **4 hours** were taken and compared to the housekeeping gene, cdc-42. The CT value was used for each, and the standard deviation was taken and graphed.*

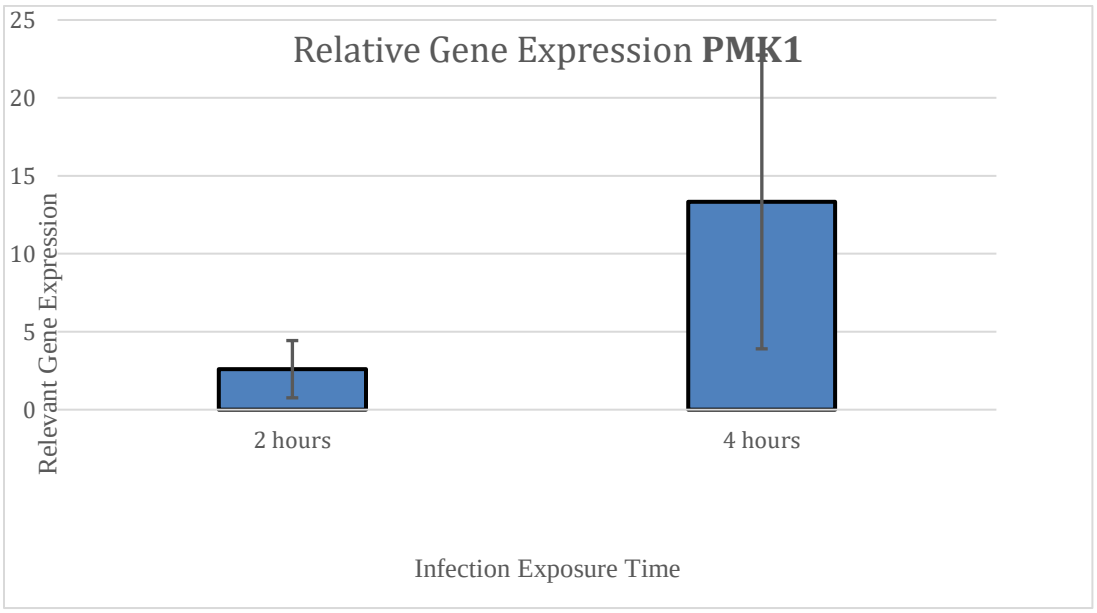


Figure 6: Graph representing the relevant gene expression of the PMK1 Primer over 2 and 4 hours of infection exposure to C. albicans.

A similar expression is seen in the *NPR1* gene as well, heavily expressed after 4 hours of infection (Figure 7). The averages of overall gene expression of these specific genes were compared to the averages of housekeeping genes that are always expressed in the worms. The *DAF7* expression was also upregulated in the presence of the fungal pathogen (Figure 8)

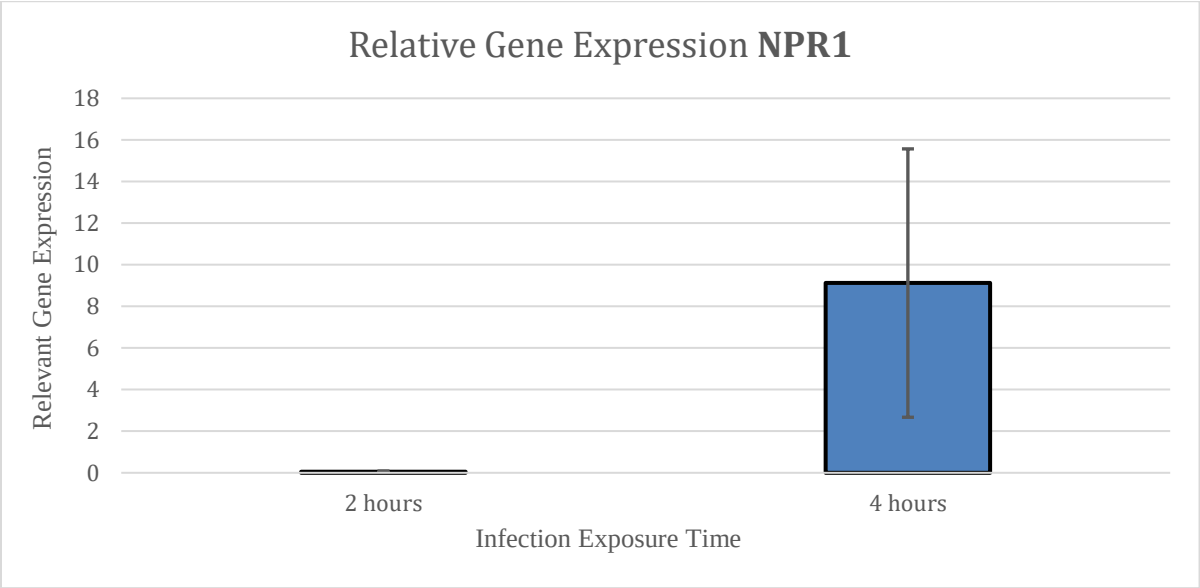


Figure 7: Graph representing the relevant gene expression of the NPR1 Primer over 2 and 4 hours of infection exposure to C. albicans.

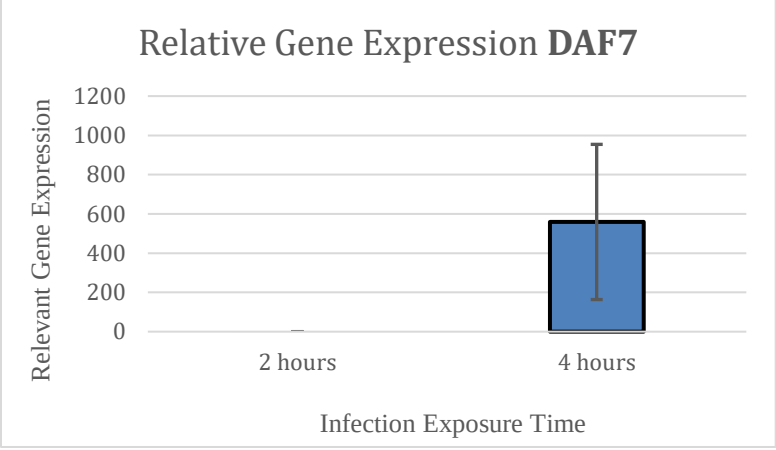


Figure 8: Graph representing the relevant gene expression of the DAF7 Primer over 2 and 4 hours of infection exposure to C. albicans.

Discussion

Overall, the gene expression of all genes increased in longer exposure to the fungal pathogen *C. albicans.* It is important to note the significant upregulation of genes when exposed. Genes are more likely to be expressed in the presence of a pathogen. The upregulation of genes directly correlates with the response pathway that may cause a cell to increase in the activity of a specific regulatory factor.

This suggests the link to pathogen avoidance and innate immunity. Recalling from earlier, *PMK1*, *NPR1*, and *DAF7* all play a significant role in the regulatory aspects of the worm. They regulate worm behavior, pathogen avoidance, neurotransmitters, and response. These genes are a part of the innate immune pathway that activates the nervous system in terms of pathogen avoidance.

These genes play a key role in this pathway linkage, but it is important to note other genes that are involved in the same process. These genes include *FLP18* and *FLP21* which are linked to sensory activity. With the testing of these genes, it can be noted that there is sensory avoidance of a pathogen in exposure to the pathogen.

General Implications and Future Directions

Through the quantitative observation of relative gene expression, initial findings can conclude that with a longer period of exposure, there is an upregulation of *PMK1, NPR1, and DAF7* genes in the *C. elegans* worm. These genes are a part of the innate immune pathway that activates the nervous system and mediates avoidance behavior. These findings align with other studies involving *C. elegans* and avoidance behavior.

In this project we developed standard protocols to probe specific innate immune genes and pathways in the gut that connect to specific neurons to illicit a response. Taking a few genes, and standardizing the process of preparing worms, infection periods, RNA extraction, and qPCR analysis. This standardization is essential for future research as the next steps would be to take more genes and perform analysis. In the next steps, it would be interesting to take worms that are deficient in specific genes and see how that changes the relative expression of genes.

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