Exploring the Impacts of Diet and Stress on Membranes in *C. elegans*

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Abstract

This study investigates how bacterial diets and stressors impact lipid metabolism in Caenorhabditis elegans (*C. elegans*). Nematodes were fed different bacterial diets and exposed to either glucose stress or food deprivation. Lipids from these animals were extracted and analyzed by HPLC and mass spectrometry. Preliminary observations for experiments analyzing dietary restriction using paraformaldehyde and oxidative stress using tert-butyl hydroperoxide were compared with the prior stress experiments. Bacterial diets influence phospholipid distribution, while stressors induce changes, from subtle to significant, in lipid composition, suggesting adaptive metabolic responses. These findings help explain the complex intersection between diet, stress, and lipid metabolism in *C. elegans*, warranting further investigation into various stressors.

Acknowledgments

We would like to extend our gratitude to Professor Carissa Olsen for her unwavering support, guidance, and mentorship throughout the duration of this project. Her expertise, encouragement, and continuous feedback have been invaluable in guiding the path and standard of our project. We are also deeply thankful to the graduate students in the lab, Mark Xatse, Alvan Okechukwu, and Paige Silvia, who generously shared their knowledge of lab procedures and provided us with the necessary training and resources to navigate the complexities of our research. Furthermore, we extend my appreciation to the dedicated undergraduate students who diligently carried out preparatory work to set up the lab, ensuring that we had a conducive environment to conduct our experiments. The collective support and contributions of Professor Carissa Olsen, graduate students, and undergraduate students have been indispensable to the success of this project, and for that, we are sincerely grateful.

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List of Terms

APCI – atmospheric pressure chemical ionization
C. elegans – Caenorhabditis elegans
E. coli – Escherichia coli
ESI – electrospray ionization
FA – fatty acid
GC – gas chromatography
HPLC – high-performance liquid chromatography
L-PC – lysophosphatidylcholine
L-PE – lysophosphatidylethanolamine
L1 – first larval stage of *C. elegans*
MS – mass spectrometry
MS/MS – tandem mass spectrometry orbitrap
MUFA – monounsaturated fatty acid
NGM – nematode growth media
O-PE – plasmanylethanolamine
PC – phosphatidylcholine
PE - phosphatidylethanolamine
PFA – paraformaldehyde
P-PE – plasmenylethanolamine
PUFA – polyunsaturated fatty acid
SFA – saturated fatty acid, has no double bond
TBHP – tert-Butyl hydroperoxide
VLC-PUFA – very long-chain polyunsaturated fatty acids

1.0 Introduction

Membranes are fundamental biological structures that act as a barrier that organizes cellular space while aiding intra and extracellular transport and signaling. Lipids are the major structural component of membranes. A specific class of lipids is crucial in the formation of biological membrane: phospholipids, which contain a polar head group with fatty acid tails attached. There are many types of head groups, including phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which are two highly abundant lipid species in many organisms. The composition of phospholipids reveals a deeper understanding of the structure and properties of biological membranes to look deeper into the complexity of metabolism. By diving deeper into studying these lipids, more discoveries can be made about various processes, like the organization of membranes and interaction of phospholipids and how these factors change in response to varying conditions like environmental stressors.

The goal of this project is to investigate the intersection of diet and stress and how these factors affect the membrane composition of *C. elegans*. *Caenorhabditis elegans (C. elegans)* is known as a model organism for studying scientific areas of metabolism, genetics, and biochemistry due to the easy to manipulate diet, small size, and ease of conducting mass spectrometry analysis methods. It's easy to control population, well studied genome, and brief lifespan determine it as a proper model organism in order to investigate various effects of diet and varying stressors to observe changes in lipid composition and metabolism. To understand the complexity of the intersection of diet and stress, it is necessary to comprehend exactly how changes in bacterial diets and stressors change the lipid composition in membranes in *C. elegans*. The lipid composition provides an insight on the metabolic shifts that the worm is conducting. In order to

investigate the intersection of diet and stress and subsequent effects on lipids, we focused on various experimental protocols, including using different strains of *Escherichia coli* (*E. coli*), excess glucose concentration, starvation conditions, dietary restriction using paraformaldehyde (PFA), and oxidative stress through tert-butyl hydroperoxide.

The selection of the dietary changes and stressors was based on previous relevant literature and relevant laboratory experiments where their ability to affect shifts in lipid membrane composition was explored. Through the use of high-performance liquid chromatography (HPLC) of lipids, we aimed to determine the significance of how the dietary changes and stressors impact the lipid profile, leading to dissection of the subsequent metabolic shifts and adaptive stress responses employed by the worms.

Through these experiments, our purpose was to gain a better understanding of the complexities in the relationship between diet, stress, and lipid metabolism in *C. elegans*, with possible future research ideas of exploring other modes of stress or applying this knowledge to study similar processes in other higher organisms, like humans. This investigation serves as a precursor to future research in metabolic regulation and aids in the understanding of how organisms respond to environmental factors, with possible explorations in the health and disease field.

2.0 Background

2.1 *C. elegans* as a Model for Studying Lipid Metabolism

C. elegans is a transparent nematode or roundworm that lives in temperate conditions of 15 to 25 °C (Rutter et al., 2019). It is pseudocoelomate, meaning that its body cavity is filled with fluid. It does not have specialized respiratory or circulatory systems and, therefore, depends on diffusion for gas exchange (Alkema, 2009). Most *C. elegans* are hermaphrodites, which are asexually reproducing organisms that possess two X chromosomes (Loxterkamp et al., 2021). *C. elegans* are relatively simple to study at a cellular level as they are transparent for the duration of their life cycle (Figure 1).

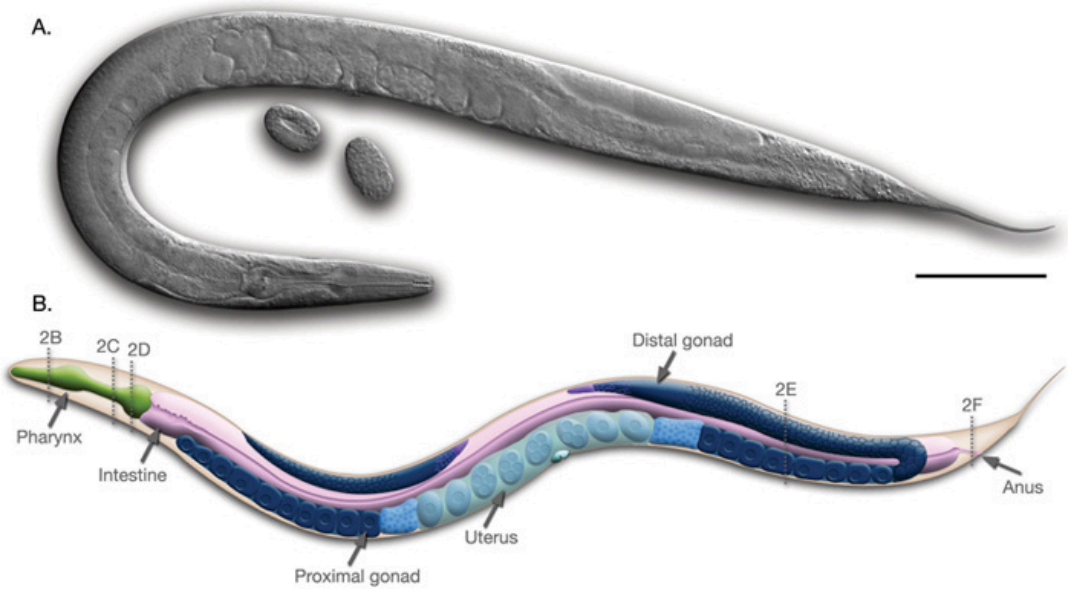


Figure 1: The Anatomy of Adult Hermaphrodite *C. elegans*.
Typical *C. elegans* used in laboratories are self-fertilizing hermaphrodites, as male worms are typically only observed in populations 0.1% of the time. The body of adult *C. elegans* has a simple anatomy, with around 1000 somatic cells, and is easy to observe with differential interference contrast microscopy due to its transparent nature (Altun, Z.F., 2009).

The laboratory strain of *C. elegans*, N2, has a 2-3 week lifespan depending on growth temperature and a 3-4 day generation time at 20°C (Urban et al., 2021). This short lifespan makes *C. elegans* a model organism for research because it can be observed throughout its several distinct phases of life within a 2-3 week time period, as shown in Figure 2. *C. elegans* are frequently used to study aging process and age-related diseases due to the advantages of a short lifespan. The diet of *C. elegans* is very easy to control or manipulate due to their ability to grow on solid plate-based media seeded with bacteria.

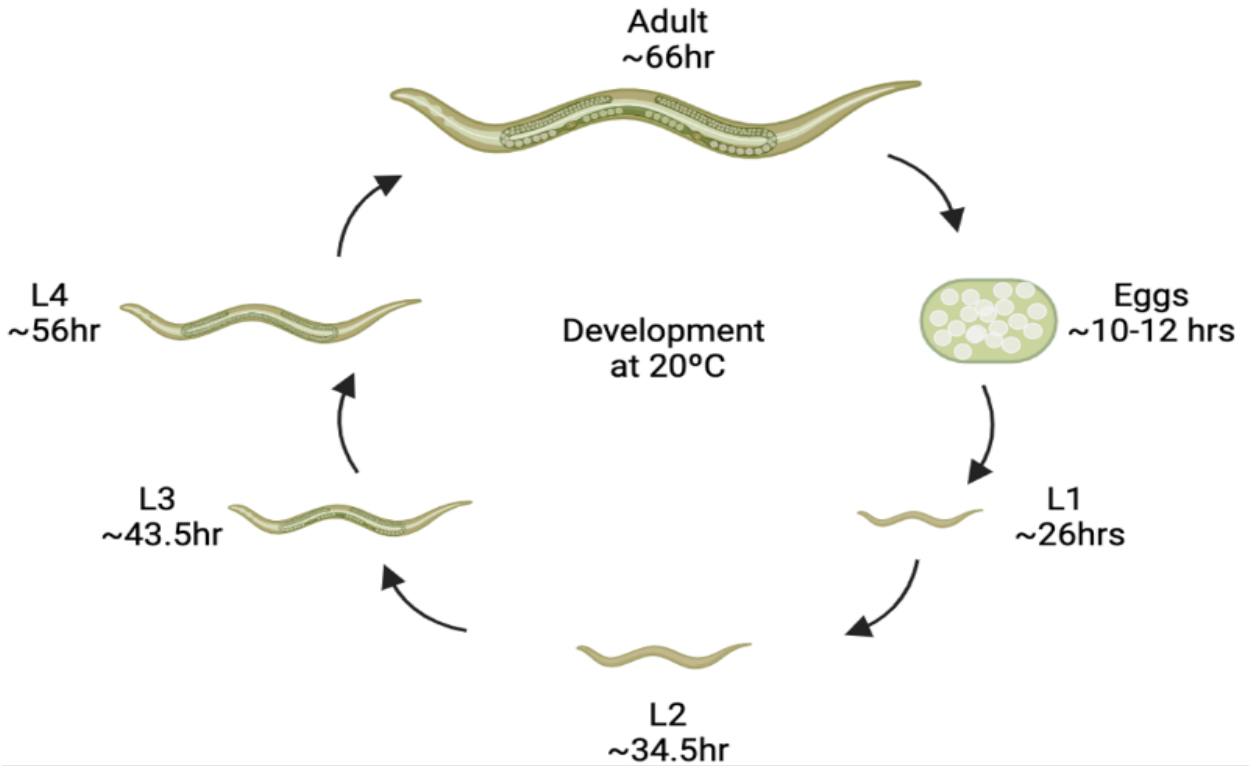


Figure 2: *C. elegans* Larval Developmental Cycle.
C. elegans go through four developmental larval stages, L1-L4, until they become adult worms. At 20 degrees Celsius, the developmental cycle of *C. elegans* unfolds with a series of key events that transition from egg to adult (Altun & Hall, 2009). First, the egg is laid, and after about 10-12 hours, it hatches into the L1 stage. In this stage, the worm's reproductive cells start to form (Altun & Hall, 2009). As it grows into the L2 stage around 34.5 hours later, its brain continues to develop, and its reproductive cells start dividing. In the L3 stage, which comes next, the worm's reproductive system grows even more, and certain cells become specialized to form the vulva (Altun & Hall, 2009). By the time it reaches the L4 stage at around 56 hours, its reproductive

system extends, and its germ cells begin to undergo meiosis. This stage also involves the shaping of tissues in the worm's body (Altun & Hall, 2009). Finally, after about 66 hours, the worm becomes an adult, with fully developed reproductive and nervous systems, ready to survive and reproduce.

In addition to the benefits of a rapid lifecycle, *C. elegans* are anatomically simpler than humans with 1000 somatic cells but have functional counterparts in humans (Silverman et al., 2009). The similarities between *C. elegans* and humans allow researchers to model human developmental and genetic experiments on *C. elegans*, making studies less expensive and time-consuming. Furthermore, *C. elegans* is easier to cultivate in a laboratory setting than other organisms because of its ease in feeding, reproducing, and maintaining. *C. elegans* has a completely sequenced genome, which provides a reference when studying genetics and molecular processes. A completely sequenced genome allows scientists to identify and analyze specific genes and their functions. *C. elegans* are used to metabolism and nutrition due to their defined bacterial diet, simple digestive system, and conserved metabolic pathways with humans.

2.2 The Lipid Membrane

Cell lipid membranes are composed primarily of phospholipid building blocks, and each phospholipid has a hydrophilic head and hydrophobic tails or fatty acid chains. The presence of hydrophilic heads and hydrophobic tails gives the molecule an amphipathic nature, which defines the ability of these phospholipids to spontaneously assemble. Although there are various other components of lipid membranes including membrane proteins, phospholipids are a large component of the lipid bilayer. The types of phospholipids included in the membrane are crucial to determine the properties of the lipid membrane including its fluidity and flexibility. Phospholipids serve as primary mediators that bridge intracellular and extracellular

environments. Neutral lipids, primarily triglycerides, serve as major energy carriers in *C. elegans,* stored within lipid droplets. Triglycerides consist of a glycerol backbone linked to three fatty acid chains. Sphingolipids play vital roles in cellular signaling, with ceramides representing a specific type crucial in stress response pathways. Other members of the sphingolipid class include sphingomyelins and glycosphingolipids, which modulate cellular responses to various stressors such as heat and oxidative stress.

In adult *C. elegans*, there are two main phospholipid headgroups species that make up the membrane. Membrane constituents of adult *C. elegans* are made of acyl linkages with cholines or ethanolamines to form PCs and PEs while alkyl-ether and vinyl-ether linkages also form with ethanolamines to form O-PEs and P-PEs. P-PEs are a plasmalogen species, which means that at the sn-1 position, they contain a vinyl-ether bond (Braverman, 2012). HPLC was used to show the typical phospholipid composition of adult worms in Figure 4. The most common phospholipids found are phosphatidylcholines, or PCs. They make up the majority of the lipid membrane, at about 82% total abundance. The next most highly abundant phospholipid group are phosphoethanolamines, or PEs, at around 7.6%. L-PCs and L-PEs are called lyso species which only contain one fatty acid tail.

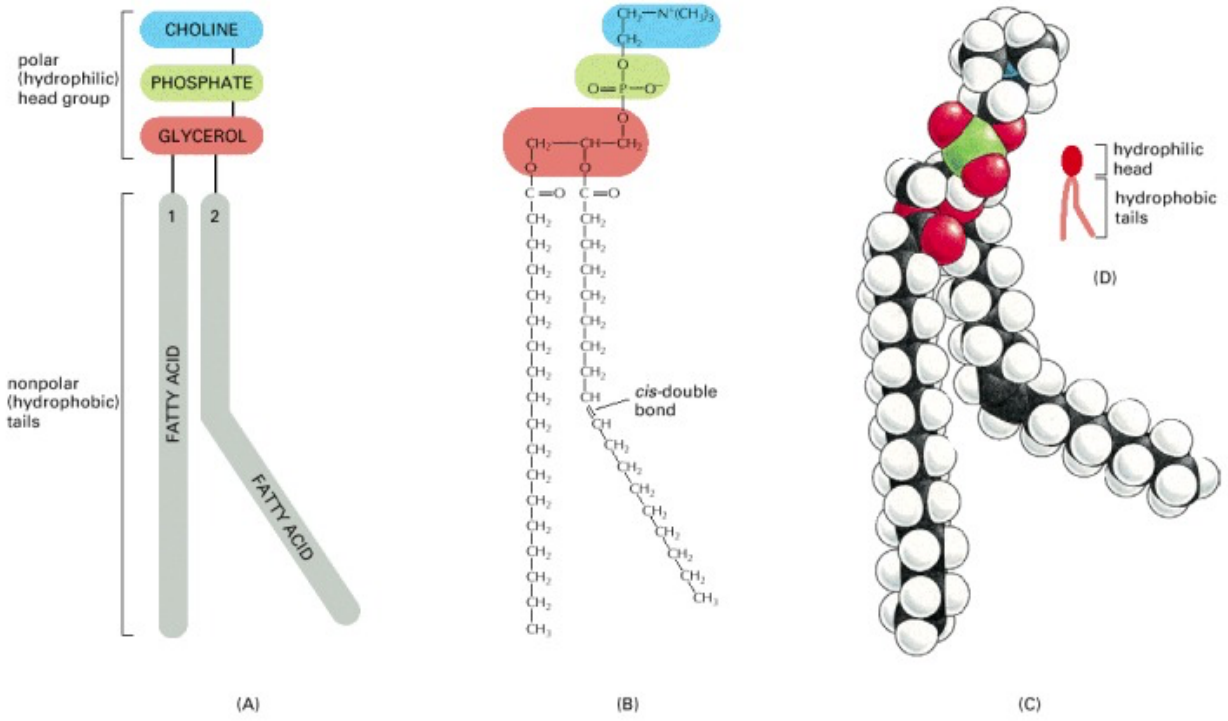


Figure 3: Parts of a phospholipid.

Phospholipid membranes are a polar hydrophilic head group and two nonpolar hydrophobic tails. (Alberts, 2002) A cis-double bond is shown, indicating a kink in the membrane, which allows for fluidity and prevents tight packing of the phospholipids. Figure from Alberts, 2002.

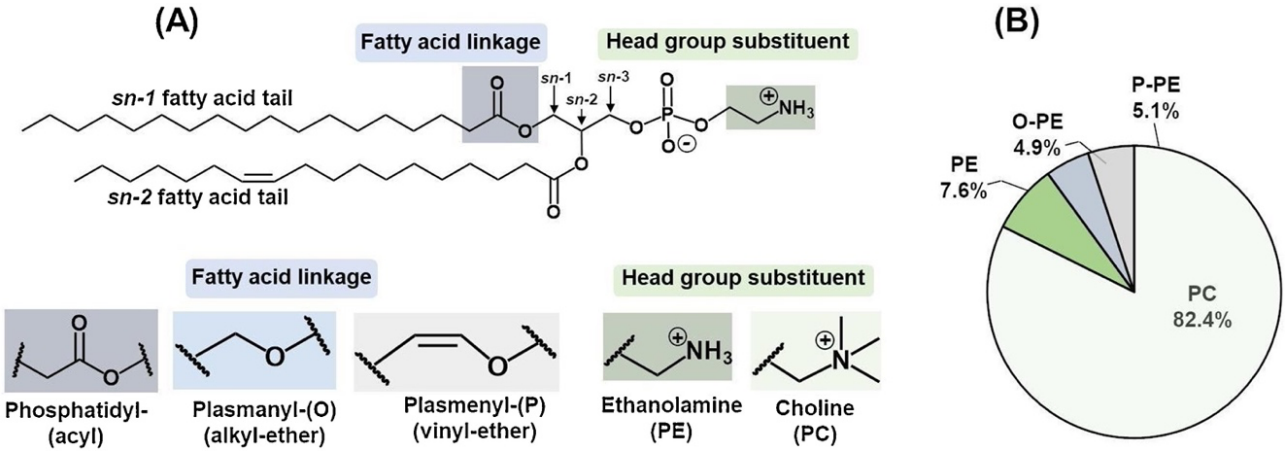


Figure 4: Common Phospholipid Groups.

Phospholipids contain a polar head group that face the aqueous environment surrounding lipid membranes. These head groups help determine the properties of lipids or membranes, including charge, solubility, membrane fluidity or dynamics, and molecular interactions. Common head groups in *C. elegans* include phosphatidylcholines (PC) or phosphoethanolamines (PE). PCs are the major constituents of *C. elegans*' lipid membrane, followed next by PEs. Head groups link with fatty acid tails to form phospholipids. Research areas of interest include looking into the chain length and double bond distribution of these fatty acids to determine fluidity and thickness of a membrane. Figure from Sultana & Olsen, 2020.

The lipid membrane serves many important roles in cells including functioning as a barrier, allowing for mobility of proteins, transmitting signals, and transporting molecules in and out of cells. Lipid membranes separate the intracellular space from the extracellular space. Selective permeability is a property of the lipid membrane that controls the movement of molecules across the membrane, which is crucial for the upkeep of the intracellular environment and cellular processes. Additionally, there are typically very many membrane proteins that aid in transport and signaling. Phospholipids are signal transducers and there are various pathways that these signals follow. Along with this, intracellular transport is conducted through the formation of vesicles to move some large molecules into their proper destination in cells (Alberts, 2002).

C. elegans have many conserved lipid metabolism pathways when compared with other organisms, but *C. elegans* also have the ability to produce long-chain omega-6 and omega-3 - polyunsaturated fatty acids through a de novo synthesis pathway with precursors from the *E. coli* bacterial strains that they eat (Perez, 2021). The membranes of *C. elegans* contain both unsaturated and saturated fatty acid chains. The amount of saturated and unsaturated fatty acids determines membrane fluidity, as well as the lengths of the fatty acid chains, or how many carbons make up the chain. Unsaturated fatty acids lead to more membrane fluidity, while saturated fatty acids cause the membrane to become more rigid (Koyiloth, 2021). Saturated fatty

acids contain no carbon-carbon double bonds, monounsaturated fatty acids contain one carbon-carbon double bond, and polyunsaturated fatty acids contain two or more carbon-carbon double bonds. The higher the degree of polyunsaturation, the more fluid a membrane will be, yet it also makes the membrane a greater target to oxidative stress and damage.

2.3. Bacterial Diet

Caenorhabditis elegans is widely used in scientific research as this type of nematode is a model organism in studying membrane composition. Previous studies examined how different bacterial diets altered lipid metabolism to understand how changes in membrane composition affect various cellular processes. Specifically, *C. elegans* was used to understand the impact of four different *E. coli* strains on fat metabolism and fat storage levels (Brooks et al., 2009).

E. coli serves as the primary dietary source for *C. elegans*, with specific strains employed to discern alterations in the worm's metabolism. Among these strains are OP50, DA837, HB101(DE3), and HT115. OP50, characterized by its thin lawn growth, facilitates the observation of *C. elegans*' developmental stages, promoting reproducibility and comparative analysis across laboratories (Brooks et al., 2009). Derived from OP50, DA837 exhibits streptomycin resistance, while HB101, a hybrid K12 x B bacterium, presents a denser lawn compared to OP50 and DA837 (Brooks et al., 2009). Although DA837 and HB101 may vary slightly in nutrient composition, they contain all essential nutrients required for *C. elegans* survival. HT115(DE3), resistant to tetracycline and derived from K12, lacks RNAse III activity and is frequently utilized in *C. elegans* research, particularly for RNAi-based experiments

(Brooks et al., 2009). A fat staining method and biochemical lipid analysis were used to show the fat stores for each type of *E. coli* strain.

2.4 Types of Mutant Worms

Mutant *C. elegans* can be used to investigate various types of laboratory studies, understanding their responses to stressors through alterations in their genetic makeup. These genetic changes allow researchers to explore a wide range of studies, delving into aspects such as metabolism and behavior.

Eat-2 Mutant Worms

Eat-2 worms are a mutant *C. elegans* strain that is characterized by a feeding behavior mutation. The pharynx of this worm strain is unable to pump at the same effective rate as wild-type worms, thus, the *eat-2* mutation is often used for dietary restriction type experiments due to the nature of the worms restricting their own food intake. Due to their inability to consume the same amount of bacteria as the wild-type worms do, they are often associated with dietary restriction studies. Due to their restricted food intake, they typically will live longer than wild-type worms as dietary restrictions have been seen to extend lifespans in *C. elegans* (Lakowski & Hekimi, 1998). Additionally, due to their restricted food intake, a shift in metabolism can be a potential research interest as their metabolism will shift from the "fed" state to the "fasted" state with a lack of food. The *eat-2* mutants are crucial in connecting dietary restriction with metabolism and feeding behavior.

CF512 Mutant Worms

CF512 mutant worms are a mutant strain that are a temperature-dependent sterile strain, which indicates that they are unable to reproduce at restricted temperatures. The mutated genes are fem-1 (hc17) and rrf-3 (b26). The worms are typically maintained at 15ºC and are sterile at 25ºC. These mutant worms are beneficial for studies due to the absence of offspring. The absence of offspring can be useful when conducting aging studies or longevity studies that focus primarily on adult worms and do not take into consideration the offspring (Kasimatis et al., 2018). The elimination of larvae allows for the metabolism of the adults to be interrogated without perturbation by the metabolism of the larvae.

3.0 Materials and Methods

3.1 Worms Maintenance

Preparing Liquid Cultures

To prepare liquid cultures, a Bunsen burner is needed to sterilize the equipment used. The bottle of LB is flamed prior to pouring. ~6mL of liquid culture is poured into a culture tube. A micropipette is cleaned using ethanol. A micropipette tip is used to transfer bacteria from a plate to the tube. This process of transferring is repeated 3 times. This culture is incubated and shaken for 16 hours. It is then stored in the 4ºC fridge before use.

Seeding Plates

To seed plates, first HG plates and liquid bacteria cultures are needed. These are prepared prior to seeding. The liquid cultures are removed from the 4ºC fridge and are vortexed thoroughly. Glass beads are poured onto the plates to spread the cultures. 100 microliters of liquid culture is

pipetted onto the plate. Plates are shaken by hand for about 30 seconds. The beads are removed from the plate and the plate is stored upside down in the 20ºC fridge.

Bleaching Worms

A solution is prepared with 1.25 mL KOH, 2.5 mL bleach, and 8.5 mL of water. Worms are collected and the remaining solution is aspirated out. The bleach solution is added to the worms, and the worms are rotated. The rotation time is dependent on the strength of the bleach. The amount of time that a bottle of bleach has been open determines the length of the rotation of the worms in the bleach. For a newer bottle of bleach, the worms are spun for about 3 minutes and for an older bottle of bleach, worms are spun for about 8 minutes. The worms are observed visually under the microscope for broken bodies. If some bodies are broken, the bleach has had enough time to affect the worms but not the eggs. The worms must then be washed as quickly as possible. The bleached worms are centrifuged for 1 minute at 2000 rpm and 20ºC. The worms are then washed with M9 buffer three times. After the final wash, the worm tube is filled with M9 and rotated in the 20ºC fridge overnight until plating.

Counting worms

To count worms, first, the bleached worms must be centrifuged in order to aspirate the M9 that the worms have been rotating in overnight. The worms are centrifuged at 2000 rpm and 20ºC for 1 minute and the M9 is aspirated to the 1mL line. A dilution is performed, so it is important to aspirate the liquid to the 1mL line to perform accurate calculations. A microcentrifuge tube is prepared and 1000 microliters of M9 are added to the tube. The worm solution is vortexed for about 10 seconds and then 10 microliters of the worm solution are transferred to the

microcentrifuge tube. This tube is vortexed for about 10 seconds and then 10 microliters of this diluted worm solution is transferred to a 3cm HG plate for counting. The worms are counted under the microscope and the number of worms is recorded. This process is repeated three times, and the average number of worms per 10 microliters is calculated. The dilution factor is accounted for by multiplying the number of worms by 10 microliters. It is necessary for experimentation to plate 5000 worms per plate, so to calculate the microliters of solution needed for this, 5000 is divided by the number of worms per 10 microliters.

average # of worms *10 µL = worms/10 µL

5000 worms/plate worms/10 µL=µL needed for plating

The worm solution is then vortexed for about 10 seconds and the number of microliters needed to plate 5000 worms per plate is dispensed onto each plate. The plates are left to dry uncovered in the fume hood for five to ten minutes, or until no liquid remains on the plates. The plates are then stored upside down in the 20ºC fridge.

Collecting Bacteria

Additional bacteria plates are seeded two dates before the beginning of the experiment, and then grown for the duration of the experiment. The bacteria from these plates are collected at the same time as when the worms are collected. First, 3mL of 1xM9 is pipetted onto the plate and the bacteria is power washed into solution. The bacterial solution is then pipetted into a microcentrifuge tube and centrifuged at maximum speed for 1 minute. The 1xM9 remaining is poured out and the bacteria pellet is snap frozen using liquid nitrogen.

Transferring Worms

To transfer worms, the worms are swirled with about 5mL of 1xM9. The worm solution is carefully poured into a centrifuge tube. This process is repeated twice to ensure minimal worms remain on the plate. The worms are centrifuged at 2000 rpm and 20ºC for 1 minute. The liquid layer is aspirated, and the worm pellet is left. The worms are washed with 1xM9 three times to ensure that the bacteria are removed from the worms. To wash, the centrifuge tube is filled to 15mL with 1xM9, the tube is vortexed, the worms are centrifuged at 2000 rpm and 20ºC for 1 minute, the liquid is aspirated, and the tube is filled again. The worm pellet is then transferred onto a new plate using a micropipette. The pellet is drawn up and expelled onto the plate, and then the tip is rinsed by pipetting the remaining M9 up and down, and which is then plated to transfer any remaining worms from inside the pipet tip.

3.2 Exploring the Impact of Dietary Composition on Fat Storage in *C. elegans*

To investigate the impact of dietary composition on fat storage, a Nile Red analysis was conducted and discussed by Brooks et al. When the *C. elegans* were first fixed in paraformaldehyde, a fixative to preserve the morphology of the worm, and then stained with Nile Red, a lipophilic dye used to stain and visualize lipid droplets in tissues, there were differences in the sizes and intensities of the stained lipid droplets for each type of *E. coli* diet (Brooks et al., 2009). The *C. elegans* that were grown on OP50 and DA837 bacteria strains had larger lipid droplets and more intense staining compared to the ones grown on HB101 and HT115 bacteria strains (Brooks et al., 2009). There were no significant differences in phospholipid levels in worms feeding on the four *E. coli* strains; however, there were significant differences in triacylglycerol (TAG) levels (Brooks et al., 2009). Both worms grown on OP50 and DA837

bacteria strains had high levels of TAGs while both worms grown on HB101 and HT115 bacteria strains had low levels of TAGs (Brooks et al., 2009). The size and intensity of the Nile Red stains of fixed worms correlated with the quantification of triacylglycerol (TAG) stores.

Furthermore, previous studies found that mutants of *C. elegans* with long lifespans had metabolic changes that impact fat storage (Kimura et al., 1997). However, work by Brooks et al determined that the different bacterial diets did not affect lifespan even though the different bacterial diets affected fat storage (Brooks et al., 2009).

To determine the cause for fat storage variability between worms feeding on the four different *E. coli* strains, the macronutrient composition of each *E. coli* strain was investigated. Notably, HB101 and HT115 bacteria strains had 3-5 times more of total carbohydrate levels as measured by TLC/GC, than OP50 and DA837 bacteria strains (Brooks et al., 2009). There is an inverse correlation between the carbohydrate levels in each *E. coli* strain and fat storage. Worms feeding on OP50 and DA837 bacteria strains had lower carbohydrate levels but higher levels of TAGs while worms feeding on HB101 and HT115 bacteria strains had higher carbohydrate levels but lower levels of TAGs in the worms.

To identify changes caused by glucose, a 5% glucose concentration was introduced to agar plates. By adding 5% glucose to the agar plates, the carbohydrate levels of OP50 and HB101 bacteria strains were changed to identify whether dietary carbohydrates regulated fat storage (Brooks et al., 2009). It was concluded that the carbohydrate composition of *E. coli* strains did not regulate fat storage. There was only a slight increase in TAG levels when the glucose content

of OP50 bacteria strain was increased, and there was no change in TAG levels when the glucose content of HB101 bacteria strain was increased (Brooks et al., 2009).

The different bacterial compositions were investigated. HB101 bacteria strain contained higher levels of monounsaturated fatty acids and lower levels of cyclopropane fatty acids (Brooks et al., 2009). On the other hand, OP50, DA837, and HT115 bacteria strains contained lower levels of monounsaturated fatty acids and higher levels of cyclopropane (Brooks et al., 2009). The worms mirrored these trends when fed the different bacteria strains (Brooks et al., 2009). The worms feeding on HT115 bacteria strain exhibited similar fatty acid composition to the worms feeding on OP50 bacteria strain, but their TAG levels were significantly different. Therefore, the differences in fatty acid composition do not correlate with fat storage.

When studying the effects of diet on worm metabolism, it is crucial to separate bacterial metabolism from worm metabolism because they represent two distinct biological systems with their own metabolic processes and responses to dietary changes.

3.3 Comparing the Effects of Different Diets on *C. elegans*

Wild-type N2

To better understand how different diets affect the metabolism of *C. elegans*, an experiment that examined the impact of different *E. coli* strains on phospholipid composition of wild-type N2 *C. elegans* via high-performance liquid chromatography (HPLC) was conducted. Specifically, OP50, HB101, and HT115 were the *E. coli* strains used in this experiment. For each condition, four HG plates were seeded with 100 μL of the bacterial culture two days before plating the

worms. HG plates are made of NaCl, peptone, agar, cholesterol, and P.P.B. OP50, HB101, and HT115 bacteria plates were grown for 2 days at 20ºC.

The day after seeding plates, a plate of gravid adult wild-type N2 *C. elegans* from the *C. elegans* Genetics Center (CGC) were collected into a 15 mL tube. To collect the worms, about 2 mL of 1xM9 buffer was poured onto the worm plate. The solution, 1xM9 is buffer that mimics the natural environment of *C. elegans* and commonly used for washing them. Typically, 1xM9 is composed of M9 salts, including NaCl, KH_2PO_4 NH_4Cl, and $MgSO_4$, which provide ions for nematode growth. It also contains a buffering agent, like KH_2PO_4, to maintain the pH of the solution. The 1xM9 solution was gently swirled on the plate to collect all the worms. The worm solution was poured from the plate to a 14 mL tube, and the tube was filled with 1xM9. The tube was centrifuged for 1 min at 2000 rpm. The supernatant was discarded, leaving the pellet of worms. The tube of pelleted worms was treated with diluted bleach solution, containing 1.25 mL of KOH, 2.5 mL of bleach, and 8.5 mL of deionized water, and spun for 3-5 min. The bleach effectively killed the adult worms while leaving the eggs unharmed. After bleach treatment, the worm eggs were collected, washed three times, and rotated overnight at 20°C in 1xM9 solution. Washing the worm eggs, detailed in Figure 5, removes the excess bleach. This process allowed the worms to be synchronized and begin at the same L1 development stage.

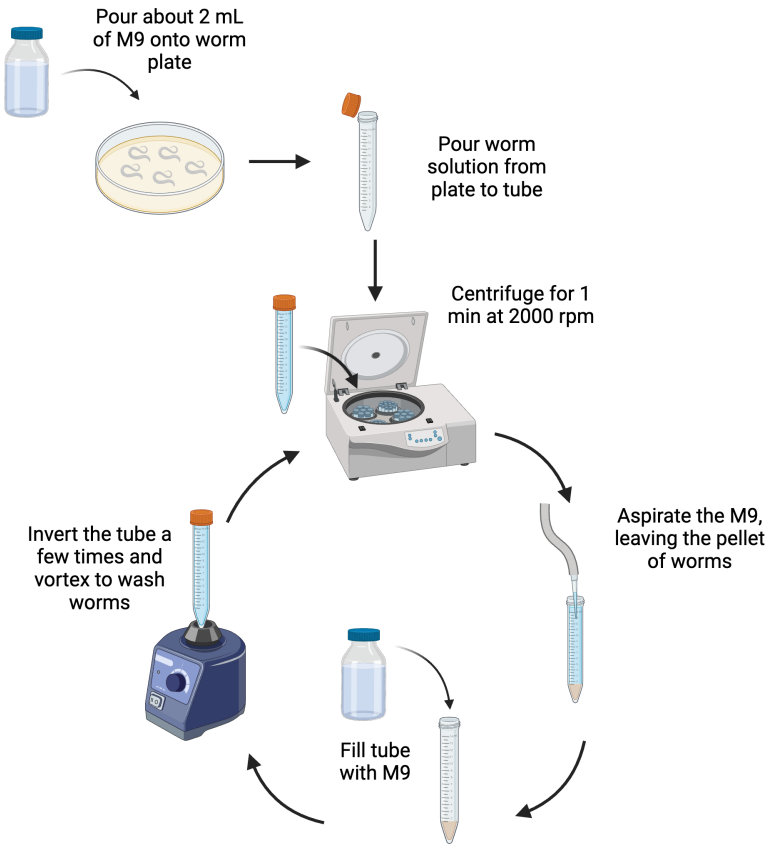


Figure 5: Flowchart for washing worms.
This flowchart illustrates the step-by-step procedure for washing worms in preparation for collection. About 2 mL of 1xM9 buffer was poured onto the worm plate. If there are multiple worm plates with the same conditions, the solution was poured from one plate to another. The solution was gently swirled to collect all the worms. The worm solution was poured from the plate to a 14 mL tube, and the tube was filled with 1xM9. The tube was centrifuged for 1 min at 2000 rpm. The supernatant was discarded, leaving the pellet of worms. The tube was filled with 1xM9. Tube was inverted a few times and vortexed to wash the worms. This process was repeated twice for a complete wash.

The day after bleaching, the synchronized L1 stage worms were plated on the seeded HG plates. Approximately, five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the L4 stage and were collected for analysis, as shown in Figure 6.

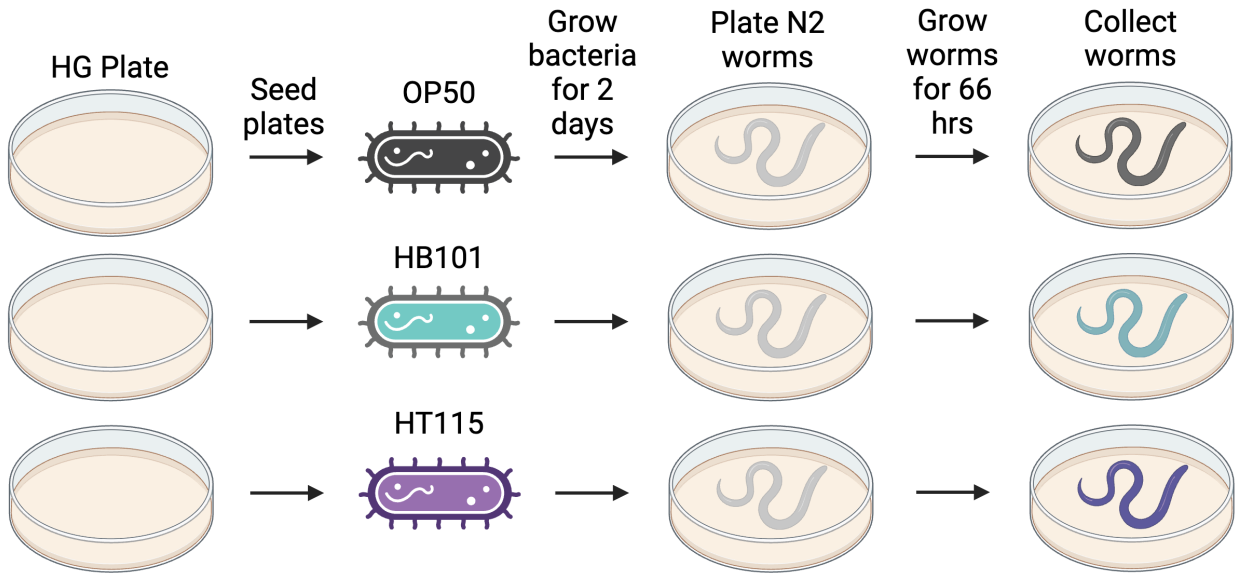


Figure 6: Experimental Design for growing wild-type *C. elegans* on different diets.
L1 stage worms were plated on seeded HG plates with OP50, HB101, or HT115 bacteria grown two days before plating. Approximately five thousand L1 stage worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were collected for HPLC analysis.

To collect the worms for analysis, each plate of worms was transferred to 15 mL tubes and washed three times with 1xM9, again shown in Figure 5. The solution, 1xM9 is buffer that mimics the natural environment of *C. elegans* and commonly used for washing them. Typically, 1xM9 is composed of M9 salts, including NaCl, KH_2PO_4 NH_4Cl, and $MgSO_4$, which provide ions for nematode growth. It also contains a buffering agent, like KH_2PO_4, to maintain the pH of the solution. After washing, the worms were transferred to Eppendorf tubes and snapped frozen with dry ice and ethanol. Once frozen, the worms were stored in -80°C until the lipid extraction process for HPLC analysis. *Eat-2* mutant worms follow the same protocol.

3.4 Liquid Chromatography-Mass Spectrometry

High-performance liquid chromatography (LC) is a method used to separate compounds in a sample based on their affinity to a solvent. In LC-based lipidomics, samples interact with the reverse phase C18 silica column hydrophobically and elute in order of increasing hydrophobicity or fatty acid chain length (Sultana & Olsen, 2020). HPLC is coupled with a mass spectrometer to quantify intact membrane lipids, enabling comprehensive assessment of phospholipids (Sultana & Olsen, 2020).

The eluting lipid molecules are ionized, using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) techniques, as they go through the mass spectrometer (Thomas et al., 2022). These ionized molecules are subsequently introduced to the mass analyzer, which separates the ions based on their mass-to-charge ration (m/z) (Thomas et al., 2022). As the ions pass through the mass analyzer, sensors quantify their abundance and produce mass spectra. Employing the Data-Dependent Acquisition (DDA) technique, the mass spectrometer autonomously chooses precursor ions for fragmentation based on predetermined conditions like intensity or abundance thresholds (Davies et al., 2021). Selected ions undergo collision-induced dissociation (CID) in the collision cell of the mass spectrometer. During CID, the ions collide with inert gas molecules, causing them to fragment into smaller ions (Thomas et al., 2022). These fragment ions undergo a second mass analysis, allowing for precise structural characterization and targeted quantitative analysis with stable isotope-labelled standards (Thomas et al., 2022).

One of the advantages of HPLC-MS-based lipidomics is its ability to quantitatively monitor hundreds of lipids simultaneously. Various software solutions, ranging from open-source

packages to commercial vendor-specific tools, are used for lipidomic data processing (Sultana & Olsen, 2020). LipidXplorer is one of these software solutions that processes fragmentation of lipid molecules within a complex mixture and is followed by the analysis of the resulting fragments using tandem mass spectrometry (Sultana & Olsen, 2020). The development of Lipid Data Analyzer by Graz University and Technology uses 3D algorithm that uses a comprehensive approach to analyze lipidomic data from multiple dimensions based on the precise mass of ions, distribution of isotopes, retention time, and fragmentation patterns of ions (Sultana & Olsen, 2020).

Expected Results from Mass Spectrometry

Mass Spectrometry data can be represented in various ways. Volcano plots are commonly produced from mass spectrometry data to compare two types of experimental conditions, as they show the statistical significance of changes or variations in lipid species present across various samples. The x value of each individual lipid species represents the change in the lipid species, and the y value represents the magnitude of the change. There are two sets of dashed lines, one vertical and one horizontal. The horizontal line is the line of interest when comparing statistical significance, as all dots above this line represent statistically significant changes, usually with a p value <0.05 (Freudenberg, 2014).

3.5 Gas Chromatography

Gas chromatography (GC) is widely used in laboratories for the separation and quantification of lipids. Typically, GC uses a fatty acid derivative of methyl-esters for analysis. In this FAME preparation, a short-chain alcohol like methanol, ethanol, 2-propanol, or butanol is used to replace a sphingosine or glycerol component of the lipid (Cruz-Hernandez, 2021).

3.6 Stressors Utilized in this Study

Dietary Stress

Paraformaldehyde can be used as a tool in experimental designs to control the food source available to worms. Paraformaldehyde (PFA) is a polymer of formaldehyde that is typically used in laboratories as a fixative in biological samples (Darvell, 2018). Previous studies using PFA have observed substantial metabolic changes when comparing worms fed live OP50 bacteria and worms fed PFA-killed OP50. The metabolome of the worms is greatly impacted by changes in bacteria (Beydoun, 2021). Paraformaldehyde can be used to kill bacteria samples as it permeabilizes the bacterial cells which renders them inviable (Beydoun, 2021). Worms were found to grow well on PFA treated E. coli strains including OP50 and this method is preferred in some labs over other methods of killing bacteria, like ultraviolet irradiation and the use of antibiotics. The bacteria will not replicate when exposed to ultraviolet irradiation and antibiotics, but they will remain metabolically active, while PFA will render the bacteria metabolically inactive (Beydoun, 2021). PFA is also preferred as UV-killing can be inconsistent, which can delay experimentation or provide inaccurate results. PFA has other benefits, including the fact that it permeabilizes bacterial cells, which makes them inviable but does not damage the inner plasma membrane, which helps provide a viable food source for the worms (Beydoun, 2021).

Oxidative Stress

Oxidative stress causes damage to cellular macromolecules including lipids. The effects of reactive oxygen species on the lifespan of *C. elegans* are not widely understood, however there are effects caused by the reactive oxygen species like the development of aging-associated

diseases (D'Amora, 2018). Additionally, lipids and DNA can be damaged by reactive oxygen species. *C. elegans* enact DNA repair mechanisms to protect the genome. Then, oxidative stress can limit the fertility of *C. elegans*, causing reproductive stress across populations. There are different types of reactive oxygen species that can cause stress within *C. elegans*. Tert-butyl Hydroperoxide, or TBHP, is a compound that induces oxidative stress in *C. elegans* and can be used in laboratories to observe oxidative stress conditions (Yang, 2020).

Glucose Stress

Glucose is typically used for energy production in cells through glycolysis to produce adenosine triphosphate, or ATP, a major energy storage molecule in cells. Glucose additionally has been found to maintain osmotic balance in cells which in turn maintains homeostasis (Sohn, 2020). In *C. elegans*, glucose has been found to shorten their lifespan (Alcántar-Fernández et al., 2018). Glucose uptake has been found to increase lipid peroxidation and the amount of bagging worms, while decreasing the number of eggs laid (Alcántar-Fernández et al., 2018). Additionally, higher concentrations of glucose results in a higher reduction in oleic acid and linoleic acid, it is hypothesized that oleic and linoleic acids may be regulated in hopes of combatting the increased glucose concentrations (Vieira, 2023). When *C. elegans* are exposed to glucose, a process called glycation may occur, which is when glucose spontaneously bind to proteins, lipids, or nucleic acids (Dubois, 2021). During this process, Advanced Glycation End Products or AGEs are produced. AGEs are complex structures that are produced as a result of the chemical transformation of biological molecules by glucose or other sugars. They can alter the structure and function of proteins, lipids, and nucleic acids, leading to various cellular dysfunctions and contributing to the development of age-related diseases. In the context of *C. elegans*, exposure to

glucose can evoke glycation reactions, which results in the formation of AGEs within the organism's cells. These AGEs may then accumulate over time and cause stress to the worms, like cellular damage, oxidative stress, and other physiological consequences associated with elevated glucose levels (Vlassara, 2014). Studying glycation and the formation of AGEs in *C. elegans* can provide information about the molecular mechanisms that contribute to glucose metabolism, aging, and age-related diseases.

Starvation

Most animals often endure periods of starvation and must adapt. For example, as *C. elegans* populations expand, they require more food, therefore decreasing nutrient availability (Baugh & Hu, 2020). Studying food restricted *C. elegans* allows for the investigation of their adaptive mechanisms, metabolic responses, and the molecular pathways that enable survival under nutrient-limiting conditions. During periods of food scarcity, *C. elegans* undergoes a series of physiological changes aimed at maximizing survival. These adaptations include changes in development, adjustments in behavior, and alterations in metabolism (Baugh & Hu, 2020). During starvation, worms may utilize the autophagy pathway to degrade proteins or organelles. Metabolic substrates can be produced through this pathway to produce cellular ATP under starvation conditions (Kang, 2007). Typically, when collecting worms, it takes about 20 minutes. The exact timeline of when worms begin to shift into starvation metabolism is unknown. One area of exploration for this project is to identify at what point the shift to starvation metabolism begins and narrow down this timeline.

3.7 Developmental and Behavioral Shifts in Response to Starvation

Changes in Development in Response to Starvation

The impact of starvation on *C. elegans* has a broader implication for aging research and longevity studies. Animals must be able to detect and adapt to changes in their environment to survive, revealing the organism's plasticity (Fielenbach & Antebi, 2008). *C. elegans*' ability to arrest at the dauer diapause in response to high population density or limited food demonstrates its plasticity and changes in development to adapt in starved conditions (Fielenbach & Antebi, 2008). In favorable laboratory environments with adequate food and at 20°C, the worm goes through reproductive development from embryo and through four larval stages (L1–L4) to an adult in about 3.5 days (Fielenbach & Antebi, 2008). Typically, hermaphrodites reproduce over 3 to 5 days and have a subsequent lifespan of 2 to 3 weeks (Fielenbach & Antebi, 2008). When the worms hatch in environments lacking food, they stop development at the L1 diapause (Baugh & Sternberg, 2006). If the worms experience overcrowding in an environment with high population density and food scarcity during early larval stage, they will divert development to dauer diapause (L3d). This stage is a specialized third larval stage, in which the worms can live for months without food (Cassada & Russell, 1975). Diapause stages reveal plasticity throughout the life cycle of *C. elegans*. Plasticity increases its likelihood of surviving in the face of environmental challenges, and it ensures that the physical development and reproductive processes align with the available resources. (Fielenbach & Antebi, 2008).

Starvation-induced Behavioral Changes in C. elegans

Not only does starvation affect the development of *C. elegans* but also their behavior such as taxis, pharyngeal pumping, egg laying, sleep, and associative learning (Baugh & Hu, 2020). For example, well-fed worms move more slowly in the presence of food than they do without food

(Sawin et al., 2000). This behavior is caused by the basal slowing response, which is controlled by dopamine expression (Sawin et al., 2000).

In addition to a basal slowing response, *C. elegans* exhibit an enhanced slowing response in response to starvation. This enhanced slowing response presents when animals are deprived of food for 30 minutes, which causes their rate of locomotion to decrease even more than well-fed animals when they encounter food (Sawin et al., 2000). Serotonergic neural signaling allows *C. elegans* to detect the presence of food and slow down locomotion (Schwartz et al., 2021). In addition, exogenous serotonin stimulates pharyngeal pumping (Horvitz et al., 1982). When well-feed worms are initially deprived of food, they have a decreased pumping rate. Decreased pumping rate indicates a lack of food, which marks a shift of the worms into starvation metabolism. On the other hand, when well-fed worms are in the presence of food, they have an increased pumping rate. However, when worms are deprived of food for 24 hours, the rate of pumping increases to a rate approximate to the rate of pumping in fed worms (Dwyer & Aamod, 2013). Autophagy is needed to continue pumping under starved conditions (Kang *et al*. 2007). Worms resume pumping to ingest nutrients to revert to normal metabolic function by increasing food intake.

Starvation impacts feeding, foraging, and pumping; moreover, starvation inhibits egg laying behavior. Starved worms hold onto their eggs, instead of laying them. With the eggs being trapped inside the mother, the eggs hatch inside the mother, which create a “bag of worms” inside the mother. The larvae that hatched inside the mother feed on the mother, eventually causing the mother to die from bagging. The larvae then arrest in dauer diapause (Chen &

Caswell-Chen, 2004). This matricide is a method of adapting to and surviving starvation. When animals are deprived of food for 16 hours, not only do moments of inactivity increase and responses to lower O2 weaken, but brief bouts of depolarization of RIS neurons increase (Skora *et al*. 2018). The depolarization of RIS neurons activate sleep behavior as a method to maintain homeostasis during long-term starvation (Skora *et al*. 2018). *C. elegans* respond to starvation conditions through utilizing stored lipids (Li, 2020). Under starvation conditions, there is an increase in the transcription of *lipl-3*, a gene that encodes a lysosomal lipase used to break fat stores from lipid droplets into fatty acids (Li, 2020). There is an additional increase in *acs-2,* a gene that codes for an enzyme to catalyze the production of fatty acyl-CoAs to be used in β-oxidation (Li, 2020). Through the increase of these enzymes, stored lipids are broken down for energy production.

3.8 Dietary Restriction

Dietary restriction has been found to impact stress resistance in *C. elegans*, which changes the ability of the worms to adapt to various types of stress (Lee, 2006). One example of stress the worms may face is oxidative stress. Dietary restriction studies have been found to increase the worm's resistance to reactive oxygen species (ROS). Reactive oxygen species are present in typical metabolic processes, and an example of an ROS is hydrogen peroxide. Reactive oxygen species also impact aging in *C. elegans*, as oxidative damage is a key factor in aging (Back et al., 2012). *C. elegans* under dietary restriction have metabolic shifts such as decreases in triglyceride production (Heestand, 2013). A lack of nutrients has been associated with a depletion lipid stores (Hellerer, 2007). Lipid synthesis is favored while glycolysis is downregulated, which is likely in response to nutrient deprivation to replenish broken down fat stores (Hellerer, 2007).

3.9 Susceptibility to Glucose Stress

In order to investigate the extent of stress caused to *C. elegans* via starvation, an experimental design was planned out to subject the *C. elegans* to starvation for periods of one hour and 24 hours. Approximately 5000 L1 worms were first plated on OP50 or HB101 seeded HG plates for 48 hours. After the 48 hours when the worms were at about the L3 larval stage, one plate of the worms were transferred to plates containing glucose, while another plate of worms were transferred to control OP50 or HB101 plates for 18 hours, making the total duration of the worms on being on experimental plates ~66 hours.

3.10 Starvation Exposure

Having investigated the susceptibility of *C. elegans* to glucose stress, another starvation became of interest as another type of stressor. To explore the effects of starvation on N2 strains, 5000 L1 worms were first plated on OP50 or HB101 seeded HG plates for 66 hours. The control worms were collected after 66 hours. One plate of experimental worms was collected and spun in 1xM9 in 15mL conical tubes at room temperature (~20ºC) for one hour and another plate of experimental worms were collected and spun for 24 hours. Spinning worms in 1xM9 serves as a method to induce starvation by removing the food source and subjecting the nematodes to a nutrient-deprived environment. Both the 1-hour and 24-hour starved worm were collected at the respective time. This design allowed for the assessment of the effects of starvation on the nematodes over different durations, providing insights into their responses to prolonged nutrient deprivation.

3.11 TBHP-Induced Stress

Preparing TBHP Plates

TBHP causes oxidative stress in *C. elegans*. First, wild-type *C. elegans* were grown for 66 hours on HG plates with OP50 as their food source. One day before the worms would grow to day 1 adults, TBHP plates were created. To make the TBHP plates, the TBHP must be added to the media about 10 minutes after autoclaving to allow the media to cool slightly, but still be warm enough to pour. The TBHP was handled in a fume hood. For one trial, four TBHP plates were needed, so 100 mL of media was prepared. 15mM TBHP was added to the media and swirled to mix before pouring. The plates were poured and then allowed to cool, as shown in Figure 7. Along with these plates, control HG plates were seeded with 100 µL of OP50 one day before the worms were grown to day 1 adults. HG plates with no food were also set aside for this trial. The worms were transferred to the TBHP plates for a short period of two hours to prevent the worms from dying.

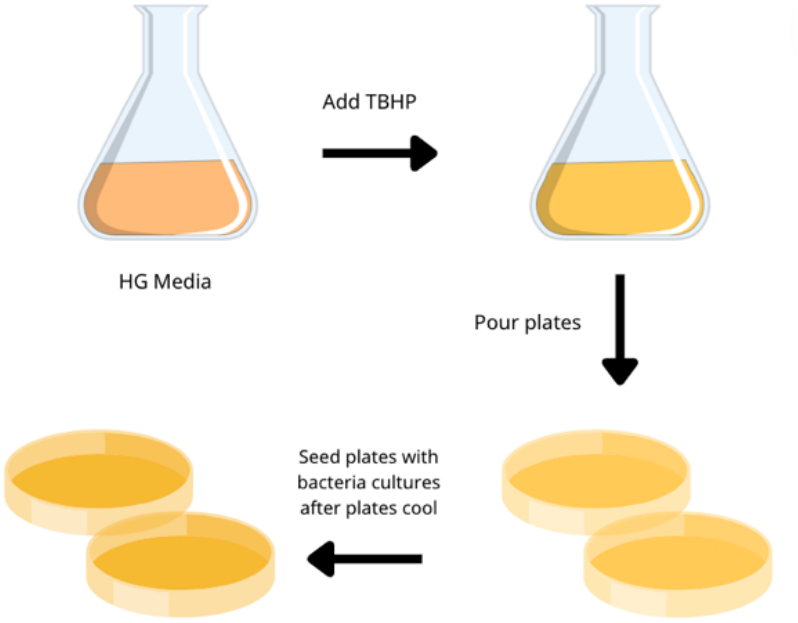


Figure 7: The comparison between tert-Butyl hydroperoxide (TBHP) induced stress and starvation of N2 worms.
TBHP is a compound that induces oxidative stress in *C. elegans*. TBHP is added to sterilized HG media and then poured onto experimental plates. The plates are left to cool for several hours before they are seeded with bacteria cultures. *C. elegans* are grown to day 1 adults consistently with previous laboratory methods and then transferred to the TBHP plates for 2 hours.

3.12 Paraformaldehyde for Dietary Restriction

Preparing PFA Cultures

Paraformaldehyde is used to kill bacteria to control the amount of bacteria that the worms are eating. This is crucial to the dietary restriction model studied, as it regulates the amount of bacteria provided to the worms in order to compare various dilution diets. First, liquid OP50 cultures were used to inoculate 100mL of LB media in a baffled Erlenmeyer flask to promote mixing and were shaken overnight for 16-18 hours. After this shaking process, 5 mL of the culture was stored and then 30% paraformaldehyde (PFA) was added to the flask to obtain a 0.5% PFA concentration, about 1.7mL. This mixture was left shaking for an additional hour in order to ensure the PFA had ample time to interact with the bacteria. At this time, 50mL conical tubes were pre-weighed. After this hour, the culture was centrifuged at 3900 rpm for 10 minutes and then washed with LB liquid to remove the remaining PFA. After washing, the PFA pellet was weighed and resuspended to a concentration of 0.15g/mL. The live culture that was stored was then streaked on an LB plate next to the PFA-killed culture and left in a 37ºC incubator overnight to ensure proper growth of the living culture, as well as no growth of the PFA-killed culture. The plate was then checked for growth in the morning, and if it looked like the cultures had grown or not grown properly, the experiment was proceeded with.

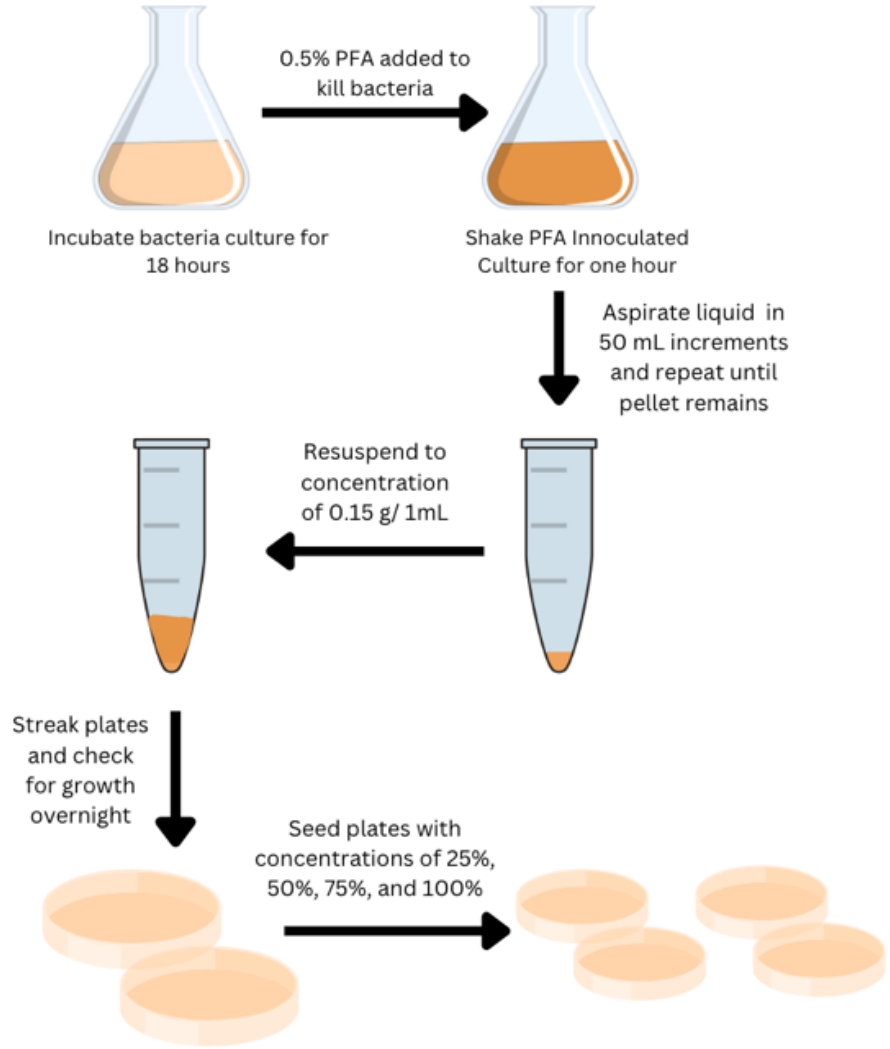


Figure 8: Experimental protocol determining the effects of paraformaldehyde for DR on *C. elegans* lipid metabolism.
0.5% Paraformaldehyde is added to liquid bacterial cultures and shaken for one hour. The cultures are transferred to 50mL comical tubes and centrifuged for 10 minutes at 3900 rpm, with additional LB liquid to wash away the remaining PFA. The pellet was weighed and resuspended to a concentration of 0.15g/1mL. The cultures were then used to streak LB plates to determine if the PFA successfully killed the bacteria. If no growth was exhibited, plates were seeded with concentrations of 25, 50, 75, and 100% PFA.

Seeding Experimental Plates at Varying Concentrations

In order to test various concentrations of PFA to observe the dietary restriction occurring, various dilutions of PFA were created using LB liquid. The dilutions began with 100%, 75%,

50%, and 25%, and then were further refined due to visual observations from the first replicate. The worms on the 100%, 75%, and 50% plates did not appear to be starved after 24 hours but the worms on the 25% did appear to be starved. The plates for the next set of replicates were prepared with the goal of continuing to use a condition where the worms did not appear to be starved as a baseline, but then to compare those worms to worms experiencing the effects of dietary restriction. The dilutions of plates were shifted to 100%, 25%, 12.5%, and 6.25%. The volume of liquid plated was 1mL to ensure proper spreading. The plates were left to dry in a hood for about 20 minutes, or until dry. Day 1 adult worms were then transferred to these plates and left for 24 hours. These plates were observed every few hours and visual observations were made, such as if the worms had laid eggs. Additionally, it was noted if the worms looked to be starved due to clumping on the plates or moving towards the edges of the plates. Since the worms were transferred to the PFA-killed bacteria at day 1 adults, they were in the process of laying eggs. Worms were then observed one final time before they were collected using gravity aspiration to eliminate the L1 worms from collection. L1 worms have a different lipid profile than the adult worms that were of interest, so it was important to try to remove as many L1s as possible through gravity aspiration.

3.6 Phospholipids Extraction by HPLC-MS/MS

Having outlined the experimental design for each experiment, the focus now shifts towards explaining the methods employed for lipid extraction in preparation for HPLC-MS/MS analysis, which is briefly outlined in Figure 11.

Figure 9: Analysis Protocol for High Performance Liquid Chromatography.
The number of worms in a 10µL diluted aliquot is counted 3 times with a microscope, the average is taken, the dilution factor is accounted for, and ~5000 worms are plated. The plates are then placed in a 20ºC incubator for 66 hours, which is consistent with the development cycle, and then lipid extraction is conducted to the worms to isolate their lipids, and finally they are analyzed on the HPLC.

For the lipid extraction process, about 4 mL of chloroform/methanol (2:1) was added into a glass vial with PTFE-cap. The thawed worm sample was added into the glass vial of chloroform/methanol (2:1). 40 µL of standard stock, made of 0.05 mg/mL of C11 PL standard and 0.05 mg/mL of C13 TAG standard stored at -20°C, was added to the glass vial. The solution was vortexed for 30 seconds and shaken for 1.5 hours at room temperature. The solution was washed with 800 µL of 0.9% NaCl and the centrifuged at 2000 RPM for 2 minutes, creating two separate phases: polar and nonpolar fractions. The nonpolar phase (bottom phase) contained the extracted lipids, which were transferred into a new glass vial and completely dried down under a nitrogen gas steam to prevent oxidation. The dried extracted lipids were resuspended in 200 µL of acetonitrile/2-propanol/water (65:30:5v/v/v) dilution buffer (Xatse et al., 2023).

The following process of loading samples, setting the conditions, and running the samples on the LC-MS/MS system was taken from Xatse et al., 2023. Next, 10 µl of resuspended lipids were injected into the LC-MS/MS system for the negative ion scanning mode analysis. Dionex

UHPLC UltiMate 3000 was the HPLC system used to separate the lipid samples. This HPLC system included Thermo Scientific instruments, C_{18} Hypersil Gold 2.1 × 50 mm, 1.9 µm column (25002-052130) and a 2.1 mm ID, and 5 µm Drop-In guard cartridge (25005-012101). The column was attached to a Dionex UltiMate 3000 RS quaternary pump, a Dionex UltiMate 3000 RS autosampler, and a Q Exactive Orbitrap mass spectrometer from Thermo Scientific, which was coupled with a heated ESI source.

The HPLC phospholipid separation used mobile phases A and B, consisting of 60:40 water (H_2O):acetonitrile plus 10 mM ammonium formate (NH_4COOH) and 0.1% formic acid and 90:10 isopropyl alcohol (IPA):acetonitrile with 10 mM ammonium formate (NH_4COOH) and 0.1% formic acid, respectively. The gradient method began with 32% B from 0–1.5 minutes; 32%–45% B from 1.5 to 4 minutes; 45%–52% B from 4 to 5 minutes; 52%–58% B from 5 to 8 minutes; 58%–66% B from 8 to 11 minutes; 66%–70% B from 11 to 14 minutes; 70%–75% B from 14 to 18 minutes; 75%–97% B from 18 to 21 minutes; 97% B up to the 25-minute mark; 97%–32% B from 25 to 26 minutes. Lastly, 32% B is maintained until 30 min for column equilibration.

For the HPLC conditions, the column temperature was set at 50°C, and the autosampler was set at 10°C with mobile phase flow rate of 300 µl/min. The MS scan range was set between *m/z* 300 and 1,200. The capillary temperature was set at 325°C. The sheath gas flow rate was set at 45 units. The auxiliary gas flow was set at 10 units. The source voltage was 3.2 kV, and the AGC target was 10^6. The acquisition used the full-scan data-dependent MS2 (ddMS2) mode. The scans were run at a resolution of 70k for MS1 profiling. MS2 analyses used six scan events. This

process had the top five ions chosen from an initial MS1 scan. A normalized collision energy of 35 was used for fragmentation. While MS1 spectra were collected in profile mode, the MS2 spectra were collected in centroid mode.

3.7 HPLC-MS/MS Data Analysis

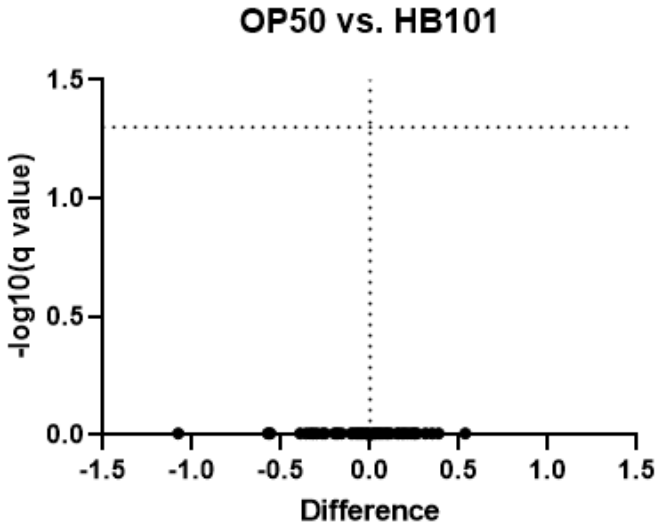
The Lipid Data Analyzer (LDA) Version 2.8.1 was the software used to conduct lipid analysis of the LC-MS/MS data. The LDA is a 3D algorithm that analyzes lipidomic data based on the precise mass of ions, distribution of isotopes from full scan MS, retention time, and MS/MS spectra (Sultana & Olsen, 2020). A 0.1% relative peak cutoff value was applied to the RAW files, so major lipid species could be examined while using the LDA for analysis. LDA mass lists were generated for phospholipids, phosphatidylcholines (PC), phosphatidylethanolamines (PE), plasmenyl-PEs (P-PE), plasmenyl-PCs (P-PC), plasmanyl-PEs(O-PE), plasmanyl-PCs(O-PC), lysophosphatidylcholines (LPC), and lysophosphatidylethanolamines (LPE), based on a previous study (Dancy et al., 2015). To compare samples, a relative quantification was used.

Additionally, GraphPad Prism 9.4.1 software was used to conduct statistical analysis for all the experimental data. To compare two conditions in the data, multiple *t*-test (unpaired) with corrected by false discovery rate, with an adjusted p- value (q) at 5% was used.

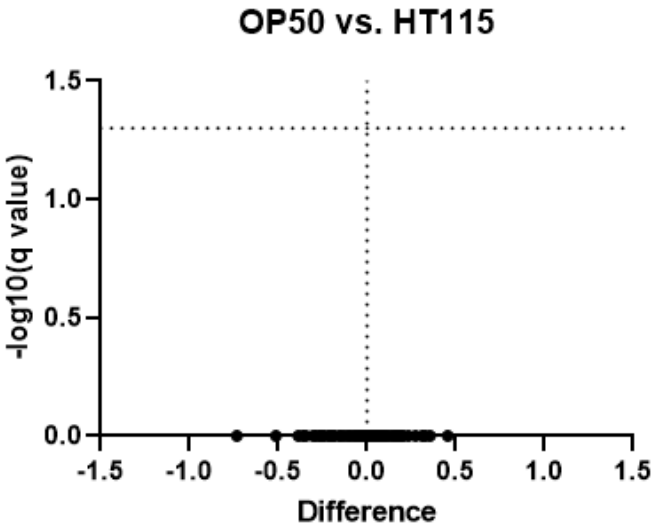
4.0 Results

4.1 The Impact of Different Diets on N2 Worms

Previous studies have distinguished changes in fat storage and metabolism of wild-type N2 *C. elegans* under different bacterial diets by mass spectrometry (Brooks at el., 2009). Therefore, the first step of this project was to confirm the differences between animals feeding on various bacterial strains. The animals grew on HG plates with OP50, HB101, or HT115 *E. coli* strains. OP50 is characterized by its thin lawn growth, and is commonly used across laboratories (Brooks et al., 2009). Derived from OP50, HB101, a hybrid K12 x B bacterium, presents a denser lawn compared to OP50 and DA837 (Brooks et al., 2009). Although DA837 and HB101 may vary slightly in nutrient composition, they contain all essential nutrients required for *C. elegans* survival. HT115(DE3), resistant to tetracycline and derived from K12, lacks RNAse III activity and is frequently utilized in *C. elegans* research, particularly for RNAi-based experiments (Brooks et al., 2009). The lipids were extracted from the bacterial and worm samples and processed through GC-MS and HPLC-MS/MS.



A



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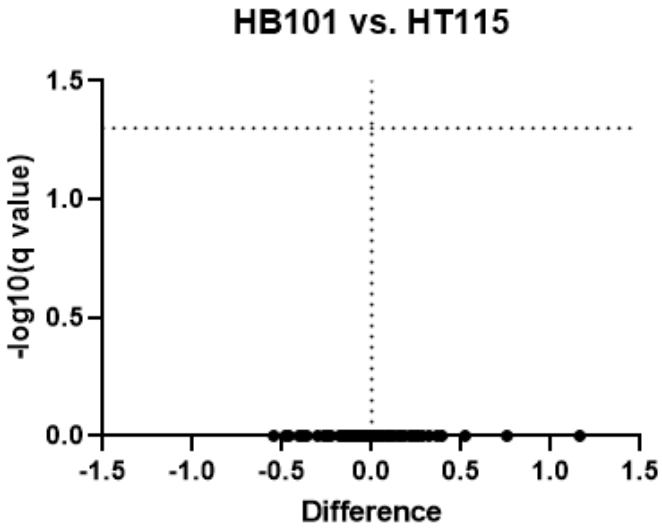
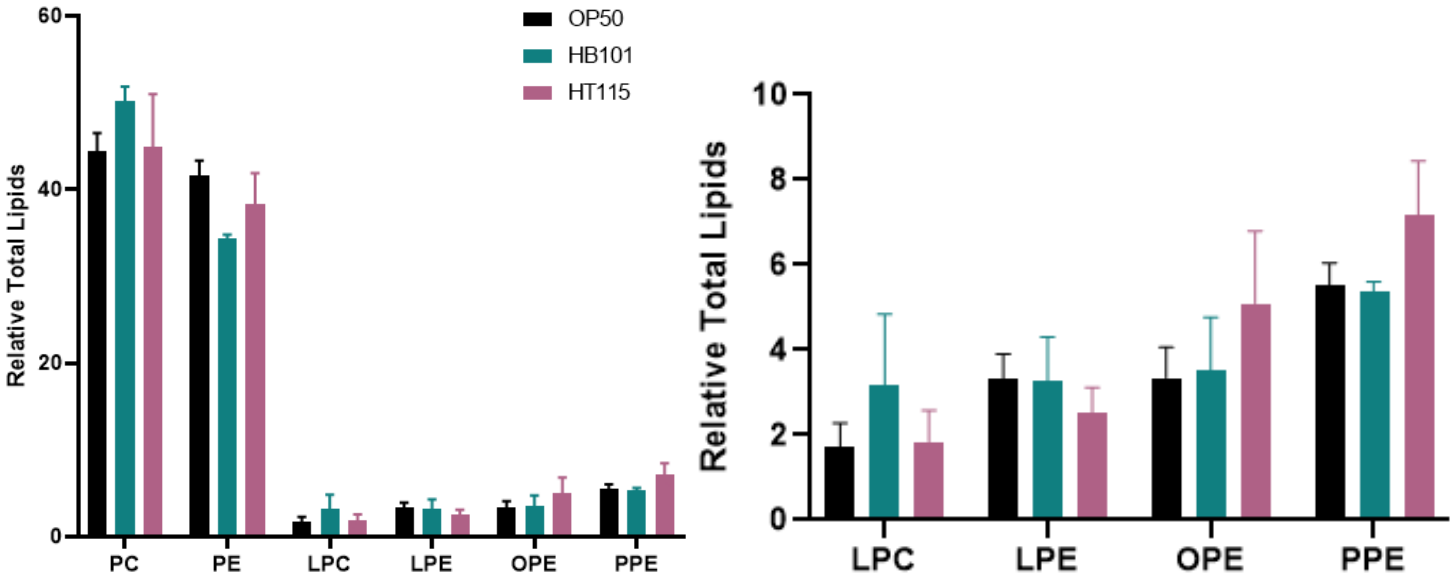


C

Figure 10: Volcano plots for wild-type nematodes under different diets.
L1 stage N2 worms were plated on seeded HG plates with OP50, HB101, or HT115 bacteria grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were collected for HPLC analysis. Volcano plots are used to show the

statistically significant changes in lipid species present between samples. There were no significant differences between the lipid species for (**A**) OP50 vs HB101, (**B**) OP50 and HT115, and (**C**) HB101 vs HT115.

Although the bacterial diets varied, there was no significant difference among the lipid species for worms feeding on each bacterial strain as there are no data points above the dotted-horizontal line, as shown in all panels of Figure 10. This result shows that the overall lipid composition is maintained. However, there are differences between the distribution of phospholipid classes, as shown in all panels of Figure 11. Despite differences in phospholipid classes, the overall lipid composition is maintained within a relatively narrow range across different bacterial strains. This result suggests that while specific phospholipid classes vary, the relative total amount of lipids or lipid species remains relatively constant.



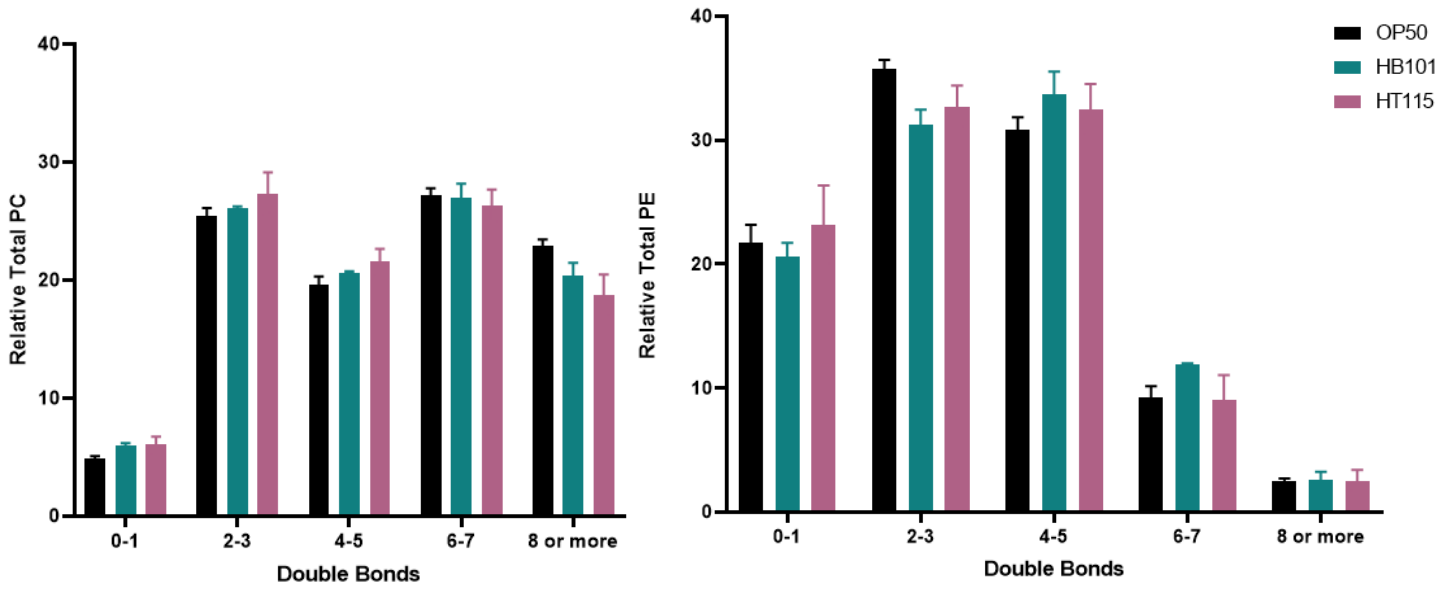
A **B**

Figure 11: Composition of phospholipid classes for wild-type nematodes under different diets.

L1 stage N2 worms were grown on HG plates with OP50, HB101, or HT115 bacteria. At the adult stage, the worms were collected for analysis. The lipid samples were observed using Lipid Data Analyzer to evaluate HPLC-MS data. (**A**) The worms that were fed HB101 bacteria had

higher PC levels compared to the worms that were fed OP50 bacteria and HT115 bacteria. The worms that were fed HB101 bacteria had lower PE levels compared to the worms that were fed OP50 bacteria and HT115 bacteria. (**B**) The magnified graph of the phospholipid class composition showed similar trends between the worms feeding on OP50 bacteria and HB101 bacteria for the LPE, OPE, and PPE classes. OPEs and PPEs are plasmalogen species, which contain ether linkages between their head group and fatty acid tails. Plasmalogens have been observed to be upregulated in response to stress conditions in *C. elegans*.

After visualizing the composition of phospholipid classes, the PC and PE classes were analyzed to identify differences among the double bond distributions for each bacterial diet. More relative total PCs with more than one double bond indicate a higher proportion of polyunsaturated fatty acids (PUFAs) in the cell membrane, as shown in Figure 12A. Furthermore, more relative total PCs with 2-3 double bonds and 6-7 double bonds suggest a specific metabolic or dietary influence favoring shorter and longer-chain PUFAs over intermediate and very long-chain PUFAs, as shown in Figure 13A. There was a decrease in relative total PE with 6-7 double bonds and those with 8 or more double bonds, indicating a reduction of very long-chain polyunsaturated fatty acids (VLC-PUFAs), as shown in Figure 12B. The increase in relative total PE with 2-3 double bonds and those with 4-5 double bonds highlights a preference for intermediate-chain PUFAs, as shown in Figure 13B.



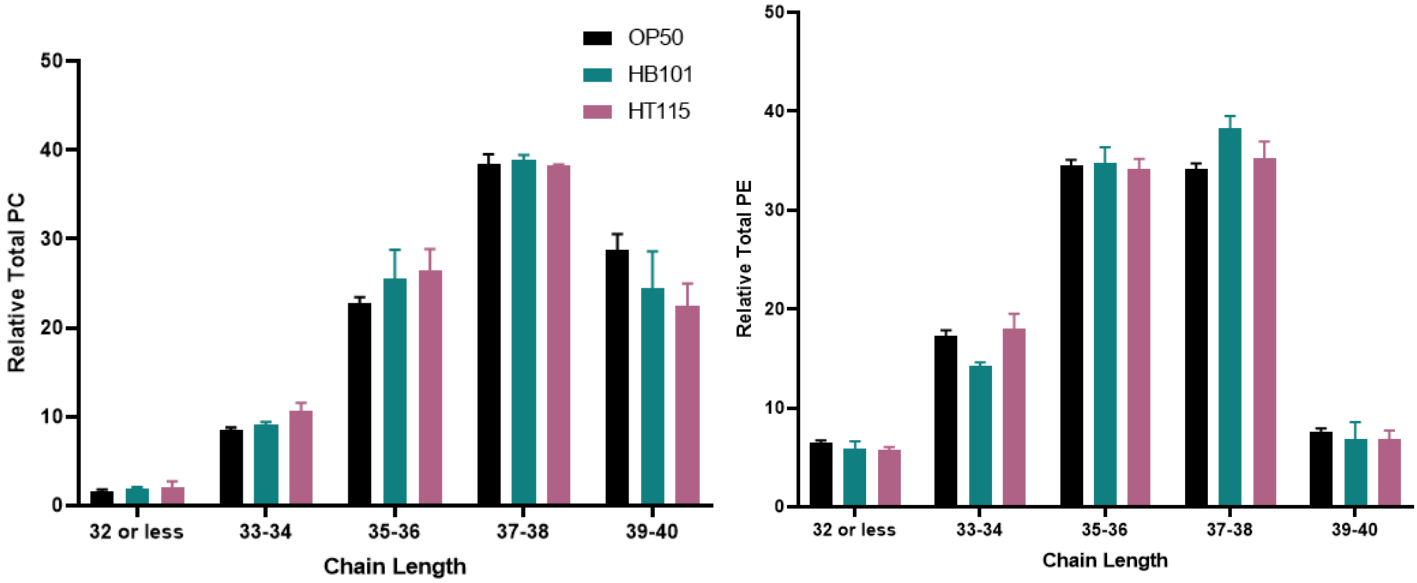
A **B**

Figure 12: Quantification of PC and PE double bonds for wild-type nematodes under different diets.

L1 stage N2 worms were grown on HG plates with OP50, HB101, or HT115 bacteria. At the adult stage, the worms were collected for analysis. (**A**) Across all worms, there were fewer relative total PCs with 0-1 double bonds in their FA tails, while there were more relative total PCs with more than one double bond in their FA tails. This result indicates a higher proportion of polyunsaturated fatty acids (PUFAs) in the cell membrane. The decrease in PCs with 4-5 double bonds and those with 8 or more double bonds suggests a specific metabolic or dietary influence favoring shorter and longer-chain PUFAs over intermediate and very long-chain PUFAs. (**B**) Worms that fed on OP50, HB101, and HT115 bacteria had fewer relative total PEs with 8 or more double bonds, suggesting a lower proportion of highly unsaturated fatty acids, such as very long-chain polyunsaturated fatty acids (VLC-PUFAs). The decrease in PCs with 0-1 double bonds and those with 6-7 bonds suggests a preference for intermediate-chain PUFAs over shorter and longer-chain PUFAs.

To better understand the lipid composition of the worm membranes, the chain length distribution of the PC and PE classes were analyzed for each bacterial diet. The reduced relative total PCs with a chain length of 32 or less suggests a shift towards longer-chain PCs within the cellular membrane, as shown in Figure 13 A. Shorter-chain fatty acids tend to increase membrane

fluidity, so a reduction in PCs with shorter chains could lead to decreased membrane flexibility. However, this shift towards longer-chain PCs stops when the chain length reaches 39-40 carbon atoms, signifying an avoidance of very long chain FAs. The higher concentration of relative total PCs with a chain length of 37-38 showed that a specific metabolic or dietary influence favored longer chain FAs, which made the membrane more rigid and reduced membrane fluidity and permeability, as shown in Figure 13 A. Similarly, Figure 15B illustrates a higher concentration of relative total PE with chain lengths of 35-36 and 37-38, signifying a preference towards longer chain FAs. Figure 13 B shows a sharp decrease in relative total PE with a chain length of 39-40. This result suggests that the worm's membrane does not contain very long chain PEs.



A **B**

Figure 13: Quantification of PC and PE chain lengths for wild-type nematodes under different diets.

L1 stage N2 worms were grown on HG plates with OP50, HB101, or HT115 bacteria. At the adult stage, the worms were collected for analysis. (**A**) An upward trend favored PCs with longer chains until a chain length of 39-40 was reached. The increase in longer chains signified a more rigid membrane and reduced membrane fluidity and permeability. (**B**) There were increasingly higher concentrations of PEs with longer chain lengths until a chain length of 39-40 was achieved. There was a higher preference for PEs with a chain length of 35-36 and 37-38, favoring longer chain FAs but not very long chain FAs.

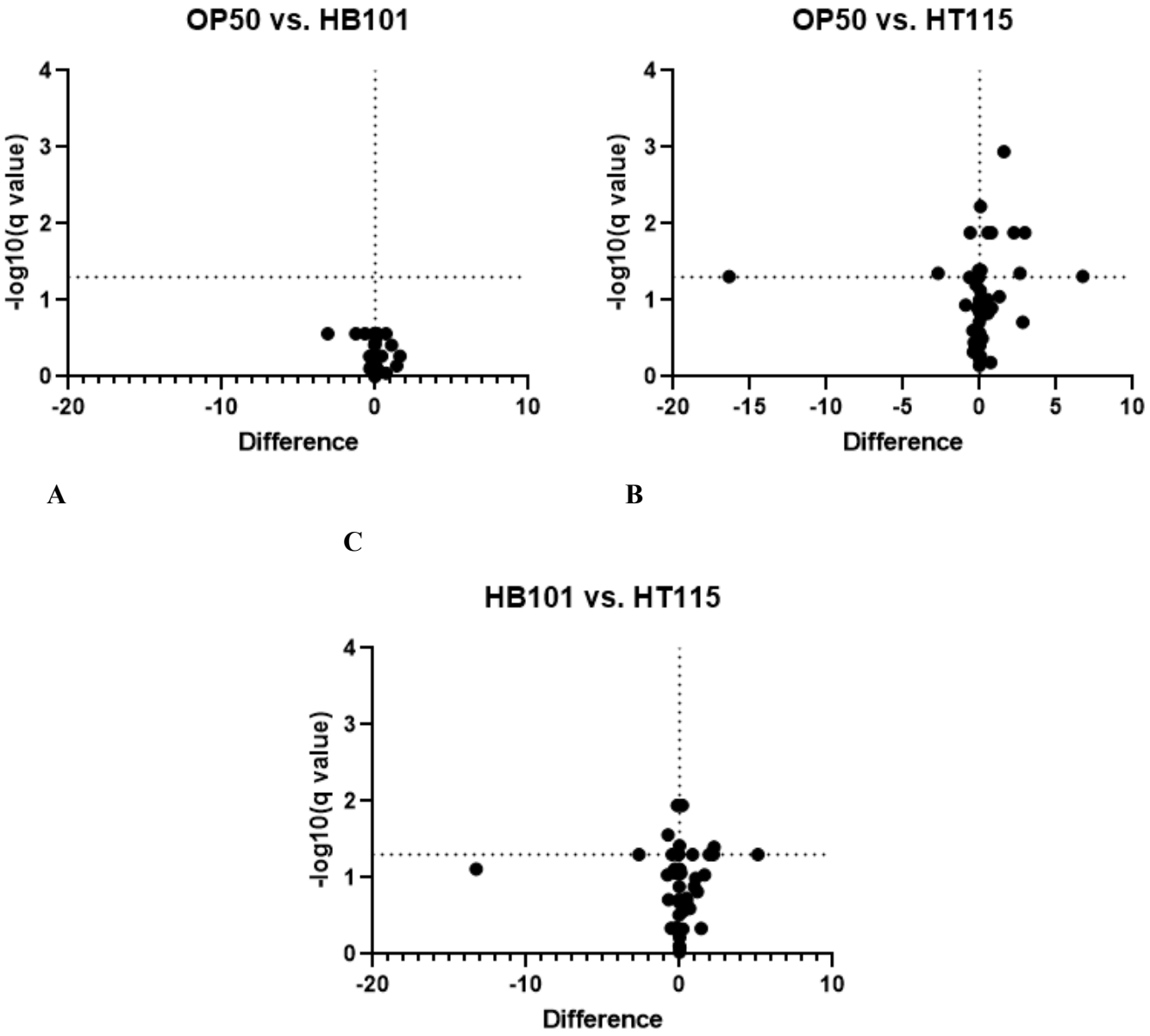


Figure 14: Volcano plots for different bacteria.

C. elegans were fed different *E. coli* strains, OP50, HB101, and HT115. There was about 100 µL of the specific bacterial culture put on the HG plates. These seeded plates were placed in 20°C for about 85-95 hours, and then the bacteria were collected for analysis. (**A**) Volcano plot of differentially expressed genes related to the OP50 bacteria compared to HB101 bacteria. No significant difference exists between the lipid species of OP50 bacteria and those of HB101

bacteria. (**B**) Volcano plot of differentially expressed genes related to the OP50 bacteria compared to HT115 bacteria. There is a significant difference between the lipid species of OP50 bacteria and those of HT115 bacteria. (**C**) Volcano plot of differentially expressed genes related to the HB101 bacteria compared to HT115 bacteria. There is a significant difference between the lipid species of HB101 bacteria and those of HT115 bacteria.

Comparing the volcano plots between the lipid composition of N2 worms, shown in Figure 14 A, and the lipid composition of the bacteria, shown in Figure 11 A, there were no significant differences. This result suggests that OP50 and HB101 bacteria are comprised of similar phospholipids. Additionally, the worms that fed on OP50 and HB101 have a similar makeup of phospholipids. In contrast, Figure 14 B shows significant differences between OP50 and HT115 bacteria, but Figure B shows no significant differences between the worms fed OP50 and HT115. While the bacteria themselves might have distinct characteristics or biochemical compositions, these differences might not be significantly reflected in the composition of the phospholipids within the worms that fed on them.

After exploring the volcano plots detailing the lipid composition disparities between N2 worms and bacteria, it is important to focus on comparing the phospholipid class composition, particularly the PE class, across N2 worms and various bacterial strains. The worms that fed on OP50 bacteria had the most relative total lipids in the PE class, as shown in Figure 11 A. However, the OP50 bacteria had the least relative total lipids in the PE class, as shown in Figure 15 A. This disparity could indicate complex interactions between worm physiology and bacterial metabolism, potentially influencing nutrient uptake and lipid metabolism pathways.

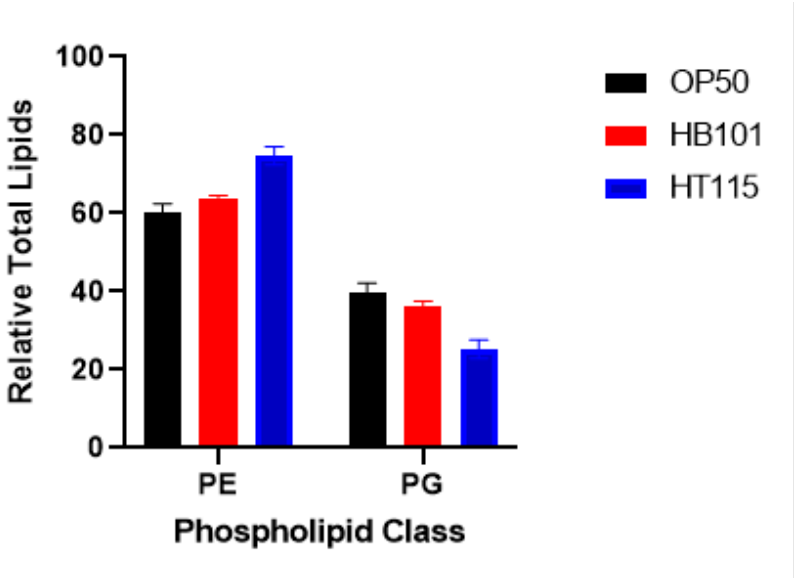
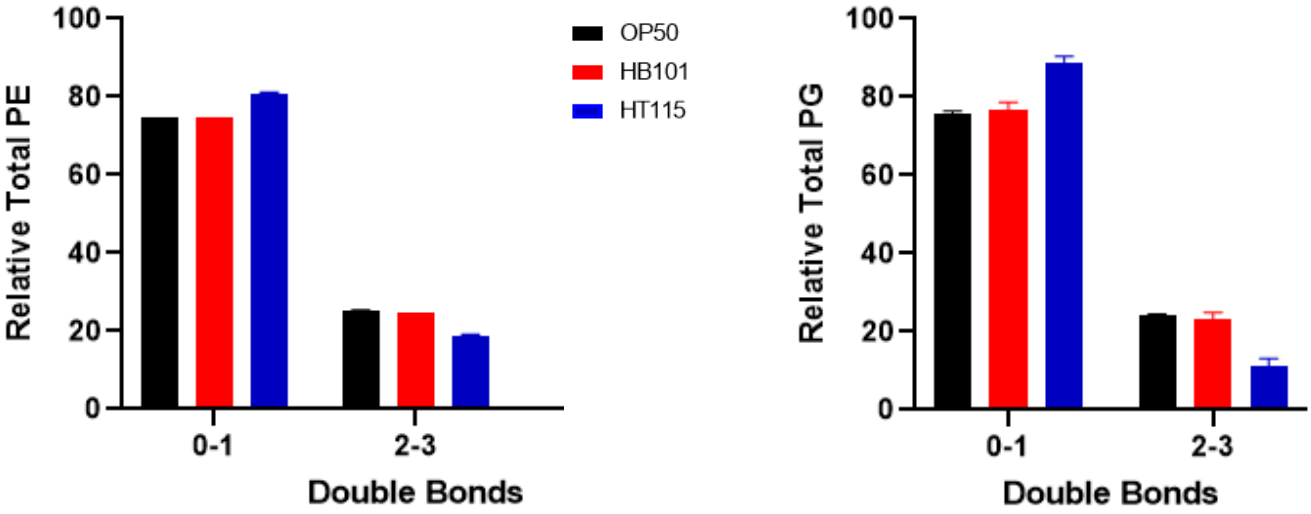


Figure 15: Composition of phospholipid classes for different bacteria.
Plates were seeded with OP50, HB101, and HT115 bacterial cultures and placed in 20°C for about 85-95 hours, and then the bacteria were collected for analysis. The distribution of the relative total lipids is slightly different in the different bacterial strains. The OP50 bacteria has less relative total lipids in the PE class compared with HT115, with similar levels to HB101. The OP50 bacteria had higher PG levels compared to the HB101 bacteria and HT115 bacteria. The HT115 had lower PG levels compared to the OP50 bacteria and HB101 bacteria. The OP50 bacteria and HB101 bacteria have similar relative total lipid distributions compared to the HT115. The relative total lipid distribution of the bacteria can be compared with that of the worms to look at the way the worms metabolize the bacteria. PEs are a major contributor to stability and fluidity, while PGs help maintain membrane function and integrity (Murzyn, 2005).

Having investigated the phospholipid class composition across OP50, HB101, and HT115 bacteria, the next step is to shift focus towards a more detailed analysis, examining the quantification of PE and PG double bonds within these bacterial strains for a comprehensive understanding of their lipidomic profiles. The overall trend of favoring phospholipids with 0-1 double bonds indicates that these *E. coli* strands have more saturated FAs and MUFAs than PUFAs, as shown in Figure 16. This saturation makes the membrane more rigid and less permeable to substances. Comparing the PE double bond distribution for worms fed different

bacteria in Figure 12 to the PE double bond distribution for the different bacteria in Figure 18 A, there are fewer relative total PEs with 0-1 double bonds for the worms compared to the bacteria. This result suggests a metabolic adaptation or modification in the worms' lipid composition. For example, the worms might selectively incorporate specific types of PEs from the bacteria they consume due to differences in enzymatic processes or selective transport mechanisms in the worms' cells. They might preferentially uptake or synthesize PEs with 2-3 and 4-5 double bonds as shown in Figure 12.

While comparing the double-bond distribution of worms (Figure 12) and the bacteria they consume (Figure 16) gives information about inter-species lipid dynamics, exploring the double-bond distribution for each individual bacteria provides insight into the microbial lipid metabolism. In Figure 18, the OP50 and HB101 bacteria have similar PE and PG double-bond distributions, which could indicate that the bacterial strains evolved similar enzymatic pathways or regulatory mechanisms related to lipid composition independently of one another due to the similarity. Further experimentation would be needed to test these hypotheses and look deeper into the pathways affecting the bacterial composition. However, the HT115 bacteria has more PEs and PGs with 0-1 double bonds and fewer PEs and PGs with 2-3 double bonds when compared to the OP50 and HB101 bacteria. The higher abundance of PEs and PGs with 0-1 double bonds in HT115 bacteria may reflect adaptation to specific environmental conditions, such as higher oxidative stress, as higher chain PUFAs are more susceptible to oxidative stress (Awada, 2012). Lipids with fewer double bonds, such as saturated or monounsaturated fatty acids, can provide greater stability and protection against oxidative damage.



A **B**

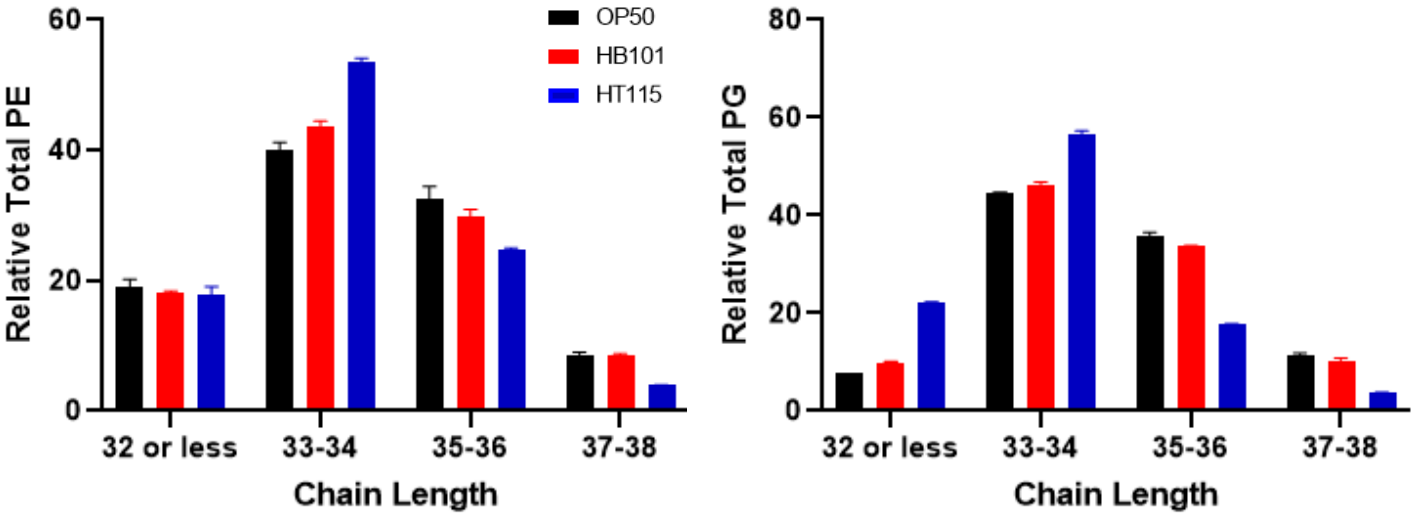
Figure 16: Quantification of PE and PG double bonds for different bacteria.
Plates were seeded with OP50, HB101, and HT115 bacterial cultures and placed in 20°C for about 85-95 hours, and then the bacteria were collected for analysis. (**A**) The relative total PEs with 0-1 double bonds for HT115 had a slight increase compared to OP50 and HB101. In contrast, the relative total PEs with more double bonds for HT115 had a slight but not statistically significant decrease compared to OP50 and HB101. (**B**) The relative total PGs with 0-1 double bonds for HT115 had a slight increase compared to OP50 and HB101. In contrast, the relative total PEs with more double bonds for HT115 had a slight decrease compared to OP50 and HB101.

After examining the double-bond distribution for each individual bacteria, it is important to analyze the chain length distribution to obtain a more comprehensive understanding of the bacterial lipid profiles. Some general trends of the PE and PG chain lengths for OP50, HB101, and HT115 bacteria include the increase from PEs and PGs with chain lengths of 32 or less carbon atoms to 33-34 carbons atoms as shown in Figure 17. The abundance of PEs and PGs decreases as the chain lengths reach 35-36 and 37-38 carbon atoms. Therefore, across all bacterial strains, PEs and PGs with a chain length of 33-34 carbon atoms were favored.

However, Figure 13 showed the worms that were fed different bacteria favored PEs with chain lengths of 35-36 and 37-38 carbon atoms. This discrepancy hints that there is a difference in lipid preference or utilization between the bacteria and the worms.

Focusing on the chain length distribution comparison between bacterial strains, HT115 bacteria had more PEs and PGs with a chain length of 33-34 carbon atoms but fewer PEs and PGs with chain lengths of 35-36 and 37-38 carbon atoms compared to OP50 and HB101 bacteria. Furthermore, HT115 bacteria had more PGs with 32 or less carbon chains than OP50 and HB101. The OP50 and HB101 had slight differences between the chain length distribution. Conversely, HT115 bacteria exhibit more pronounced differences in lipid composition compared to the OP50 and HB101 strains. This observation suggests that HT115 may have distinct metabolic pathways or regulatory mechanisms governing lipid metabolism, leading to unique lipid profiles. Based on the data comparing the chain length distribution of phospholipids among different bacterial strains, HT115 bacteria exhibit distinct lipid composition compared to OP50 and HB101 strains. Specifically, HT115 bacteria show higher abundance of certain phospholipids with shorter chain lengths, particularly in the case of PEs and PGs. Alternately, OP50 and HB101 strains display slight differences in chain length distribution, indicating a more similar lipid profile between these two strains. These findings suggest that the choice of bacterial diet may influence the lipid composition of *C. elegans*, with HT115 exerting a more pronounced effect on phospholipid chain length distribution. Further investigations into the metabolic implications of these differences may provide insights into the mechanisms underlying dietary effects on lipid metabolism in *C. elegans*. It is possible that these differences in lipid composition may influence membrane fluidity or energy in *C. elegans*. Further studies

investigating the functional impact of these differences may provide valuable information about the mechanisms that dietary bacteria aid lipid metabolism in *C. elegans*.



A **B**

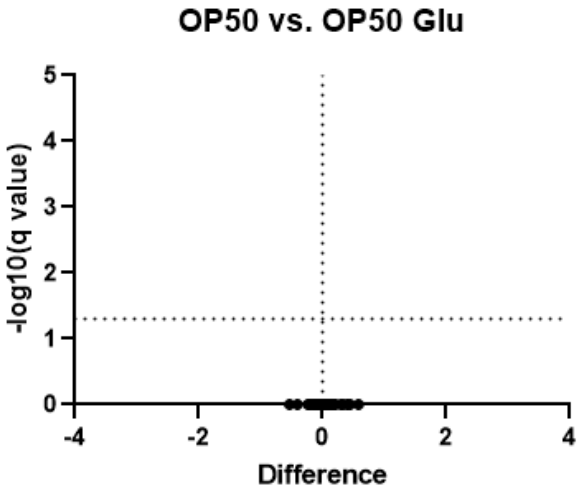
Figure 17: Quantification of PE and PG chain lengths for different bacteria.
Plates were seeded with OP50, HB101, and HT115 bacterial cultures and placed in 20°C for about 85-95 hours, and then the bacteria were collected for analysis. Across all bacterial strains, PEs and PGs with a chain length of 33-34 carbon atoms were favored. (**A**) The relative total PEs with a chain length of 33-34 carbons for HT115 had an increase compared to OP50 and HB101. In contrast, the relative total PEs with a chain length of 35-36 and 37-38 for HT115 had a decrease compared to OP50 and HB101. Stats were run and the changes between OP50 and HB101 were not statistically significant. Changes between OP50 and HT115 in the 33-34, 35-36, and 37-38 categories were significant. Changes between HB101 and HT115 in the same categories were also statistically significant. (**B**) The relative total PGs with chain lengths of 32 or less and 33-34 carbon atoms for HT115 had an increase compared to OP50 and HB101. In contrast, the relative total PEs with chain lengths of 35-38 and 37-38 carbon atoms for HT115 had a decrease compared to OP50 and HB101. Changes in PG chain lengths of 32 or less between OP50 and HB101 were statistically significant. All changes seen were statistically significant when comparing OP50 and HT115 as well as comparing HB101 and HT115.

Overall, this experiment aimed to explore the impact of different bacterial diets on the lipid composition of N2 worms. While previous studies highlighted changes in fat storage and metabolism of *C. elegans* under different bacterial diets, the experiment intended to confirm these differences through lipid analysis. Despite the varied bacterial diets, no significant

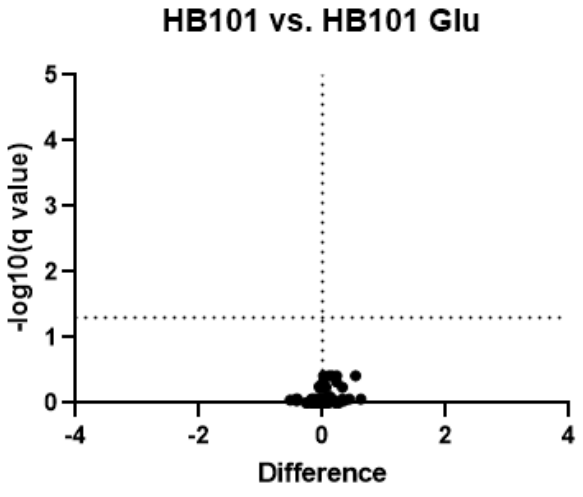
differences were observed among the lipid species for worms feeding on each bacterial strain. However, differences in phospholipid class distribution for PEs such as decreased relative total PEs in the worms compared to the bacteria, were evident, suggesting nuanced metabolic or dietary influences. The statistical significance of these changes was not investigated. Particularly, the preference for certain number of double bonds and fatty acid chain lengths varied between the worms and bacteria, indicating complex interactions between worm physiology and bacterial metabolism. This result emphasizes the importance of understanding both bacterial and worm metabolic processes when studying the effects of diet on worm metabolism.

4.2 Dietary Influence on Glucose Stress Susceptibility

Investigating glucose stress in *C. elegans* fed various diets can provide insight about how metabolic shifts occur in response to stress and if those changes are impacted by dietary shifts. This can be studied to understand how certain diets help *C. elegans* deal with stress through changes in lipid composition.



A



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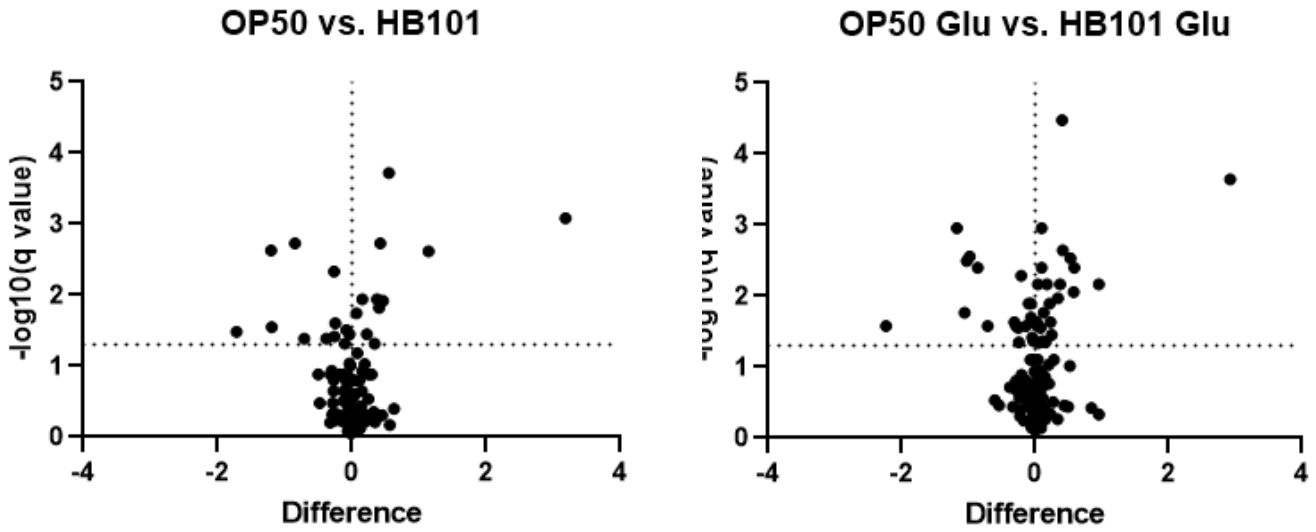


C **D**

Figure 18: Volcano Plots for Experiment 3: The Effect of Different Diets on Susceptibility to Stressors Like Glucose for N2 (Seed ALL plates 2 days before plating).
L1 stage N2 worms were plated on seeded HG plates with OP50 or HB101 bacteria compared with OP50 and HB101 bacteria and glucose grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were collected for analysis. Volcano plots are used to analyze the statistical significance of changes or variations in lipid species present across various samples. Panel A compares the worms that were fed OP50 with the worms that were fed OP50 + Glucose. There do not appear to be statistically significant differences in the lipid species present between these conditions. Panel B compares worms that were fed HB101 with worms that were fed HB101 + Glucose. There do not appear to be statistically significant differences in the lipid species present between these conditions. Panel C compares the worms fed OP50 with the worms fed HB101. There do appear to be statistically significant differences in the lipid species present between these conditions. Panel D is comparing worms fed OP50 + Glucose with worms fed HB101 + Glucose. There do appear to be statistically significant lipid species present between these conditions.

Overall, there do not appear to be major differences in lipid species present when glucose is fed to the worms compared to the absence of glucose. The major changes observed are when

different bacterial diets are introduced to the worms, like comparing OP50-fed to HB101-fed worms. There may be subtle changes or trends observed that were not detected.

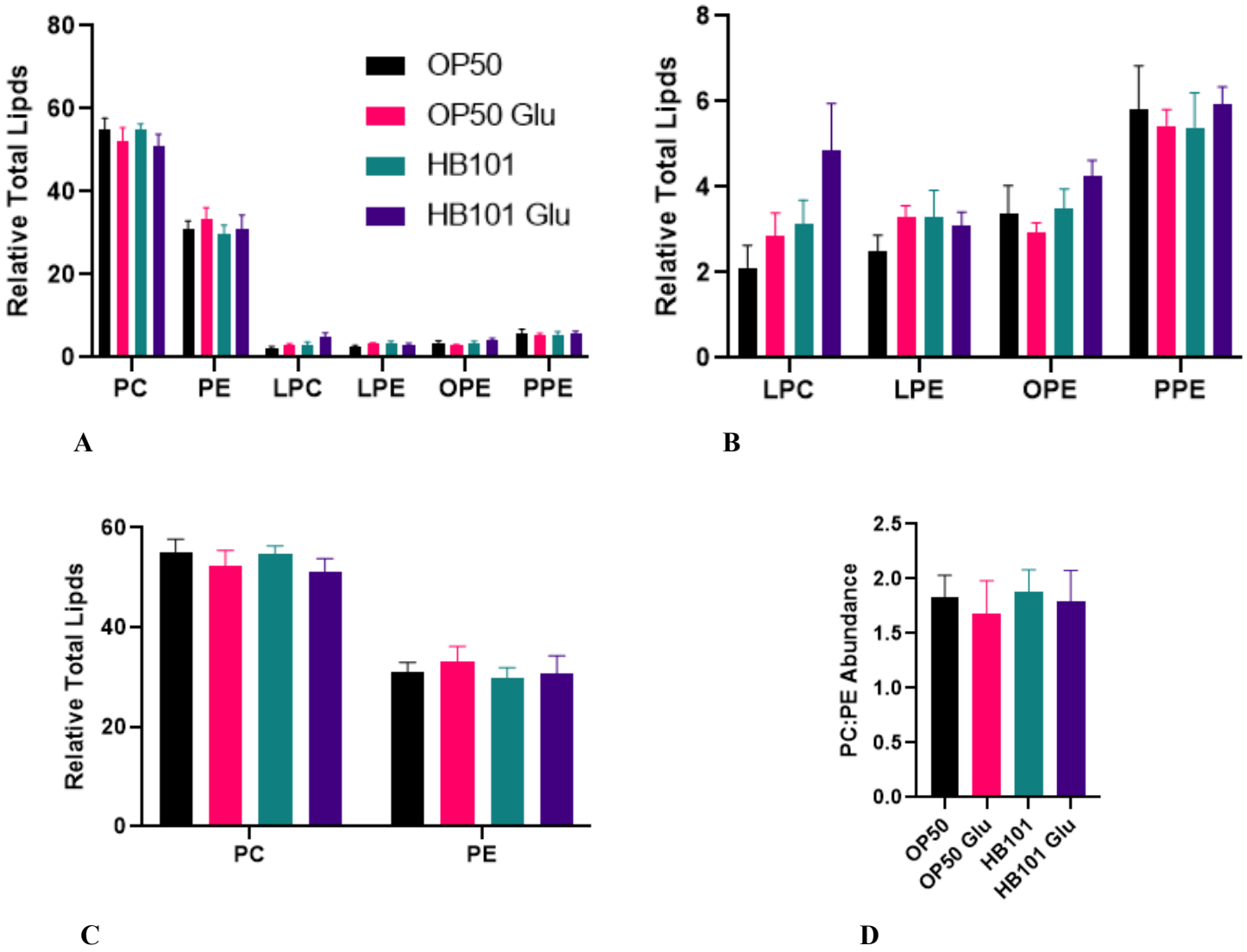
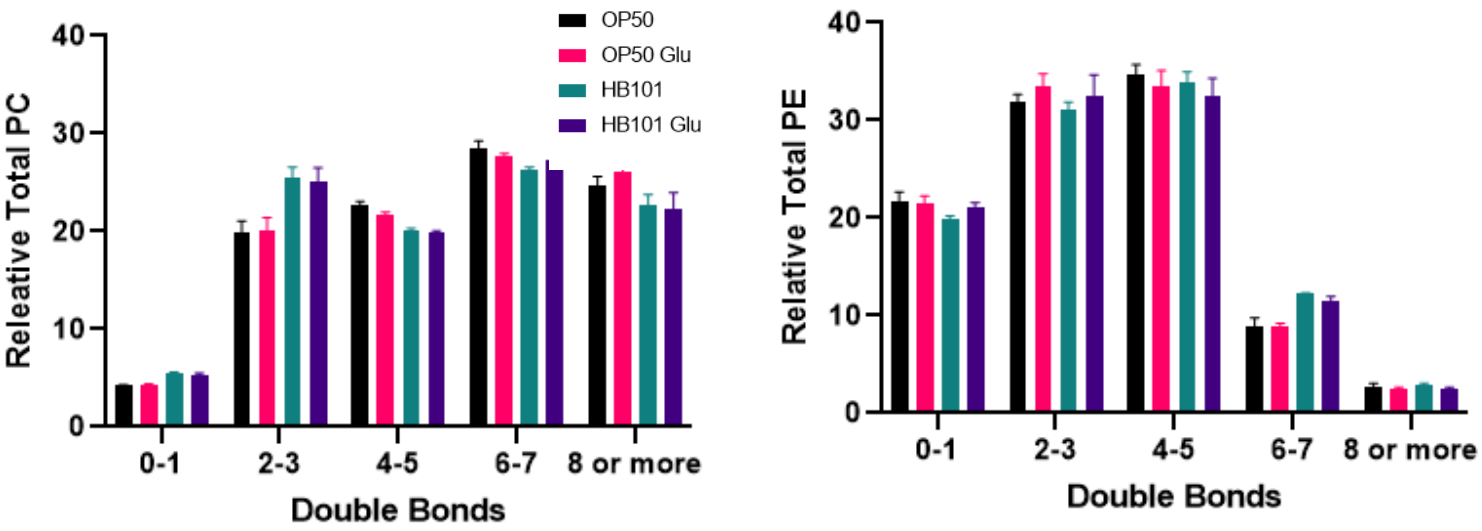


Figure 19: Relative Total Lipid Composition for wild-type nematodes under glucose stress. L1 stage N2 worms were plated on seeded HG plates with OP50 or HB101 bacteria compared with OP50 and HB101 bacteria and glucose grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were collected for analysis. Panel B is extracted from Panel A and is simply a zoomed-in version of the less abundant classes of lipid species. There appears to be an increase in LPCs in OP50 + Glucose-fed worms compared to

OP50-fed worms. This also appears to be consistent in HB101 + Glucose-fed worms compared with HB101-fed worms. Panel C is extracted from Panel A, focusing specifically on the PCs and PEs present. There appears to be a decrease in PCs in OP50 + Glucose-fed worms compared to OP50-fed worms. This trend appears consistent in HB101 + Glucose and HB101-fed worms. Additionally, there appears to be a slight increase in PEs in OP50 + Glucose-fed worms when compared with OP50-fed worms. This looks to be consistent in HB101 + Glucose and HB101-fed worms, though it is less substantial. Panel D compares the abundance of PC:PE head groups. There appears to be a decrease in the PC:PE ratio in OP50 + Glucose-fed worms when compared with OP50-fed worms. This decrease appears to be consistent when comparing HB101 + Glucose-fed worms with HB101-fed worms.

In glucose-fed worms, the amount of phosphatidylcholine (PC) groups is decreased when compared with worms not fed glucose (Figure 19 C). This trend is observed in both worms that were fed OP50 as well as worms fed HB101. This indicates that the worms are producing less PCs, so there must be an increase in other lipid species to compensate for this loss. It appears that there is an increase in phosphatidylethanolamine (PE) groups in glucose-fed worms (Figure 19 C). When looking at Figure 19 D, it is apparent that there is a decrease in the ratio of PC:PE in glucose-fed worms, indicating an increase in the PEs produced by glucose-fed worms. Generally, there do not appear to be many other consistent trends across the other lipid species analyzed. This indicates that the major consistent changes between worms occur in PCs and PEs. Glucose causes stress and metabolic changes in *C. elegans* (Alcántar-Fernández, 2018) which could be the explanation for the varying levels of PCs and PEs. PCs and PEs are some of the most highly abundant head groups in membranes. The production of PEs may be increased in glucose-fed worms as they are smaller than PCs, so they may increase the fluidity of the membrane. The membrane fluidity may need to be increased due to the stress caused by glucose, so the worms may undergo this metabolic change for self-preservation.



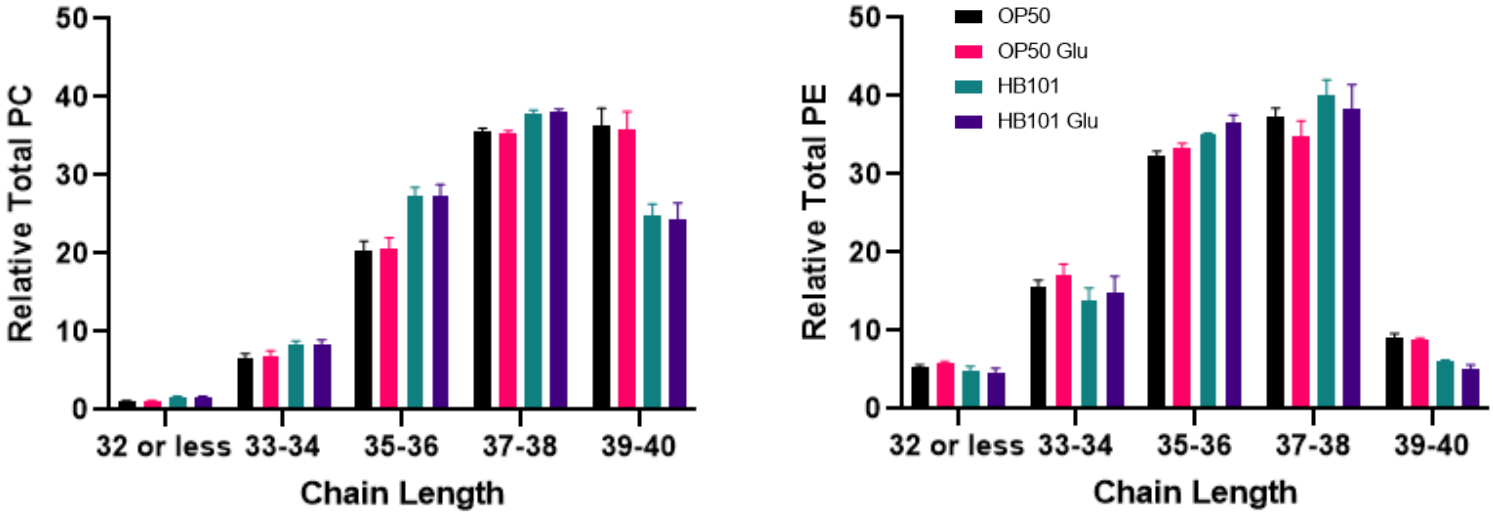
A **B**

Figure 20: Quantification of PC and PE double bonds for wild-type nematodes under glucose stress.

L1 stage N2 worms were plated on seeded HG plates with OP50 or HB101 bacteria compared with OP50 and HB101 bacteria and glucose grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were collected for analysis. Panel A shows the number of double bonds for lipids in the PC class. There do not appear to be any major changes when comparing OP50 to OP50 + glucose. The same can be said when comparing HB101 to HB101 + glucose. There do appear to be changes when comparing OP50 to HB101, like in the 2-3 double bonds, as well as in OP50 + glucose and HB101 + glucose. Panel B shows the number of double bonds for lipids in the PE class. There do appear to be slight changes occurring in the 2-3 double bonds category when comparing OP50 + glucose and OP50. The same appears to be consistent for HB101 + glucose compared to HB101.

As seen in the volcano plots in Figure 18, there do not appear to be major statistically significant changes in the lipid classes present when comparing OP50-fed worms to OP50 + glucose-fed worms, as well as when comparing HB101-fed worms to HB101 + glucose-fed worms. This is reflected as well in the PC double bonds (Figure 20 Panel A), as there are no major observable

changes. To properly identify if there are any changes present, more replicates can be conducted. However, there do appear to be slight changes in the PE double bonds (Figure 21 Panel B). The extent to which the *C. elegans* are affected by glucose stress may be limited.



A **B**

Figure 21: Quantification of PC and PE chain lengths for wild-type nematodes under glucose stress.

L1 stage N2 worms were plated on seeded HG plates with OP50 or HB101 bacteria compared with OP50 and HB101 bacteria and glucose grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were collected for analysis. Panel A shows the fatty acid chain lengths of lipids in the PC class. There are no distinct changes in fatty acid chain lengths in the PC class when comparing OP50 to OP50 + glucose-fed worms, and the same is reflected in the HB101 and HB 101 + glucose-fed worms. Panel B shows fatty acid chain lengths of lipids in the PE class. In the PE class, do not appear to be any distinct changes.

4.3 Metabolic Adaptations in Response to Starvation

The investigation of starvation can help to identify the significance of metabolic changes in response to another type of stress.

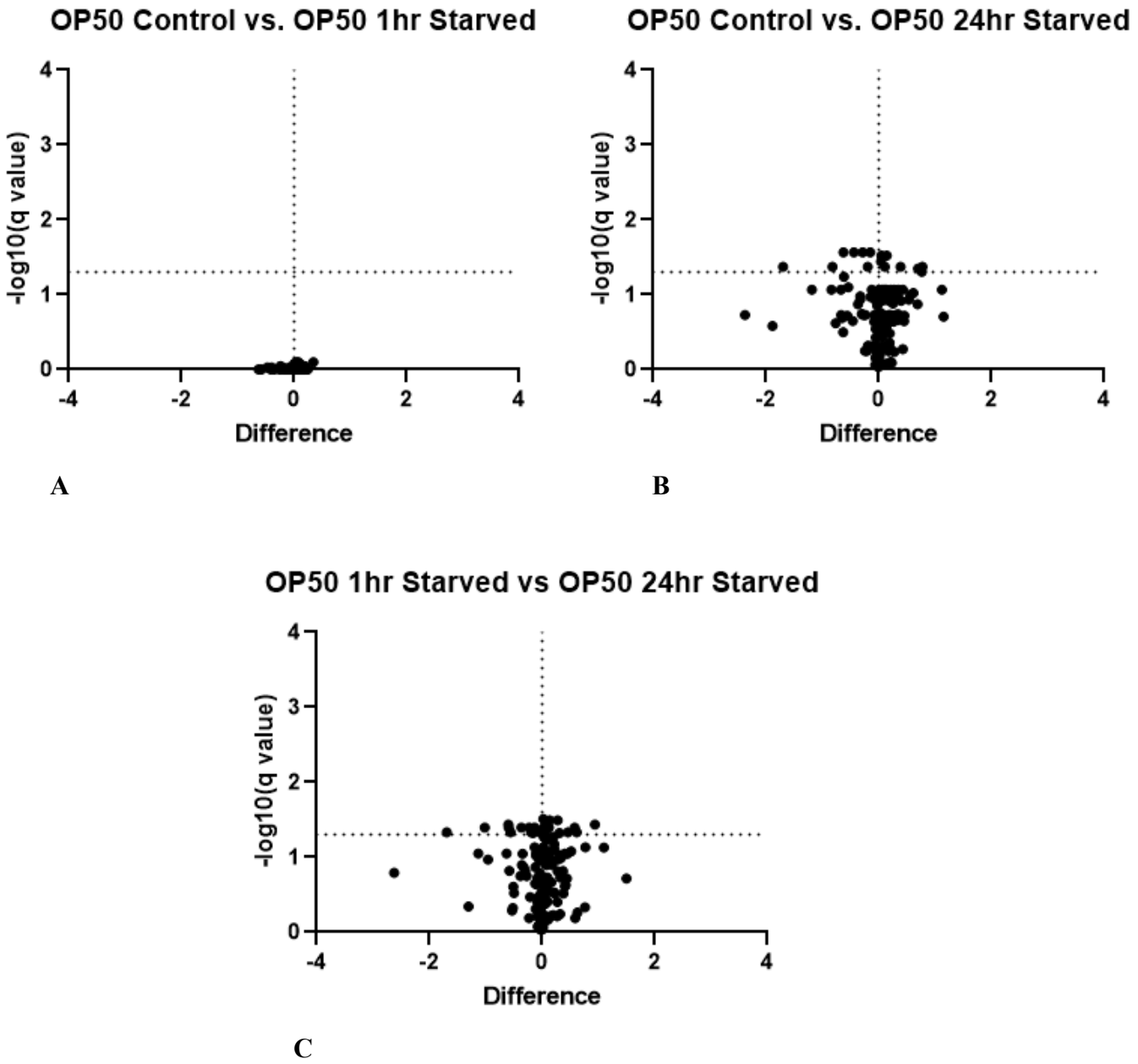


Figure 22: Volcano plots for wild-type nematodes under Starvation Stress with an OP50 diet.

In this experiment, worms were grown for 66 hours on HG plates with OP50. Control worms were collected after 66 hours. Starved worms were spun in 1xM9 in 15mL conical tubes at room temperature (~20ºC) for one hour and 24 hours respectively, and then collected. The similarities and differences in lipid species of the worms are shown in these volcano plots. In panel A, there are no statistically significant differences in the lipid species present between control worms and one hour starved worms. In panel B, there are statistically significant changes in the lipid species present between control worms and 24 hour starved worms. In panel C, there are some

statistically significant changes between control worms and one hour starved worms.

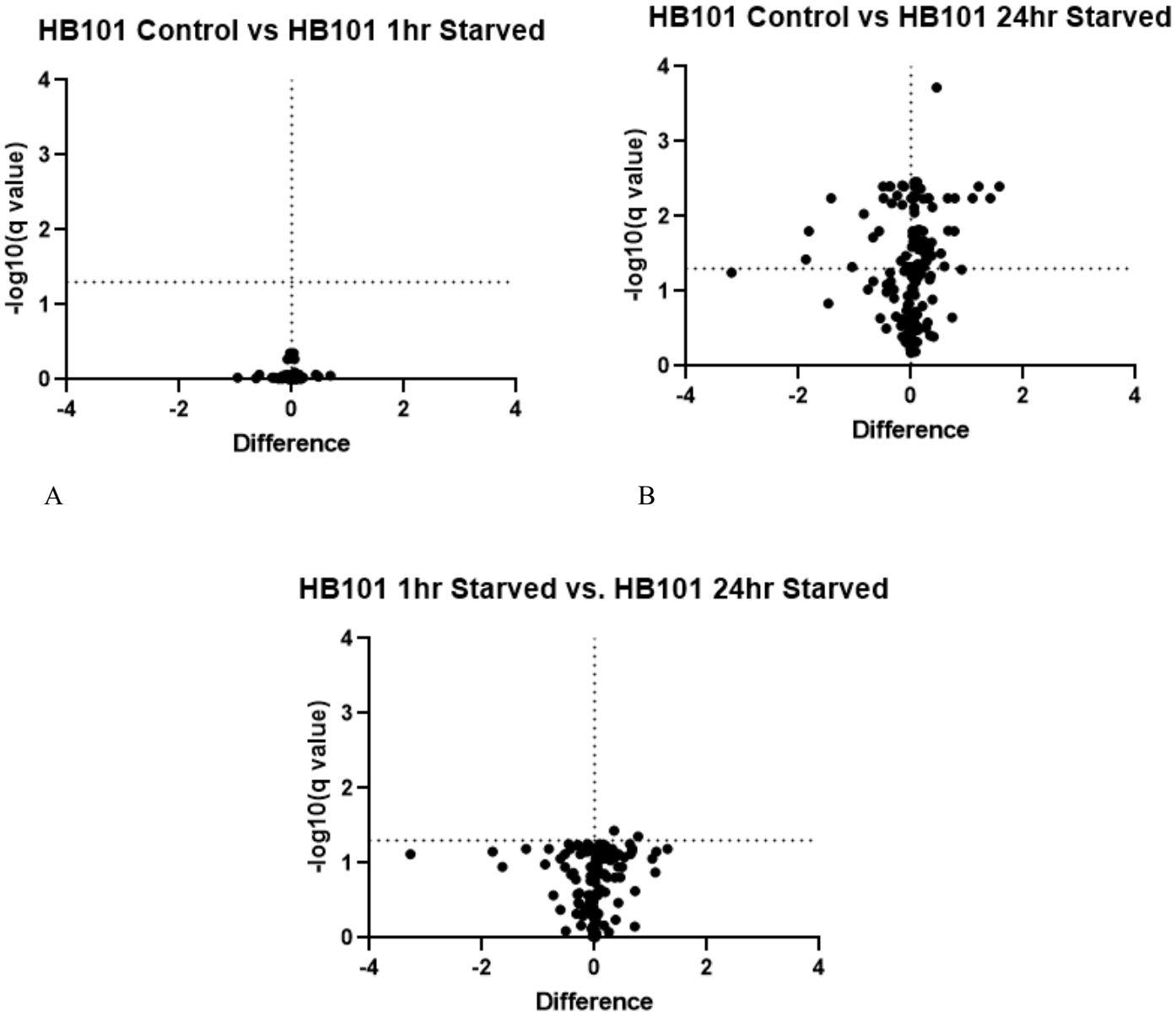


Figure 23: Volcano plots for wild-type nematodes under Starvation Stress with an HB101 diet.

In this experiment, worms were grown for 66 hours on HG plates with HB101. Control worms were collected after 66 hours. Starved worms were spun in 1xM9 in 15mL conical tubes at room temperature (~20ºC) for one hour and 24 hours respectively, and then collected. The similarities and differences in lipid species in the worms are shown in these volcano plots. In panel A, there are no statistically significant differences in the lipid species present between control worms and one hour starved worms. In panel B, there are many statistically significant changes in the lipid

species present between control worms and 24 hour starved worms. In panel C, there are some statistically significant changes between control worms and one hour starved worms.

As seen in Figures 22 and 23, major changes in the worms were observed between the control and 24-hour starved worms. This indicates that the longer starvation conditions greatly affect the worm's lipid metabolism and composition. The lack of food can trigger the worms to go into starvation metabolism. Fatty acid breakdown can be triggered through the mechanism of fatty acid beta oxidation to promote survival in worms while they are starving (Harvald, 2016). Changes in worms might be more significant after 24 hours of starvation compared to one hour due to the nature of starvation metabolism. During the initial period of food deprivation, the metabolic response may be more subtle as the worms utilize readily available energy reserves. However, as time progresses and the duration of starvation increases, the metabolic demand for energy becomes more urgent. After 24 hours of starvation, the worms may have depleted their immediate energy stores and need to ramp up fatty acid breakdown more significantly to sustain vital cellular functions. Therefore, the effects on lipid metabolism and composition may be more pronounced and readily observable after a longer duration of starvation.

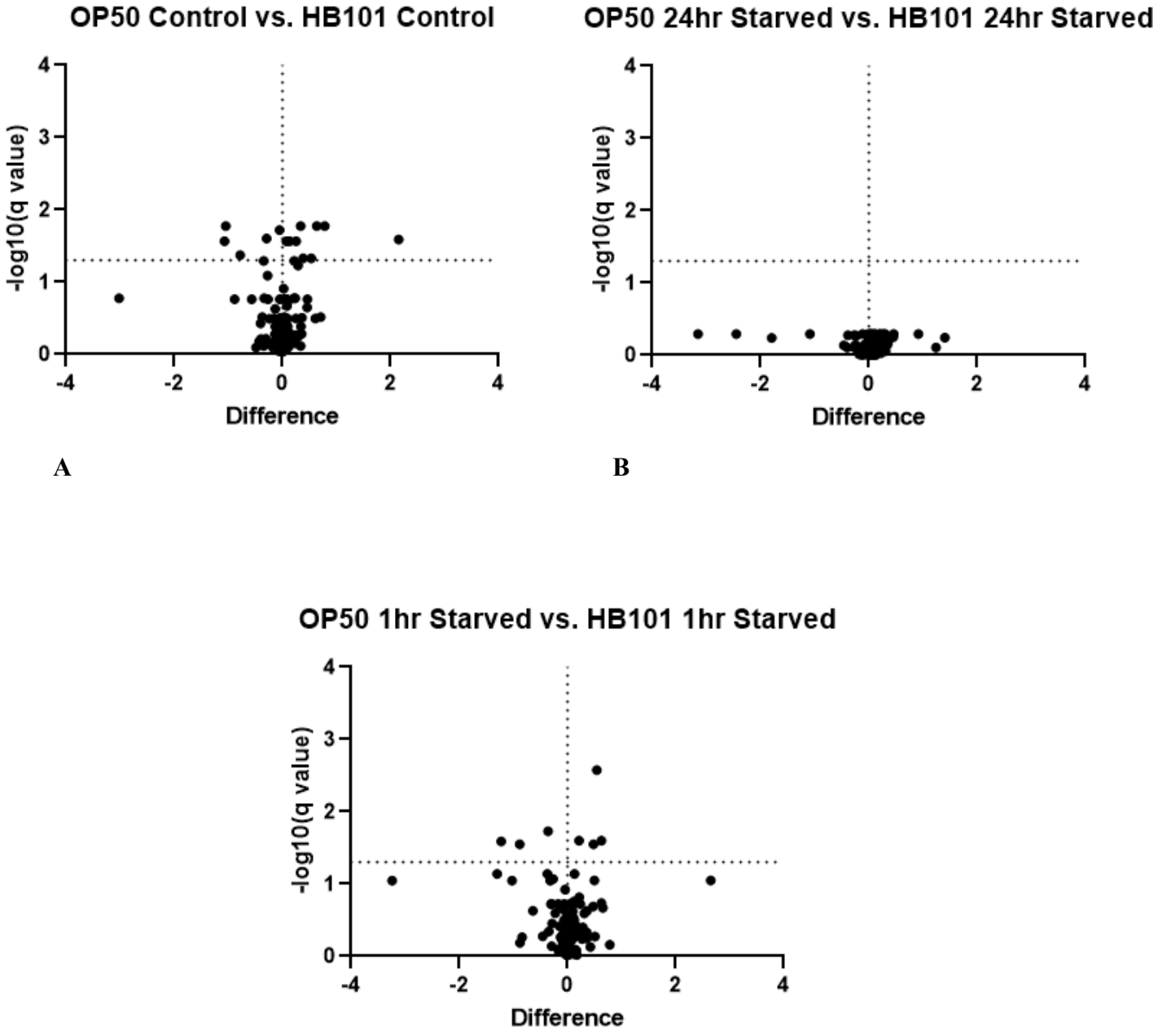


Figure 24: Volcano plots for wild-type nematodes under Starvation Stress comparing OP50 and HB101 diets.

In panel A, it is shown that there are statistically significant changes in lipid species when comparing OP50 control and HB101 control worms. Additionally, there are some statistically significant changes in the OP50 one hour starved worms and the HB101 one hour starved

worms, as seen in panel B. In panel C, there are no statistically significant changes in lipid species between OP50 24 hour starved worms and HB101 24 hour starved worms.

As seen in Figure 24, there are statistically significant changes in lipid composition between OP50 control worms and HB101 control worms, as well as OP50 one hour starved worms and HB101 one hour starved worms, as seen in Figure 24 panels A and B. In panel C, there are no statistically significant changes in the lipid species present between OP50 24 hour starved worms and HB101 24 hour starved worms. The worms may shift into starvation metabolism after 24 hours and the starvation conditions may affect the worms more greatly than the change in diet affects the worms.

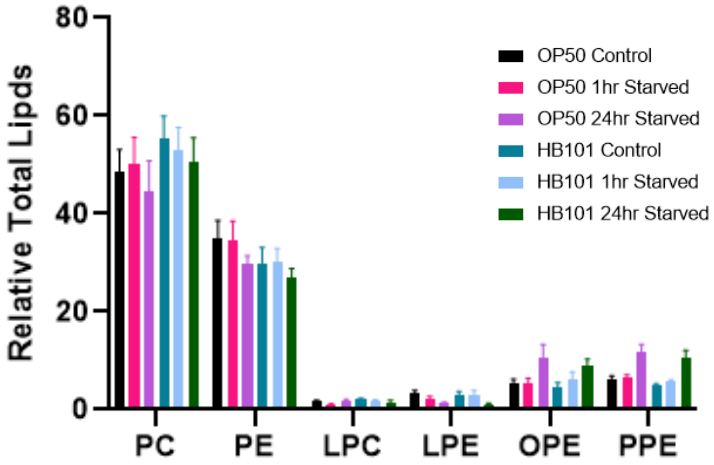


Figure 25: Relative Total Lipid Composition for wild-type nematodes under starvation stress.

L1 stage N2 worms were plated on seeded HG plates with OP50 or HB101 bacteria grown two days before plating. After the 66-hour growth period, the worms reached the adult stage and were either collected immediately, starved for one hour, or starved for 24 hours and then collected for analysis. The major changes in lipid species appear to be between the OP50 control and OP50 24-hour starved worms and between the HB101 control and HB101 24-hour starved worms.

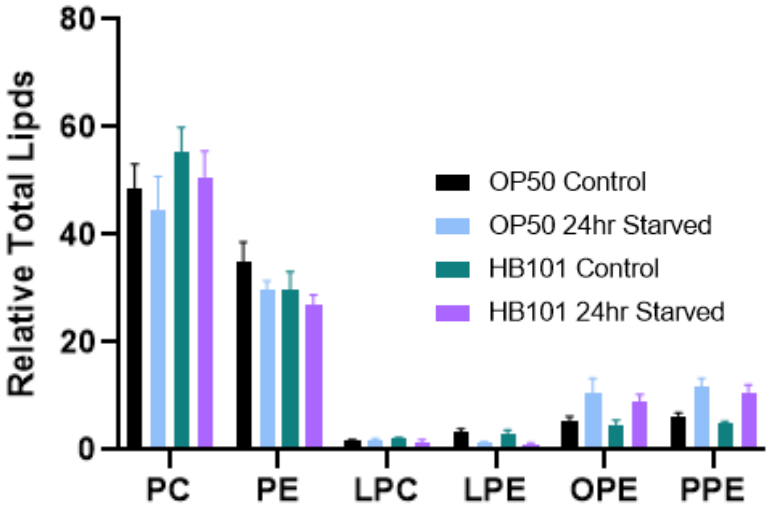
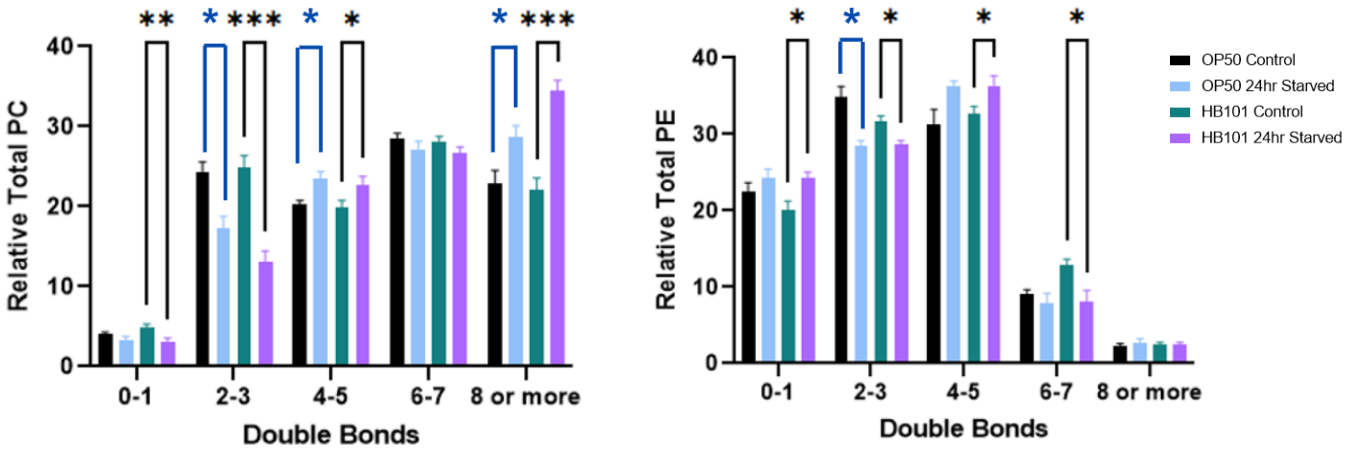


Figure 26: Relative Total Lipid Composition for wild-type nematodes fed OP50 and HB101 and subjected to starvation stress for 24 hours.

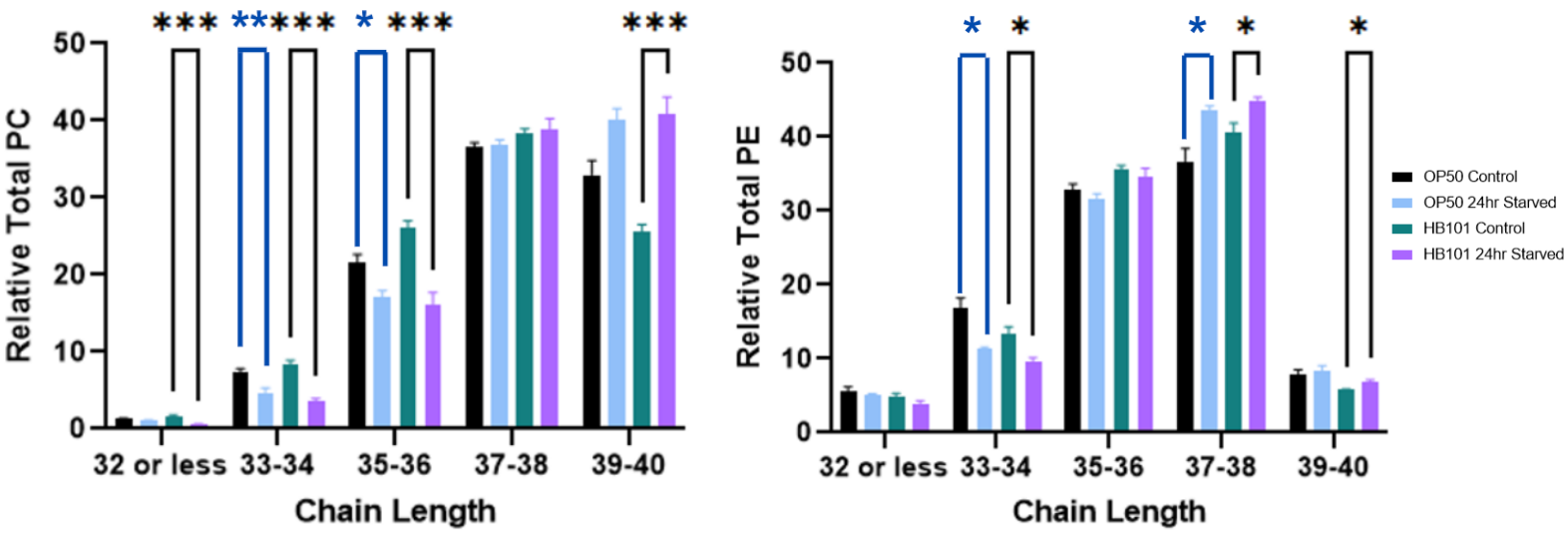
Figure 26 is adapted from Figure 25, but the one hour starved samples were removed to get a better understanding of the changes occurring in the control and the 24 hour starved worms. There is a decrease in PCs and PEs in both starved OP50-fed and starved HB101-fed worms. There is a subsequent increase in OPEs and PPEs in both starved OP50-fed and starved HB101-fed worms.



A B

Figure 27: Quantification of PC and PE double bonds for wild-type nematodes under starvation stress with stars indicating statistically significant changes.

Panel A shows stars indicating the significance value of the changes in lipid species for PC Double Bonds in HB101 fed control worms and HB101 fed 24 hour starved worms. The most significant changes observed are a decrease in 2-3 double bonds and an increase in 8 or more double bonds for HB101 fed 24 hour starved worms. There was also a decrease in 0-1 double bonds and an increase in 4-5 double bonds in HB101 fed 24 hour starved worms. Panel B shows stars indicating the significance value of the changes in lipid species for PE double bonds in HB101 fed control worms and HB101 fed 24 hour starved worms. There were decreases in 2-3 and 6-7 double bonds, and increases in 0-1 and 4-5 double bonds in HB101 fed 24 hour starved worms. One star indicates a P value of <0.5. Two stars indicate a P value <0.05. Three stars indicate a P value of <0.005. The smaller the P value, the more statistically significant the result.



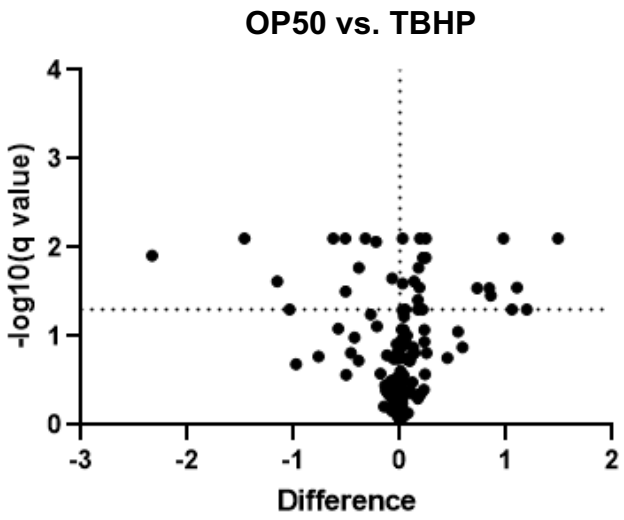
A **B**

Figure 28: Quantification of PC and PE chain lengths for wild-type nematodes under starvation stress.

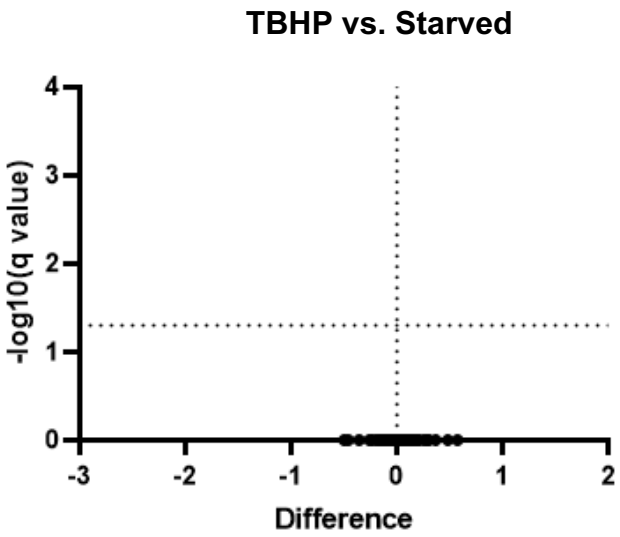
Panel A shows stars indicating the significance value of the changes in lipid species for PC Chain Length in HB101 fed control worms and HB101 fed 24 hour starved worms. There was a decrease in chain lengths of 32 or less, 33-34, and 35-36 in HB101 fed 24 hour starved worms. There was also an increase in chain lengths of 39-40 in HB101 fed 24 hour starved worms. Long-chain fatty acids have the potential to be more polyunsaturated as they have more availability to make carbon-carbon double bonds. Long-chain polyunsaturated fatty acids increase the fluidity and stability of the membrane, so an increase in these 39-40 chain lengths may make the membrane more fluid and stability. Panel B shows stars indicating the significance value of the changes in lipid species for PE Chain Length in HB101 fed control worms and HB101 fed 24 hour starved worms. There was a decrease in chain lengths of 33-34 for HB101 fed 24 hour starved worms, and an increase in chain lengths of 37-38 and 38-40. One hour starved worms One star indicates a P value of <0.05. Two stars indicate a P value <0.01. Three stars indicate a P value of <0.001.

4.4 The Comparison Between TBHP Induced Stress and Starvation

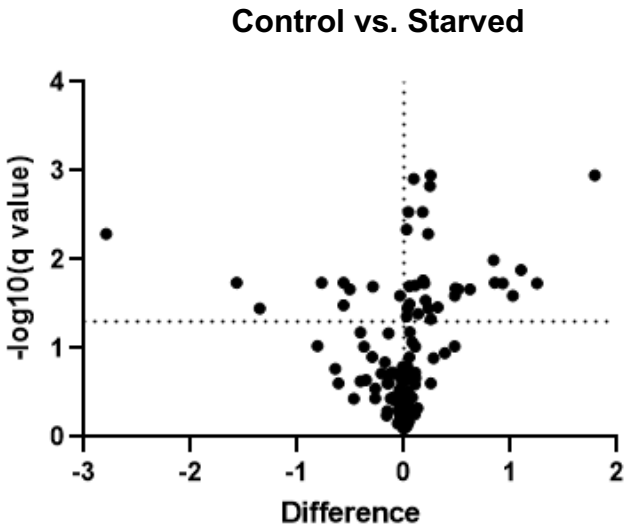
Comparing oxidative stress and starvation stress in *C. elegans* fed OP50 bacteria can provide insight about how metabolic shifts occur in response to different types of stress.



A



B



C

Figure 29: Volcano plots for wild-type nematodes under TBHP Induced Stress and Starvation.

In this experiment, worms were grown for 66 hours on HG plates with OP50 and then transferred to various plates for two hours. The different conditions the worms were transferred to were OP50 control plates, TBHP plates to induce oxidative stress, and plates with no food to induce starvation. Above are volcano plots showing the statistically significant changes in lipid species present with varying conditions. In panel A, worms transferred onto control OP50 plates were compared with worms transferred onto TBHP plates. There appear to be statistically significant differences in the lipid species present between OP50-fed worms and TBHP-fed worms. In panel B, worms transferred onto TBHP plates were compared to worms transferred to plates with no

food present. There do not appear to be any statistically significant differences between TBHP-fed worms and starved worms. In panel C, worms transferred onto control OP50 plates were compared to worms transferred onto plates with no food. There do appear to be statistically significant changes in lipid species present between OP50-fed worms and starved worms. These volcano plots were made with data from two replicates of this experiment.

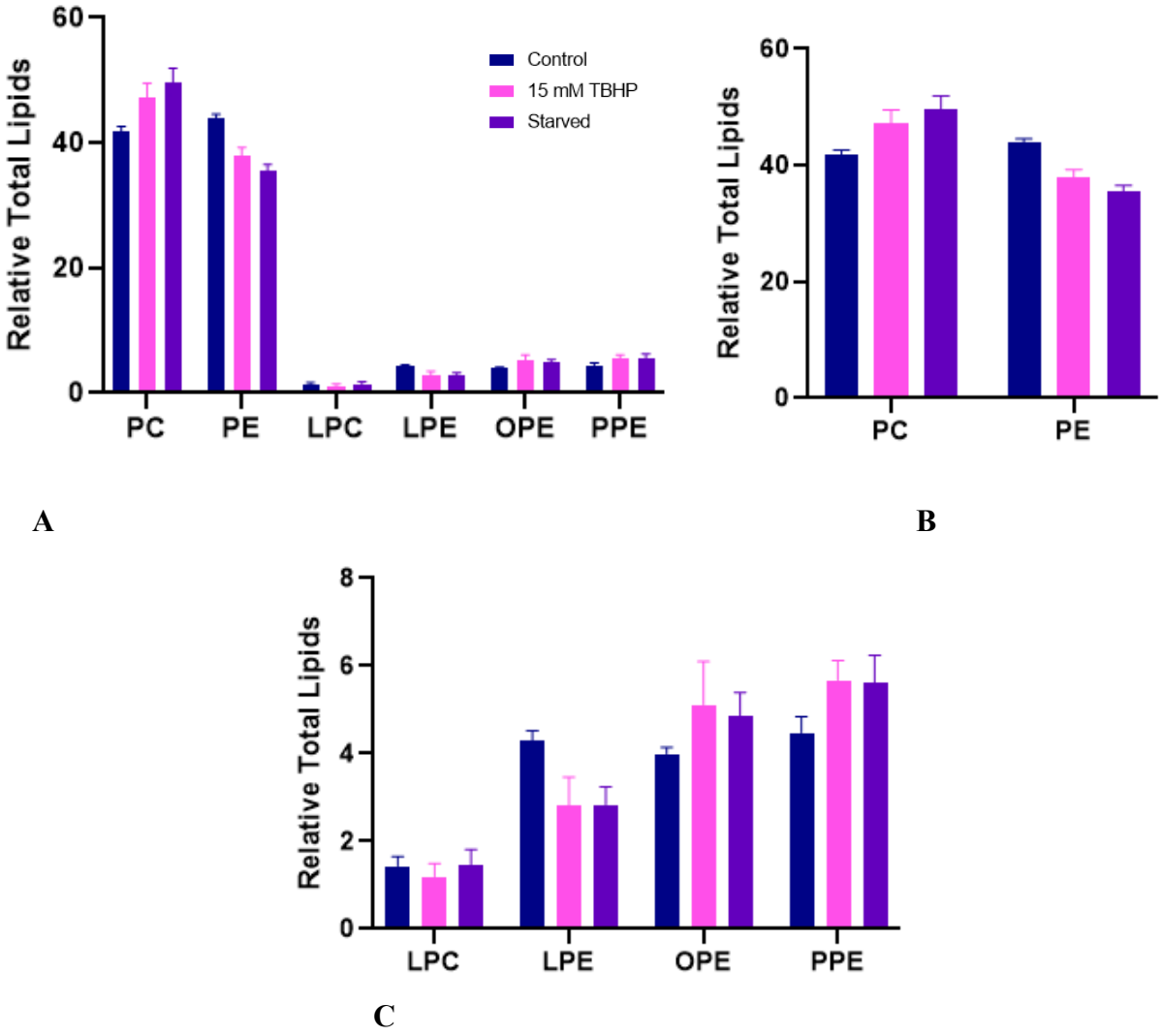
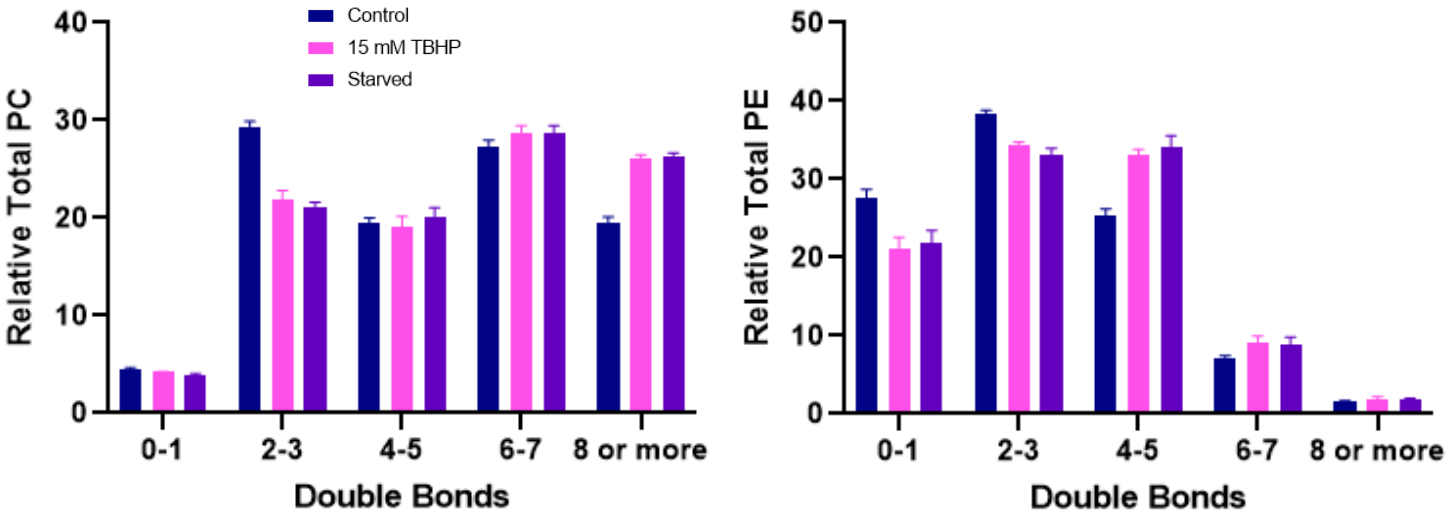


Figure 30: Relative Total Lipid Composition for wild-type nematodes under TBHP stress. Panel A shows an overview of all the relative total lipids present in the nematode samples collected. Panels B and C are zoomed-in versions of this figure. Panel B focuses on specifically the PC and PE groups present. Panel C focuses on the less abundantly present lipid classes, lyso species including LPCs and LPEs, and plasmalogen species including OPEs and PPEs. In Panel B, the worms transferred to control OP50 plates appear to have fewer PCs present than the worms transferred to 15mM TBHP plates as well as those transferred to plates with no food. The loss must be compensated for somewhere, and it appears that there are more relative total PEs

present in worms transferred to OP50 plates than in the worms transferred to 15mM TBHP plates and plates with no food. In Panel C, there appears to be a loss of OPEs and LPEs in worms transferred to control OP50 plates. This loss seems to be compensated for in the presence of LPEs. There do not appear to be any other major changes in the lipid species present.

There are major changes between the lipid species present in worms transferred to control OP50 plates when compared to worms transferred to both TBHP plates and plates with no food (Figure 31). There is a decrease in PCs and an increase in PEs in worms transferred to control OP50 plates when compared to the worms transferred to both TBHP plates as well as plates with no food. There also appears to be a decrease in OPEs and PPEs in worms transferred to OP50 control plates and an increase in LPEs. LPEs have roles in the signaling of molecules (Croda, n.d.). The increase in LPEs could be due to the worms having a change in their signaling and regulation pathways. The changes in the lipid species present in worms transferred to TBHP plates appear to be similar to the changes observed in the worms transferred to plates with no food, which can be best seen in some of the less abundant lipids like the LPEs, OPEs, and PPEs in Panel C of Figure 32.



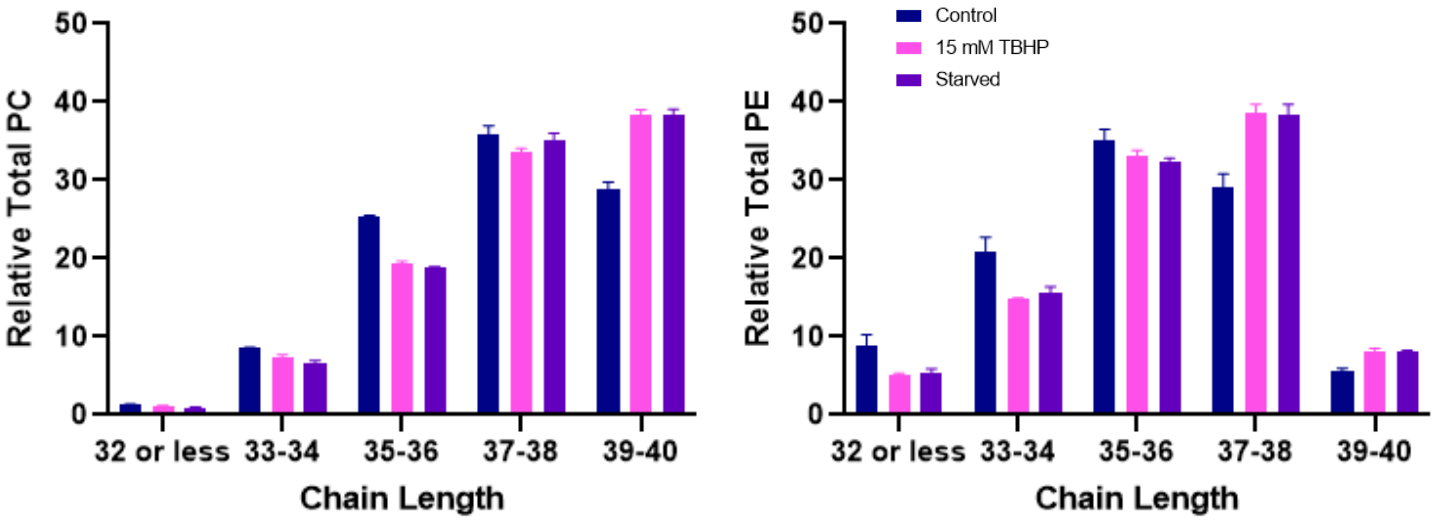
A **B**

Figure 31: Quantification of PC and PE double bonds for wild-type nematodes under starvation and TBHP stress.

L1 stage N2 worms were plated on seeded HG plates with OP50 bacteria grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were transferred to OP50 control plates, TBHP plates, or plates with no food or starved plates for two hours and then collected. There are distinct changes between PC and PE double bonds across the worms. In the PC class (A), there are distinctly fewer 0-1 double bonds observed in worms compared with the other classes. Compared with the PE class (B), which appears to have fewer worms with 8 or more double bonds. In both the TBHP-stressed worms and starved worms, there is a decrease in relative total PCs with 2-3 double bonds when compared with control worms and a subsequent increase in relative total PCs with 8 or more double bonds. In both TBHP-stressed worms and starved worms, there appears to be a decrease in relative total PEs with 0-1 double bonds when compared with control worms, and a subsequent increase in relative total PEs with 4-5 double bonds.

In both THBP-stressed worms and starved worms, there appears to be an increase in relative total PCs with 8 or more double bonds when compared with control worms. This indicates that TBHP-stressed and starved worms may favor the production of longer-chain PUFAs over shorter-chain PUFAs. In addition to this, there appears to be a decrease in relative total PEs with 0-1 double bonds, and an increase in relative total PEs with 4-5 double bonds, which indicates

that TBHP-stressed and starved worms may favor the production of intermediate-length PUFA chains rather than shorter or longer-chain PUFAs.



A **B**

Figure 32: Quantification of PC and PE chain lengths for wild-type nematodes under starvation and TBHP stress.
L1 stage N2 worms were plated on seeded HG plates with OP50 bacteria grown two days before plating. Approximately five thousand worms were plated on each plate. They were grown for 66 hours at 20°C. After the 66-hour growth period, the worms reached the adult stage and were transferred to OP50 control plates, TBHP plates, or plates with no food or starved plates for two hours and then collected. In the PC class (A), there appears to be a decrease in relative total PCs in the 35-36 chain length for both TBHP-stressed and starved worms and a subsequent increase in the 39-40 chain length. In the PE class (B), there appears to be a decrease in relative total PEs with chain lengths of 33-34 for both TBHP-stressed and starved worms and a subsequent increase in the 37-38 chain length.

Typically, during fasting, *C. elegans* break down long-chain fatty acids via β-oxidation to provide up to 80% of their energy (Olpin, 2013). The exact chain lengths broken down during β-oxidation are not specified, but it is suggested that longer chain fatty acids are broken down during fasting conditions. It would be expected that the *C. elegans* subjected to TBHP stress and starvation would break down and oxidize the longer fatty acid chains, like in the 37-38 or 39-40

categories, but that is not what is seen in Figure 34. Some of the more intermediate chain lengths for both PCs and PEs are decreased, like the 35-36 length in panel A, and the 33-34 length in panel B, leading to the subsequent production of other longer chain fatty acids, like in the 39-40 length in panel A and the 37-38 length in panel B. This may indicate that *C. elegans* are breaking down the fatty acids in these categories, so their metabolism pathway is shifting to replenish their membrane with these fatty acids to ensure proper function.

5.0 Expected Results

5.1 The Impact of Different Bacterial Diets on *Eat-2* Mutants

After observing the significant impact of prolonged starvation on lipid metabolism and composition in *C. elegans*, it becomes intriguing to delve deeper into the metabolic alterations induced by dietary restriction. The starvation experiment provided information about adaptive responses triggered by acute food deprivation. However, to understand the long-term metabolic adaptations related to restricted food intake, it would be valuable to conduct a dietary restriction experiment with *eat-2* mutant worms. *Eat-2* mutant worms present a compelling model for studying dietary restriction due to their inherent feeding behavior mutation, which limits their food consumption compared to wild-type worms.

Methods for a dietary restriction experiment with *eat-2* mutants are outlined in the methods section of this report. Previous studies have found that *eat-2* mutants have reduced food intake that extends their lifespan compared to wild-type N2 worms (Lakowski & Hekimi, 1998). In the anticipated results of the dietary restriction experiment, it is expected that the *eat-2* mutant worms will exhibit distinct metabolic profiles compared to wild-type worms under the same feeding conditions. Specifically, the analysis of lipid metabolism in *eat-2* mutants is anticipated to reveal alterations indicative of a metabolic shift towards promoting longevity and survival under restricted food availability. This shift may manifest as changes in lipid composition, with potential increases in lipid stores or alterations in lipid species reflective of enhanced stress resistance and energy conservation mechanisms. Dietary restriction increases lifespan of *C. elegans* (Lenaerts, 2008), while long periods of starvation may cause worms to pause development, or death may be induced through bagging (Carranza-García, 2020). Bagging

occurs when offspring cause death to their parent when starved parents have embryos in their uterus that hatch and then begin to consume the parent for nutrients (Mosser, 2011).

Moreover, volcano plots analysis is expected to reveal significant changes in lipidomic profiles between *eat-2* mutant worms and wild-type controls. Because starvation and dietary restriction both limit nutrient availability, the general trends for the lipid composition, double bond and chain length distribution of N2 wild-type worms starved for 24 hour and the *eat-2* mutants might be similar. For example, similarly to the N2 wild-type worms starved for 24 hours, the *eat-2* mutants might have a decrease in PC abundance due to the reduced availability of components for phospholipid synthesis under dietary restriction conditions. Moreover, the *eat-2* mutants might be expected to exhibit an increase in PCs and PEs with higher degrees of unsaturation, or more double bonds. This change may enhance membrane fluidity and flexibility, facilitating cellular processes under dietary restriction-induced stress. On the other hand, the *eat-2* mutants might be expected to exhibit a decrease in the abundance of PC and PE species with lower degrees of unsaturation, or less double bonds as these lipid species may be less adaptive to the metabolic demands imposed by dietary restriction.

The N2 wild-type worms that were starved for 24 hours had a decrease in PCs and PEs with shorter chain lengths and an increase in PCs and PEs with longer chain lengths compared to N2 wild-type control worms. The *eat-2* mutants might have a similar trend because under restricted food availability, the worms might prioritize processes to membrane stability by increasing the longer chain fatty acids while breaking down shorter chain fatty acids.

Although the comparison between *eat-2* mutants and N2 control worms might be similar to the comparison between starved and control N2 worms, the differences in lipid composition might be less drastic for the *eat-2* mutants vs. N2 wild-type control worms. While acute starvation includes a sudden and severe nutrient deprivation for the worms, dietary restriction in *eat-2* mutants has a gradual and chronic reduction in food intake. Due to *eat-2* mutants having this gradual decrease in nutrient availability, it allows them to exhibit adaptive metabolic responses. Therefore, the slower food reduction might make the changes in lipid composition less extreme compared to acute starvation. Another consideration would be that *eat-2* mutants may already have metabolic adjustments for their feeding behavior mutation that might resemble those induced by dietary restriction, leading to a less drastic shift in lipid composition compared to N2 worms under to acute starvation.

The expected results from the dietary restriction with *eat-2* mutants' experiment will yield valuable information about metabolic adaptations to dietary retraction and *eat-2* mutants' membrane structure. With these results, a comparative analysis with the starvation experiment can be conducted to understand different dietary patterns and feeding behaviors influence lipid metabolism in *C. elegans*.

5.2 The Impact of Paraformaldehyde for Dietary Restriction

General observations of the experimental *C. elegans* exposed to PFA for dietary restriction were noted. *C. elegans* exhibit certain behaviors when there is a lack of food. The *C. elegans* were observed to be bunching and clumping together in areas where food was present on the plates, as well as moving to the edges of the plates. After the first trial of dietary restriction, the worms on the plates with 1000 microliters of PFA-killed bacteria, or the undiluted plates, appeared not to

be starved. There appeared to still be food on the plates, as the worm's tracks could be observed. The 75% and 50% plates looked similar to the undiluted plates. The only condition where the worms appeared to be starved after a period of 24 hours were the 25% plates, or the worms containing the smallest amount of food: 250 microliters of PFA-killed bacteria diluted with 750 microliters of LB liquid. The worms on the 25% plates appeared to have no food remaining, were clumped in distinct areas of the plates, and were moving near the edges of the plates. Knowing this, a new experimental design was planned to have one set of conditions where the worms were known not to be starved in order to compare to smaller dilutions of the PFA-killed bacteria. The new dilutions to observe were 100%, 25%, 12.5%, and 6.25%.

Observations from the new dilutions were noted, but this data was also not analyzed. After 24 hours, worms on the 100% plates did not appear to be starved as there was still food on the plates and the worms were not in distinct clumps. The worms on the 25% plates were starting to clump together and moving to the edges of the plate. The worms on the 12.5% plate were surrounding the edges of the plate. The worms on the 6.25% plates were mostly clumped towards the center of the plate. There additionally was a control OP50 plate with worms grown under the same conditions in order to ensure that the control worms were not starved. The worms on the control plate did not appear to be starved. These animals are collected for lipidomic analysis in the future.

6.0 Discussion

This project led to various conclusions, including worms responding to stress in different ways. PCs and PEs change when the worms are exposed to various stress conditions, the lipid profiles of the worms shift when they are fed OP50 vs. HB101, trends from the glucose stress experiment indicate that PC:PE ratio decreases slightly when worms are exposed to glucose stress, and changes in lipids for starvation experiments are seen more strongly in animals fed HB101 diets.

In the introductory experiment, it was observed that the PCs and PEs of *C. elegans* shift the most when they are fed OP50 versus HB101, compared with HT115. OP50 is a typical bacterial strain used in laboratories, and some other commonly used strains are HB101 and HT115. When proceeding through experimentation, the plate count was increasing with each experiment, so a decision to continue only using two bacterial strains was made to limit the plate count. Due to the fact that there were the most differences in lipid composition between OP50 and HB101, those bacterial strains were chosen to proceed through the duration of the project to observe the differences between shifting the diets as well as comparing those diets to the exposure of the worms to different stressors.

If conducting the glucose experiments in the future, it would be helpful to include more replicates to assess the statistical significance of the changes between glucose-fed and non-glucose-fed worms. There appeared to be general trends and changes observed, but these changes may become more apparent with more replicates included. The general trends from the glucose stress experiments show that glucose does not shift the metabolism of *C. elegans* by much.

When conducting starvation experiments, a similar protocol was followed to research conducted over the summer in our lab. The controls for this experiment were not grown for the same amount of time as the experimental worms and they were also on a solid plate versus introduced into a liquid media. The one hour starved worms and 24 hour starved worms were spun in 1xM9 for the duration of their starvation. The worms themselves may have become stressed simply from the switch from plates to liquid, and some metabolic shifts may be associated with this change rather than with the starvation stress. To more properly investigate starvation stress, the control worms could have also been spun in a liquid solution containing food. A better experimental design would be for all of the worms to be transferred onto plates rather than into tubes and spun in liquid. The control worms could be transferred to control plates, and the experimental worms could be transferred to plates that contained no food for the duration of their starvation.

In the first PFA experiment, plates were seeded with concentrations of 25, 50, 75, and 100% PFA. Dilutions were prepared with LB media. For instance, for the 25% PFA plate, 250 microliters of PFA-killed OP50 was mixed with 750 microliters of LB liquid. Once the worms from these plates were observed, further experimental conditions were designed to narrow down the dilution window. Plates were diluted to concentrations of 100%, 25%, 12.5%, and 6.25%. CF512 worms were used for this experiment due to the fact that they are a temperature-sensitive sterile strain. At 25ºC, the worms lay eggs, but the eggs are not viable, so they do not hatch. However, after observing the worms for 24 hours, there appeared to be viable eggs on the plate that hatched into L1 worms. The goal was to use CF512 to observe how the metabolism of adult

worms shifted when exposed to dietary restriction for 24 hours, rather than observe the metabolism of L1 worms. The worms may have laid viable offspring due to introduction of an N2 worm accidentally or the reversal of the mutation. It was important to remove as many L1 worms as possible to analyze just the metabolism of the adult worms. In order to remove the L1s, the worms were collected via gravity collection. The worms were collected into a 15mL centrifuge tube, and the adult worms settled to the bottom more quickly than the L1 worms. The tubes were not centrifuged, but the 1xM9 containing the L1 worms was aspirated before they settled to the bottom. This process was repeated three times to remove as many L1s as possible and just collect the adult worms. To ensure that the mutation of CF512 worms is not reversed, a new frozen worm sample may need to be used in the future and the worms can be stored in separate boxes and maybe even separate incubators.

To explore various avenues of stress, paraformaldehyde and TBHP can be further explored. TBHP is an oxidant, which induces stress in the worms. General trends from the TBHP-induced stress experiments were identified, but there are no conclusions drawn. To make definite conclusions about the comparison of TBHP-induced stress to starvation, more replicates are likely needed to ensure the consistency between replicates. Paraformaldehyde is used to kill bacteria to investigate dietary restriction. General observations from the paraformaldehyde experiments were taken, but the data was not analyzed.

C. elegans samples were collected and stored for GC-MS analysis but were not analyzed due to timeline constraints and GC-MS repairs.

7.0 Future Considerations

There are various avenues for future directions based on different forms of change in diet and stress in *C. elegans*. Due to the exploration of stress in *C. elegans*, an interesting aspect of diet to look at could be dietary restriction. One research question to be explored is how does dietary restriction affect the membrane composition of *C. elegans* using paraformaldehyde? An experimental method for experimentation with paraformaldehyde is outlined in the methods section of this report. General observations of the worm's behavior during this experiment were noted, but the data was not analyzed. The next research question that can be investigated is how does the membrane composition of *C. elegans* shift if stress is introduced with an oxidizing agent? An oxidizing agent used in laboratories is TBHP, and the procedure of introducing *C. elegans* to TBHP is outlined in the methods section of this report.

Pairing the impact of different bacterial diets on *eat-2* mutants experiment with a lifespan of *eat-2* mutants experiment should be considered for future research. For example, both *eat-2* mutants and N2 mutants could be fed different bacteria, like OP50 and HB101 strains, and worm survival could be monitored over time. In addition, both worm types could be fed various bacteria and then collected at various time points throughout their lifespan, including early adulthood, mid-life, and late life stages. By comparing the lifespan of *eat-2* mutants to N2 wild-type worms with both worm types subjected to different bacterial diets, the results can interconnect bacterial diet, food restriction, and lifespan in *C. elegans*. Building upon previous studies that show a large benefit to dietary restriction on lifespan in *C. elegans*, using *eat-2* mutants as a model for dietary restriction (Lakowski & Hekimi, 1998), this future research could provide understanding how specific bacterial diets interact with the *eat-2* mutation to modulate longevity. Looking at the

lipid composition of *eat-2* mutants and N2 worms, feeding on different bacteria, at various time points throughout their lifespan, could provide valuable insights into how dietary factors modulate lipid metabolism under conditions of restricted food intake. Additionally, the worms collected at early adulthood, mid-life, and late life stages can be compared to identify lipidomic biomarkers associated with extended lifespan or enhanced health span in *eat-2* mutants. On a larger scale, the findings from this future research potentially have practical implications for identifying optimal dietary strategies that promote healthy aging and longevity in humans.

There is another future direction to explore regarding investigating starvation stress and conditions of *C. elegans*. The exact timeline of when *C. elegans* shift into starvation metabolism is not entirely known. From the data outlined in this report, there were very few major statistically significant changes in the lipid species present in control *C. elegans* when compared with one hour starved *C. elegans*. However, there were more major statistically significant changes in the lipid species present in control *C. elegans* when compared with 24 hour starved *C. elegans*. This indicates that the worms have not fully shifted into starvation metabolism after one hour of starvation, but they have shifted into starvation metabolism after 24 hours of starvation. The exact window of time when the worms shift into starvation metabolism can be further investigated. Worms could be subjected to starvation for different windows of time between one and 24 hours. An experiment could be designed starving the worms for 1, 6, 12, 18, and 24 hours. This way, the data could be analyzed, narrowing down the window of when exactly the worms are shifting into starvation metabolism. Once the window is further specified, a new experimental design could be conducted, narrowing the windows even further. This would allow for the timeline of starvation metabolism to be discovered.

8.0 Conclusion

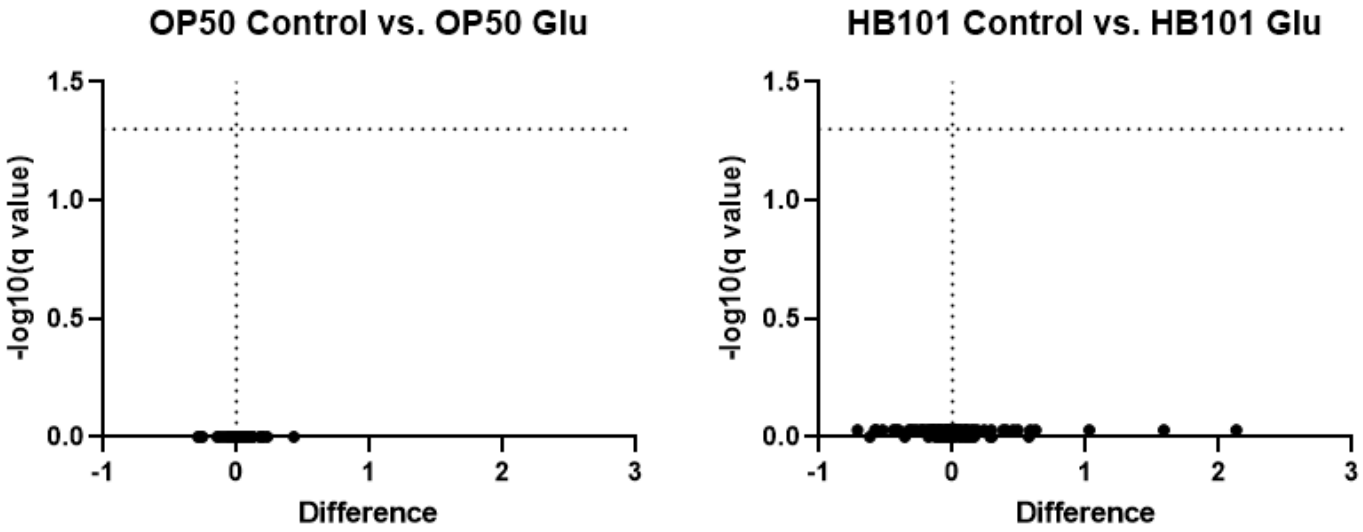
In conclusion, this research project investigated the intersection between diet, stress, and lipid metabolism in the model organism *C. elegans*. Through a series of experiments, the project aimed to dissect the complexities of how dietary changes and stressors impact the membrane composition of *C. elegans*, informing of the adaptive metabolic responses utilized by these animals. Our findings showed the nuanced relationship between bacterial diets and lipid profiles in *C. elegans,* emphasizing the importance of considering both worm and bacterial metabolism when examining the effects of diet on lipid metabolism. Despite no significant differences among lipid species under different bacterial diets, slight differences between lipid composition in the worms compared to the bacterial lipids highlight the complexity of these interactions.

Furthermore, the exploration of stressors such as glucose, starvation, and TBHP revealed information about how *C. elegans* adapt their lipid metabolism in response to different kinds of stress. Glucose stress induced metabolic changes, particularly in the increase of PEs, possibly to enhance membrane fluidity and cope with stress-induced metabolic shifts. Starvation of 24 hours triggered significant alterations in lipid composition, highlighting the worm's metabolic adaptations under prolonged food deprivation conditions. Specifically, the 24 hour starved worms had an increase in PCs and PEs with higher degrees of unsaturation that might enhance membrane fluidity and flexibility and an increase in PCs and PEs with longer chain lengths as long chain fatty acids undergo a slower metabolic breakdown. Additionally, TBHP-induced stress led to changes in lipid profiles similar to starvation, suggesting that more research must be done to identify if there are similar that responses to oxidative stress compared to starvation.

Future research could go in many directions to further elucidate the complex mechanisms regulating lipid metabolism in *C. elegans*. Investigating the impact of dietary restriction on membrane composition using paraformaldehyde to limit food availability and exploring the time-dependent changes of starvation metabolism represent compelling directions for future research. Additionally, combining lifespan studies with dietary restriction, particularly utilizing *eat-2* mutants fed various bacteria, allows researchers to study the intersection between diet, lipid metabolism, and longevity in *C. elegans*. Ultimately, the insights gained from this research project enhances the understanding of biological processes in *C. elegans* and offers perspectives that may have broader implications for health and disease research in both model organisms and higher species.

Appendix A: Additional Glucose Stress Experiment

An additional experiment was conducted with bacterial plates being seeded at a different time inconsistent with previous experiments. Previous experiments were conducted seeding bacteria onto glucose transfer plates four days before the transfer to allow the bacteria to process and uptake the glucose. Here, we seeded two days before the transfer, rather than four days before to test whether the duration of exposure to glucose impacts metabolism. Additionally, this experiment was conducted with another bacterial strain, HT115, for one replicate. The data is shown in Figures 34-37.



A **B**

Figure 33: Volcano Plots for Experiment 3: The Effect of Different Diets on Susceptibility to Stressors Like Glucose for N2 *C. elegans* (seeding plates on different days).
In Panel A, the differences in lipid species between Control OP50 fed worms and OP50 and glucose fed worms are shown. There are no lipid species above the horizontal line at ~1.25, indicating that there are no statistically significant differences between lipid species. Panel B shows the differences in lipid species between Control HB101 fed worms and HB101 and glucose fed worms. There are no lipid species above the horizontal line at ~1.25, indicating that there are no statistically significant differences in lipid species.

The differences in lipid species present between OP50 control and OP50 and glucose fed worms are not statistically significant. The same can be said for HB101 control and HB101 and glucose fed worms. This is consistent with the previous experiment and the volcano plots in Figure 20, where all the experimental plates were seeded on the same day, which is consistent with previous laboratory experiments. The changes in lipid species are not significant, but greater trends are explored when looking at the changes in total lipid composition, PC and PE double bonds, and PC and PE chain length in Figures 33-35.

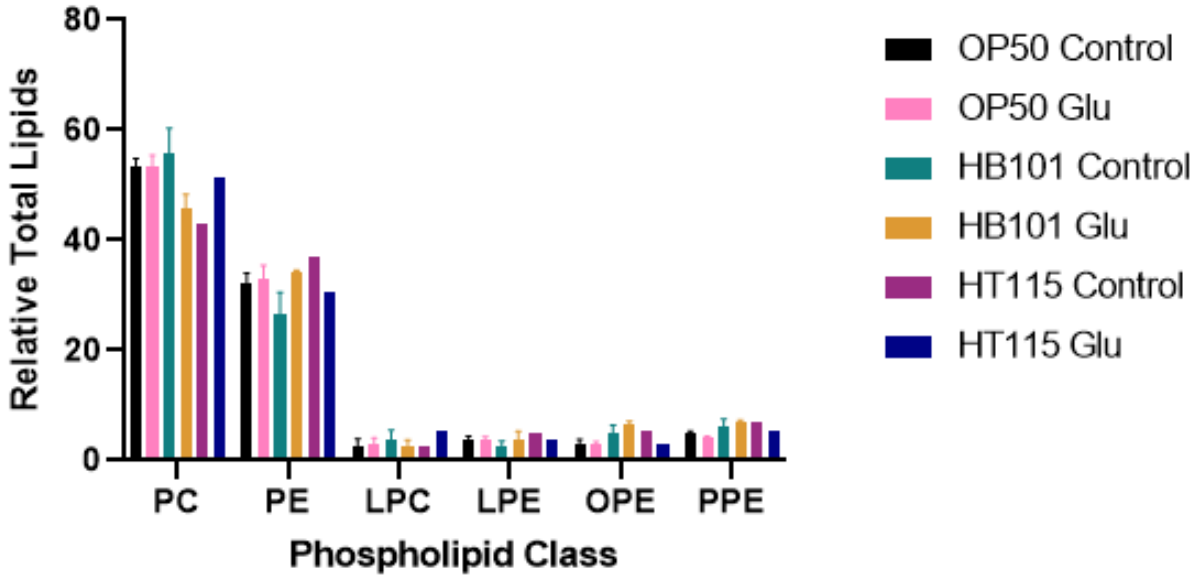
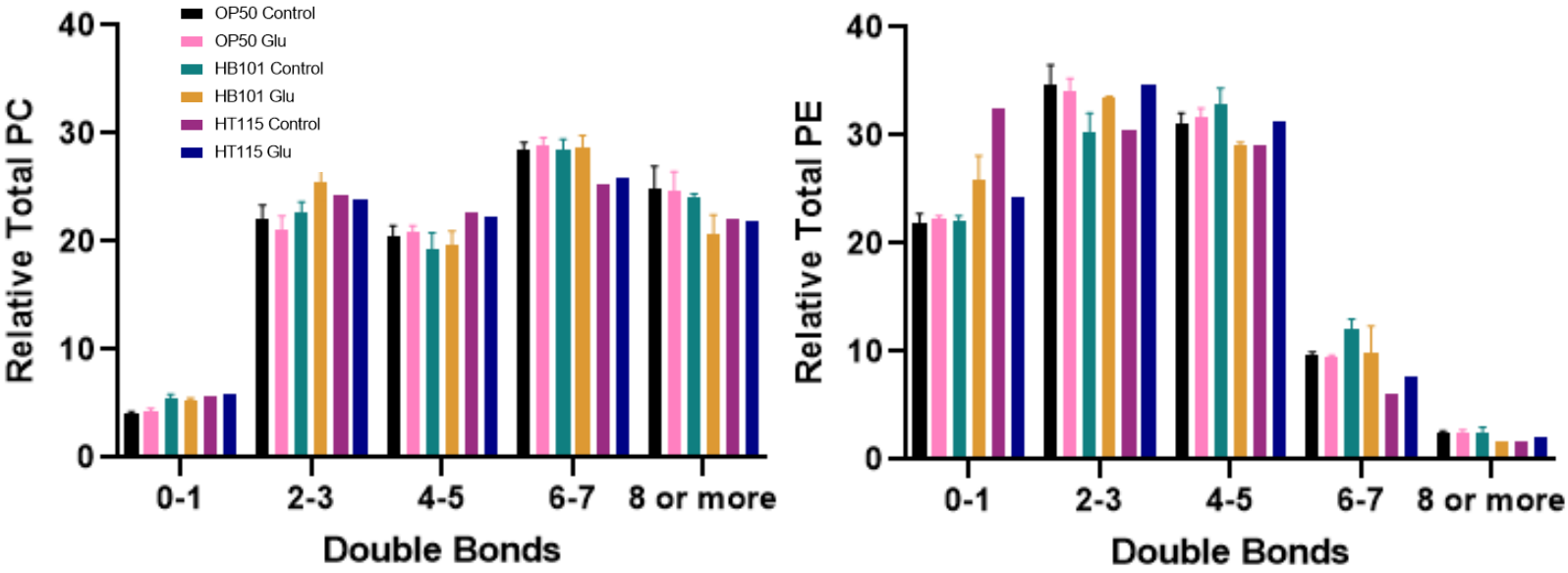


Figure 34: Relative Total Lipid Composition for wild-type nematodes under glucose stress (seeding plates on different days).
When comparing the total lipids in OP50 Control fed worms to OP50 and Glucose fed worms, there do not appear to be any general shifts or changes in total lipids. When comparing HB101 Control fed worms to HB101 and Glucose fed worms, there appear to be some slight shifts. There appears to be a decrease in PCs and an increase in PEs in HB101 and Glucose fed worms compared with HB101 Control worms. There also appear to be general shifts in the HT115 and Glucose fed worms when compared with the HT115 Control worms. There appears to be an increase in PCs and a decrease in PEs in HT115 and Glucose fed worms.

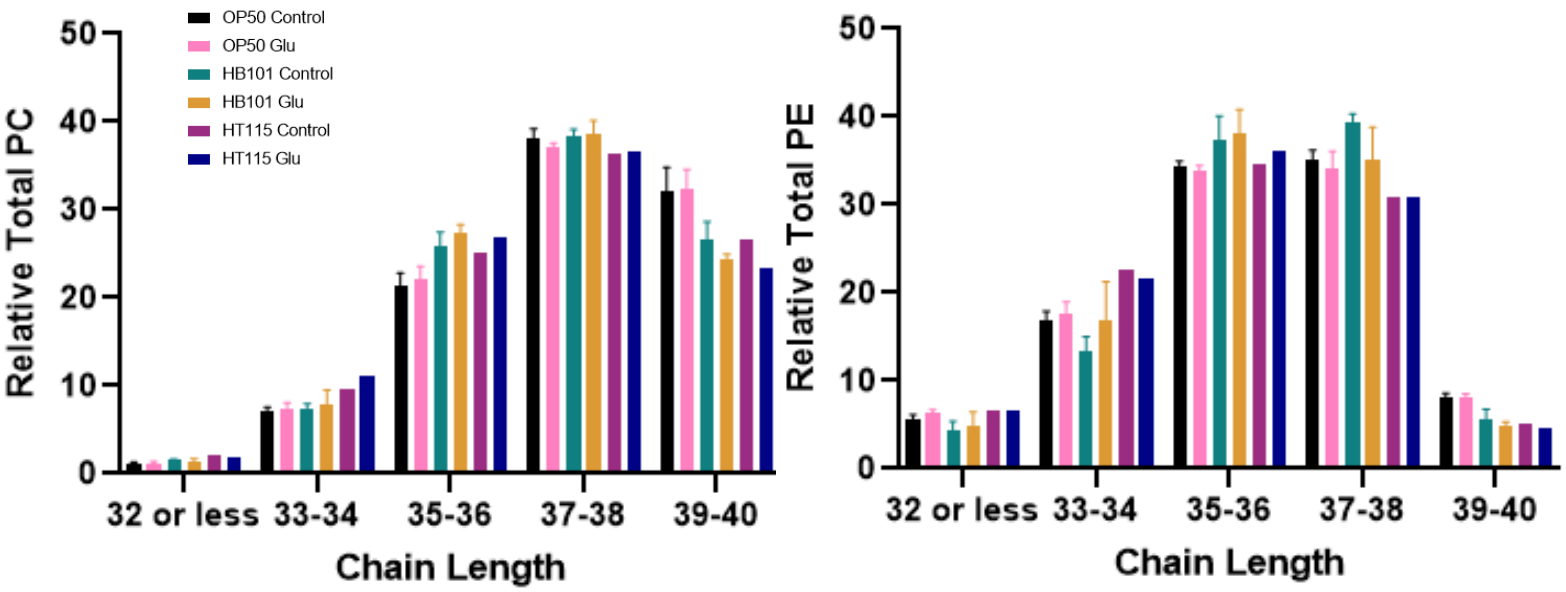
The changes seen in Figure 35 are a bit different than those seen in Figure 19. The changes in Figure 19 appear to be fairly consistent when comparing OP50 and HB101. However, in Figure 33, the changes between OP50 and HB101 are very different. This may be due to the fact that the bacteria did not have enough time to uptake all of the glucose, leading to inconsistences in the data.



A **B**

Figure 35: Quantification of PC and PE double bonds for wild-type nematodes under glucose stress (seeding plates on different days).

Panel A shows the PC double bond distribution for worms subjected to glucose stress on plates that were seeded two days prior to transfer. Generally, the HT115 and HT115 and Glucose fed worms appear to have the most differences from the OP50 and HB101 fed worms. There do not appear to be many differences in Control or Glucose fed worms in any condition. The HB101 and HB101 and Glucose fed worms have the most shifts, with the Glucose fed worms having more 2-3 double bonds and less 8 or more double bonds. Panel B shows the PE double bond distribution for worms subjected to glucose stress on plates that were seeded two days prior to transfer. The OP50 Control and Glucose fed worms appear to be fairly consistent. The HB101 and Glucose worms appear to have a slight increase in 0-1 and 2-3 double bonds, and a slight decrease in 4-5 and 6-7 double bonds compared with the HB101 Control worms. The HT115 and Glucose fed worms appear to have a decrease in 0-1 double bonds with a subsequent increase in 2-3, 4-5, and 6-7 double bonds.



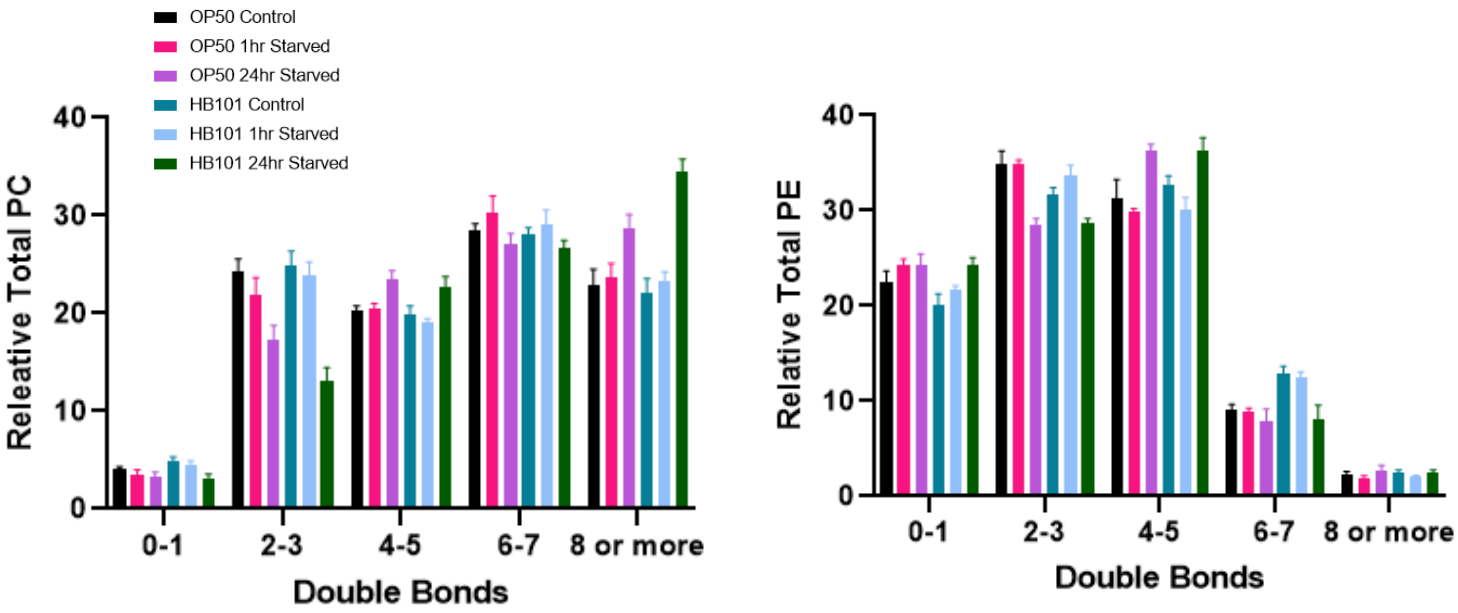
A **B**

Figure 36: Quantification of PC and PE chain lengths for wild-type nematodes under glucose stress (seeding plates on different days).

Panel A shows the distribution of PC chain length comparing control and glucose stressed OP50, HB101 and HT115 fed worms. There do not appear to be any major changes in PC chain length for worms fed any of the bacteria. Panel B shows the distribution of PE chain length comparing control and glucose stressed OP50, HB101, and HT115 fed worms. There appear to be slight trends in differences between control and glucose fed worms. There is a slight increase in chain lengths of 33-34 for HB101 glucose stressed worms compared to HB101 control worms, as well as a decrease in chain lengths of 37-38. However, there are large error bars. Due to the large error bars, more replicates may be needed in order to confirm these changes.

Appendix B: Additional Starvation Data

Additional figures for the starvation experimentation were created but were not put into the overall report. Figures 37 and 38 were used to extract smaller and easier to read figures – Figures 27 and 28. Figures 27 and 28 eliminate the one hour starved data and indicate statistically significant changes in HB101-fed control and 24 hour starved worms, which are easier to read and contribute better to the overall goal of exploring the impact of stress on membranes in *C. elegans*. However, Figures 37 and 38 show an overview of all of the starvation data collected to contextualize why figures 27 and 28 were extracted from these figures.



A **B**

Figure 37: Quantification of PC and PE double bonds for wild-type nematodes under starvation stress.

Panel A shows the PC double bond distribution for control, one hour starved, and 24 hour starved worms. Statistics were run and the differences in PC double bonds between control and one hour starved worms were not statistically significant. There are some general shifts in the double bond distribution. For instance, there is a slight decrease in 0-1 and 2-3 double bonds for OP50 one hour starved worms compared with OP50 control worms, and a slight increase in 6-8 and 8 or more double bonds. Panel B shows the PE double bond distribution for control, one hour starved, and 24 hour starved worms. Statistics were run and the differences in PE double

bonds between control and one hour starved worms were not statistically significant. However, there are some general trends observed, There is a slight increase in 0-1 and 2-3 double bonds when comparing HB101 one hour starved worms with control HB101 worms, and a slight decrease in 4-5 double bonds.

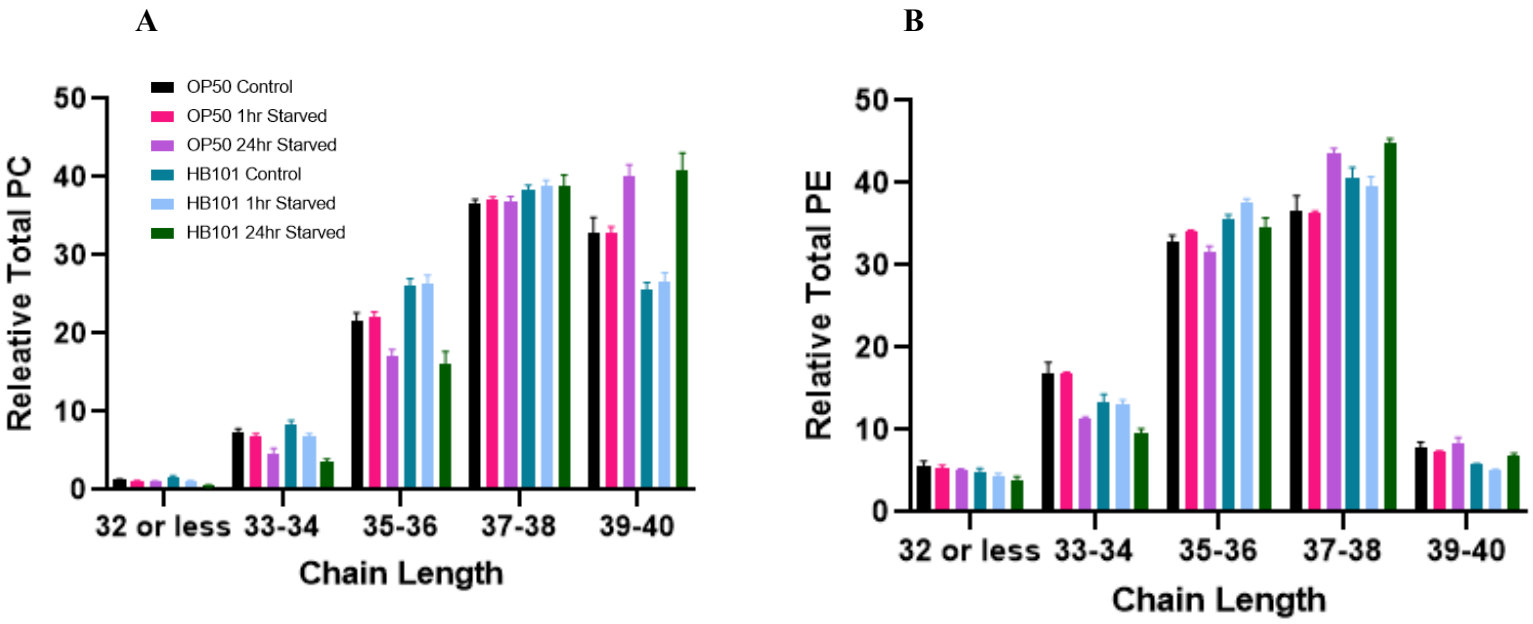
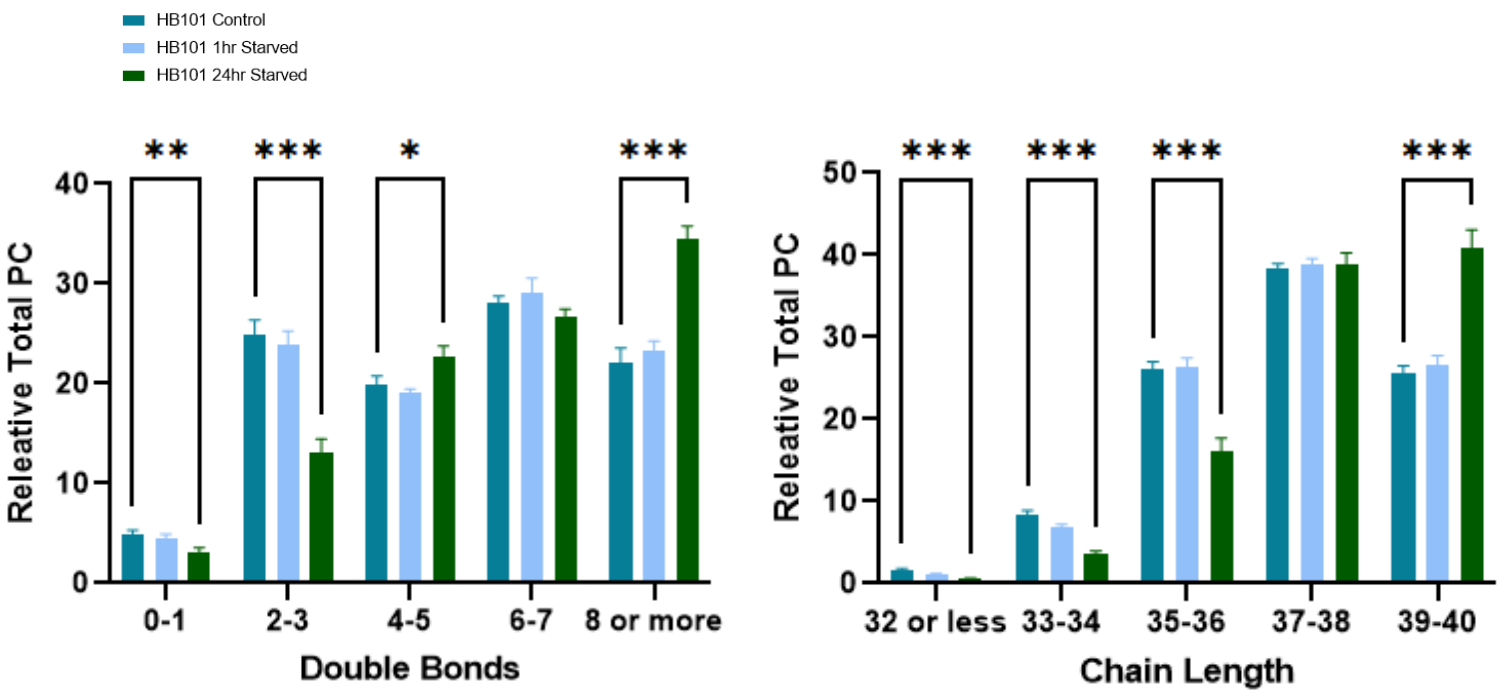


Figure 38: Quantification of PC and PE chain lengths for wild-type nematodes under starvation stress.

Panel A shows PC chain length distribution for control, one hour starved, and 24 hour starved worms. Statistics were run and the differences in PC chain lengths between control and one hour starved worms were not statistically significant. There do not appear to be any observable shifts in chain lengths between one hour starved and 24 hour starved worms. Panel B shows PE chain length distribution for control, one hour, 24 hour starved worms. Statistics were run and the differences in PE chain lengths between control and one hour starved worms were not statistically significant. The major shifts in Panels A and B are between control worms and 24 hour starved worms.



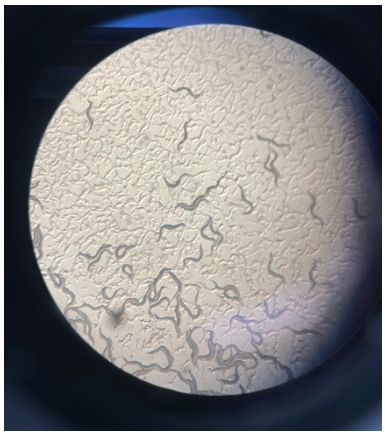
A B

Figure 39: Quantification of PC double bonds and chain lengths for wild-type nematodes under starvation stress, indicating statistically significant changes in HB101 control compared with HB101 24 hour starved worms.

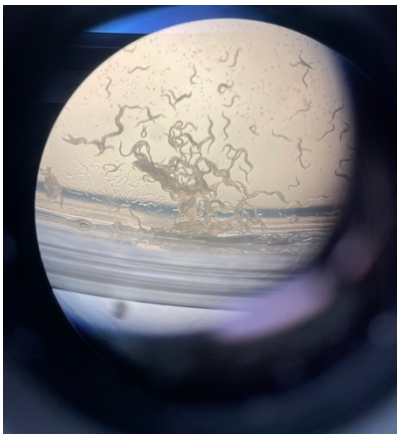
This figure is made of just HB101-fed worms control worms and worms subjected to starvation stress. This figure is extracted from Figures 31 and 32, just narrowing in on HB101, as the differences were seen more significantly in HB101-fed worms. Panel A shows the PC double bond distribution between control, one hour starved, and 24 hour starved worms. The control worms and one hour starved worms do not appear to have major changes. Panel B shows the PC chain length distribution between control, one hour starved, and 24 hour starved worms. The control worms and one hour starved worms do not appear to have any major changes in chain length. Because there are no major changes seen between control worms and one-hour starved worms, Figures 28-30 were created to compare control worms to 24-hour starved worms.

Appendix C: Qualitative Observations from PFA Dietary Restriction

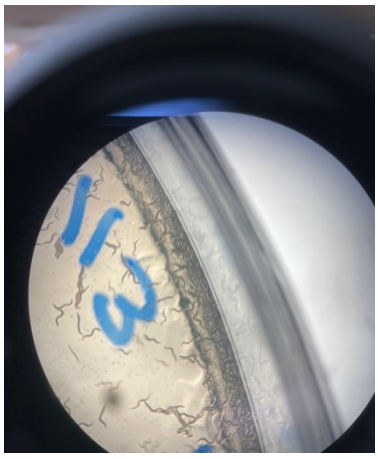
Due to time constraints and scheduling, the data from the dietary restriction experiment using paraformaldehyde was not able to be analyzed. However, there were general qualitative observations made by looking at the worms throughout the 24-hour duration of their dietary restriction window.



A



B



C D

Figure 40: Qualitative Observations of *C. elegans* exposed to dietary restriction via paraformaldehyde.

Panel A shows worms that are on a plate containing 1000µL of PFA-killed bacteria, or 100% plates. There are visible tracks of the worms left on the plates, which indicate that there is still food present. Along with the tracks, the worms do not appear to be clumped in groups or moving to the edges of the plates, indicating that they are not starved. Panel B shows worms that are on a plate containing 250µL of PFA-killed bacteria and 750µL of LB liquid, or 25% plates. The worms are very clearly forming clumps near the edges of plates and there are not clear tracks from the worms. Panel C shows worms that are on a plate containing 125µL of PFA-killed bacteria and 875µL of LB liquid, 12.5% plates. The worms were moving to the edges of the plates and even appeared to be trying to climb up the sides of the plates. Panel D shows worms that were on plates containing 62.5µL of PFA-killed bacteria and 937.5µL of LB liquid, 6.25% plates. The worms are very clearly in clumps toward the center of the plate and there does not appear to be any food on the plate behind them.

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