The Impact of Bacterial Metabolism on the Response to High Glucose Diets in *C. elegans*

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Abstract:

The plasma membrane is an essential structure that protects the cell from the environment, provides structure, regulates the movement of materials, and carries signals and receptors for cellular communication. Previous work has shown changes in the lipid profile of *Caenorhabditis elegans* under glucose stress. There are increased concentrations of saturated fats and increased turnover of monomethyl branched-chain fatty acids (mmBCFAs) following glucose exposure. In these studies, *C. elegans* incorporated glucose by consuming OP50 bacteria grown on glucose-enriched culture media. Here, we wanted to investigate if bacteria are essential agents for digesting glucose and generating glucose-related phenotypes. This comparison allowed us to define whether the lipid changes are a result of nematode or bacterial metabolism and would allow for a further understanding of the response to glucose stresses. For these studies, we examined the lipid population by mass spectrometry which revealed that polyunsaturated fatty acids in PE lipids decreased after 12 hours of 100mM glucose exposure, while monounsaturated fatty acids increased. When presented with the same concentration of glucose, *C. elegans* that were fed heat-killed bacteria did not exhibit the same decreases in polyunsaturated fatty acids. We conclude that most changes in PC and PE species in high glucose environments require a living bacterial diet. We propose that as *C. elegans* consume the glycosylated products from OP50, reactive oxygen species are generated that attack the double bonds present on PE lipids of the inner plasma membrane. The loss of these double bonds reduces the fluidity of the membrane and hinders the growth and development of the nematode.

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Background:

The Plasma Membrane

Membranes are essential for protecting our cells, providing structure, receiving and sending signals, and separating the external environment from our cellular environment. Known as the plasma membrane, these structures are formed from three major classes of lipids: glycerophospholipids, sphingolipids, and sterols (Cooper, 2000). Glycerophospholipids are the most abundant lipid classes found in membranes, as they make up 50% of the total mass of a membrane (Cooper, 2000). Moreover, glycerophospholipids are highly diverse and variations in head groups and the fatty acid chains can result in over 1000 potential lipid molecules in any given eukaryotic cell (van Meer et al., 2008). However, functionally we only observe hundreds of unique lipids in a given species.

Glycerophospholipids have a typical structure comprised of two fatty acid chains attached to the *sn-1* and *sn-2* positions of a glycerol backbone (Sultana and Olsen, 2020). Chains in the *sn-1* position usually lack double bonds, as opposed to the 2-6 double bonds present in chains at the *sn-2* position (Sultana and Olsen, 2020). These chains are attached to the glycerol backbone via acyl or ester linkages. Lastly, a phosphate-containing head group attaches to the *sn-3* position of a glycerol backbone (Sultana and Olsen, 2020). The resulting glycerophospholipid structure thus contains a hydrophilic region (the phosphate head group) and a hydrophobic region (hydrocarbon tails) that spontaneously aggregates to form the plasma membrane (Cooper, 2000, Yang et al., 2018). The head groups are highly variable, allowing glycerophospholipids to be further subdivided into several other subclasses.

The two most abundant lipid subclasses are phosphatidylcholine and phosphatidylethanolamine. Phosphatidylcholine lipids, or PCs, have a choline group attached to

the phosphate and makes up more than 50% of the lipids found in eukaryotic membranes and 32.3 % in nematodes (van Meer et al., 2008 and Satouchi, 1993). This lipid class has an overall cylindrical structure (FIGURE 1C), participating in the regulation of bilayer fluidity. Likewise, PCs are believed to be enriched in the outer leaflet of the plasma membrane, where they interact with cholesterol to promote fluidity (Kamp, 1979). Phosphatidylethanolamine lipids, or PEs, have a phosphate-ethanolamine head group and are the second most abundant class of lipids in the membrane (Yang et al., 2018). Due to the reduced molecular diameter of their head group, these lipids adopt a more conical geometry (FIGURE 1B) (Yang et al., 2018). When combined with the linear PCs, PE lipids induce a level of curvature stress that allows the membrane to bend, bud, fuse, and undergo fission (van Meer et al., 2008). Unlike PC lipids, PE lipids are more localized on the inner leaflets of the plasma membrane and have been shown to have drastic impacts on mitochondrial metabolism (Yan et al., 2018, Koyiloth and Gummadi, 2022).

Compared to PC and PE lipids, phosphatidylserine lipids and sphingomyelins are much less abundant in plasma membranes but are still important for signaling and structure. Phosphatidylserine lipids, or PSs, contain a phosphate-serine head group (FIGURE 1A). PSs play an essential role in external and internal cellular signaling events (Hishikawa et al., 2014, Perez and Watts, 2021). Finally, sphingomyelins are a unique glycerophospholipid class with a structural role (FIGURE 1D). These lipids are similar in geometry to PCs, but often lack any double bonds on their fatty acid tails. By associating with cholesterol, sphingolipids give the plasma membrane a gel-like quality (van Meer et al., 2008).

As mentioned previously, cellular membranes are highly fluid structures. The distribution of phospholipids in these membranes is variable depending on cell type, organelle, and even depending on the leaflet of the membrane (Kamp, 1979, van Meer et al., 2008 and Yang et al.,

2014). Additionally, membrane composition continues to adapt in response to environmental, internal, or other stress conditions. An optimal level of fluidity is vital to cell survival, as significant changes in a cell's lipid profile can lead to apoptotic events (Legrand and Rioux, 2010). Cells tightly regulate this fluidity by manufacturing lipids with varying levels of double bonds. Herein this report, lipids with no double bonds will be called "saturated" and those with double bonds will be called "unsaturated." Unsaturated fatty acids adopt highly kinked shapes that increase membrane disorder, promote bending, regulate protein membrane interactions, and influence gene transcription (Watts and Browse, 2002 and Hishikawa et al., 2014). Conversely, saturated fatty acids can be packed together to decrease membrane fluidity (Dancy et al., 2015). For example, the plasma membrane in *E. coli* readily shifts its composition to adapt to fluctuations in temperature. Decreases in temperature cause an increase in phospholipids synthesized from the unsaturated fatty acid cis-vaccenic acid. These new phospholipids are more kinked than those constructed with palmitic acid, the saturated fatty acid commonly found in *E. coli*. As a result, membrane fluidity is increased, and homeostasis is achieved (Mansilla et al., 2004).

In addition to maintaining fluidity, cells must constantly replenish lipid species consumed during vesicle formation, β-oxidation, and damage for optimal membrane health (Dancy et al., 2015). As a result, membrane lipids are constantly synthesized and incorporated into these membranes to replace lost populations, highlighting the dynamic properties of plasma membranes. This replenishment is so rapid that it is estimated that all membrane lipids are newly synthesized every 24 hours in young animals (Dancy et al., 2015). An adequate membrane composition is necessary for effectively responding to external and internal stress. For instance, by controlling the dynamics of specific lipid species, such as the decreased saturated fatty acid

turnover and increased monomethyl branched-chain fat acid turnover in response to glucose stress (Vieira et al., 2021), plasma membranes can manage potential deficiencies and promote homeostasis.

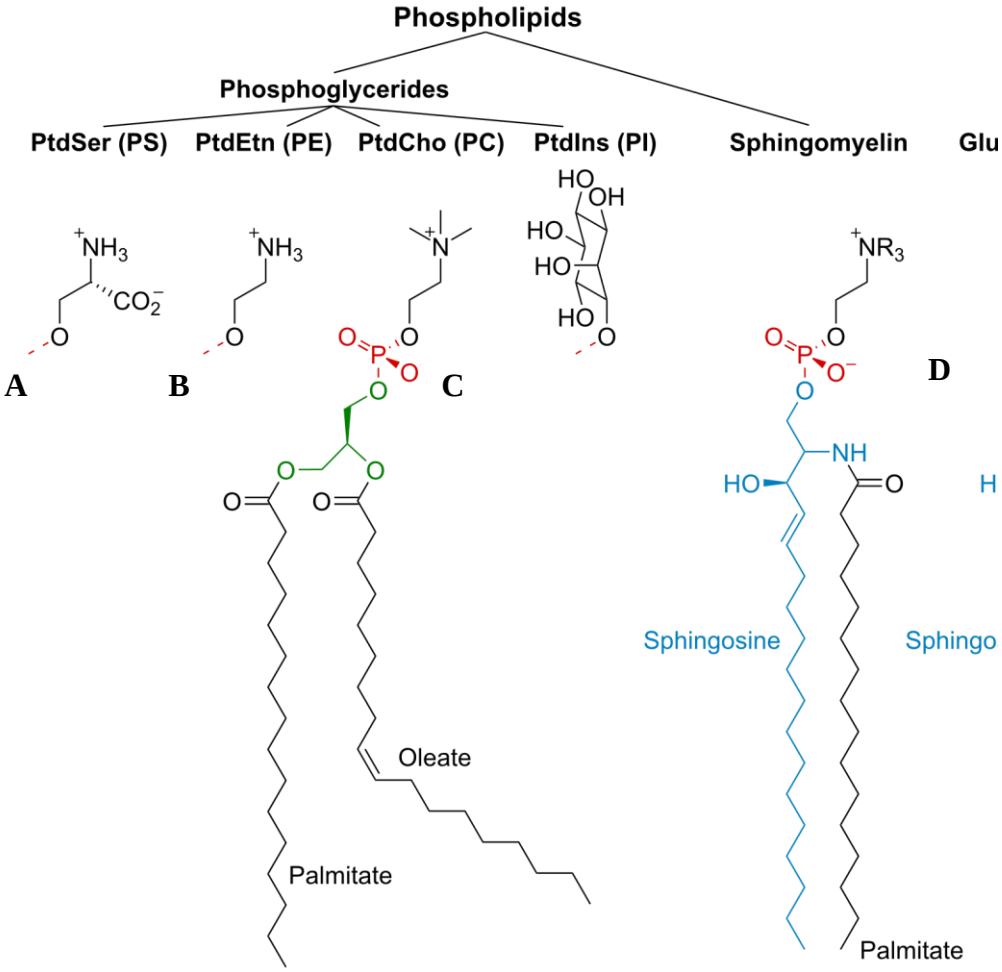


Figure 1: Structures of Four Glycerophospholipid Classes

(A) Head group of phosphatidylserines (PtdSer or PSs). Mainly used as a singling molecule. Fatty acid tail structure like PCs. (B) Head group of phosphatidylethanolamines (PtdEtn or PEs). Mainly found on inner membrane leaflets. Fatty acid tail structure like PCs. (C) Overall structure of phosphatidylcholines (PtdCho or PCs). Mainly found on the external membrane leaflets. Generally, have one saturated fatty acid tail and one unsaturated fatty acid tail to promote fluidity. (D) Overall structure of sphingomyelin phospholipids. There are less kinks in their structure, increasing the rigidity of the membrane.

Mass Spectrometry and Analyzing the Lipidome

Mass spectrometry was developed in the early 20th century to analyze compounds based on their mass-to-charge ratios (Thomson, 1910). Since then, it has become a vital analytical tool for the study of metabolomics, proteomics, and lipidomics. When combined with high-performance liquid chromatography (HPLC) or gas chromatography (GC), mass spectrometry (MS) provides both structural and quantitative information of analyzed molecules (Griffiths and Wang, 2009). A fundamental component of all mass spectrometers is the ion source, as a molecule must be charged to be analyzed. Likewise, having two sequential detection systems can provide additional information on the composition and abundance analyzed molecules (Harkewicz and Dennis, 2011). In the Olsen lab, and others like it, charged particles are created via electrospray ionization (ESI) and quantified in a triple quadruple/ORBITRAP mass spectrometer set to operate in tandem mass spectrometry mode (MS/MS).

Lipidomic assays fall into one of two categories: targeted or untargeted. Targeted approaches are best conducted when trying to identify specific lipid populations. Because the lipids are defined prior to analysis, targeted approaches tend to have higher accuracy and precision. This high specificity comes at a cost, as experiments using this approach are limited in their qualitative survey of the overall lipid profile. Untargeted approaches, conversely, are more exploratory and provide general information on a sample's overall lipid profile, but do not provide highly specific details about defined lipid populations (Harkewicz and Dennis, 2011). Despite this, discovery-based non-targeted approaches can quickly characterize the lipid profile of a sample. Samples can be easily re-analyzed as needed without knowing the biological importance or overall lipid complexity to examine changes in the presence of stresses and signals (Bird et al., 2014).

C. elegans as A Model Organism

Caenorhabditis elegans, *C. elegans*, is a species of nematode found in all soil systems that has become a valuable model for lipidomic studies. Though initially used for genetics in 1963, *C. elegans* research has grown dramatically to cover every aspect of cellular biology (Brenner, 1974 and Corsi et al., 2015). Their size (~ 1 mm in length for an adult) makes them easy to observe under a microscope, allowing researchers to document their movement, development, phenotypes, and behaviors more easily than cellular or bacterial models (Corsi et al., 2015 and Meneely et al., 2019). The basic anatomy of *C.* elegans is highlighted in Figure 2. Additionally, the worms are transparent and have well-documented organ systems that can be exploited for fluorescence imaging (Corsi et al., 2015 and Meneely et al., 2019). What makes them especially appealing for metabolism studies though is their rapid lifecycle, ability to self-fertilize, genetic malleability, and their many homologous metabolic pathways and proteins to those seen in humans (Corsi et al., 2015 and Lai et al., 2000). Most importantly, *C. elegans* diets can be easily manipulated such as by enriching it with stable isotopes of ^{13}C to allow for assaying the metabolism directly (Perez and Van Gilst, 2008).

The Olsen lab, among others, has conducted research into understanding the effects of genetics and metabolism on the lipidome, or the total composition of cellular lipids. *C. elegans* are capable of synthesizing most of their fatty acids *de novo* from acetyl-CoA and the fatty acid synthase enzyme. C. *elegans* are capable of synthesizing 100% of their monomethyl branched-chain fatty acids which are essential for development but not present in the food source (Perez and Van Gilst, 2008). For example, fatty acid synthase produces C16:0 lipids from lipid precursors. Subsequent proteins, such as Δ 9 desaturase and homologues to Elo1p, are used to

generate other fatty acids with varying amounts of double bonds and carbon chain lengths (Watts and Browse, 2002).

As mentioned previously, *C. elegans* are especially useful for genetic studies. Their genome is well-mapped and easily manipulated, so various genes can be knocked down through RNAi or knocked out, as in the case with mutants, depending on the experimental design (Corsi et al., 2015). More importantly, the *C. elegans* genome contains many genes that encode homologous proteins in humans. It is estimated that 83% of the protein sequences in *C. elegans* have human homologs, allowing researchers to make inferences on human metabolism through worm studies (Lai et al., 2000). Previous studies in the Olsen lab have focused on the role of *fat-6, fat-7,* and *elo-5* on lipid metabolism in the presence of glucose and other environmental stresses. But for this study, genetically modified strains were not used as we wanted to examine unimpeded lipid metabolism. Herein this report, these wild-type worms will be known as "N2" worms per naming conventions in the field (Caenorhabditis Genomic Center).

Catabolic pathways are highly conserved among eukaryotic cells. The breakdown of sugars, amino acids, and fats in *C. elegans* follow the TCA cycle, and the subsequent products are processed by the mitochondria to provide ATP and other necessary molecules (Watts and Ristow, 2017). The digestion of glucose, its associated catabolic pathway, regulatory genes, and shift in gene transcription must all be accounted for when trying to understand lipid metabolism. For example, mutations in the insulin-like receptor gene, *daf-2,* causes *C. elegans* to survive longer in anoxic conditions and promote fat metabolism. However, this anoxic survival rate is removed once the worm is fed glucose (Garcia et al., 2015). Similarly, genes like *pst-4* and *glod-4* encode for proteins that reduce the amount of oxidative stress within the cell (Kingsley et al.,

2021). As powerful genetic models, *C. elegans* allow researchers to observe the interplay between genes, metabolites, and development in the context of any entire organism.

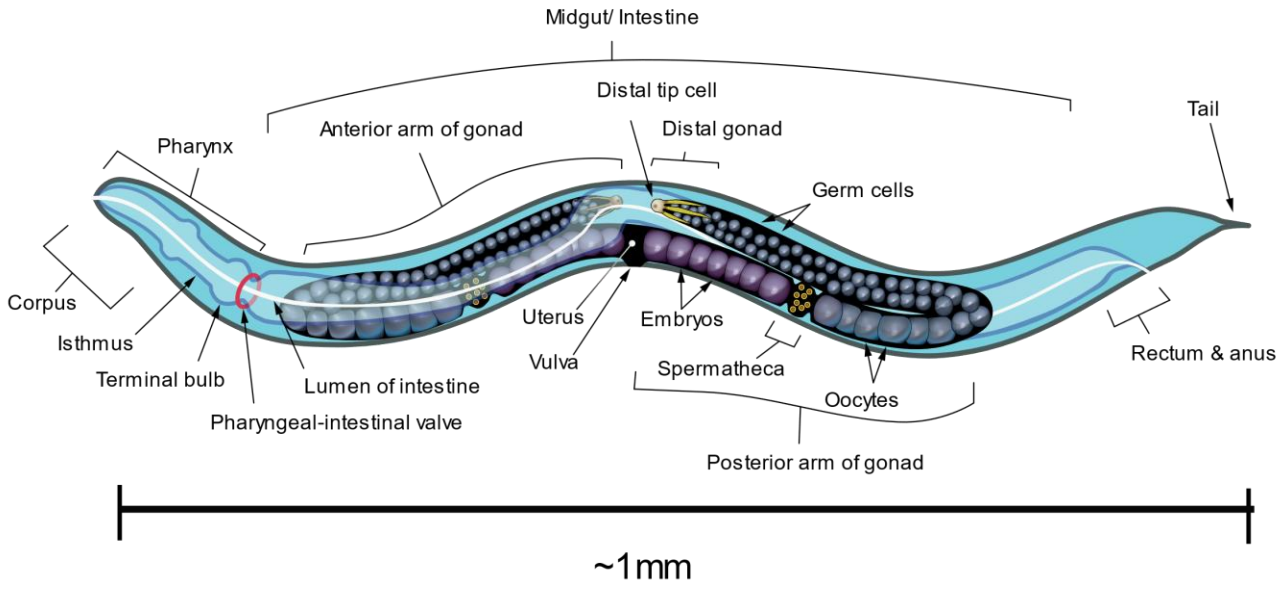


Figure 2: Anatomy of a *Caenorhabditis elegans* Hermaphrodite

An anatomical diagram of a *C. elegans* hermaphrodite. Hermaphrodites are capable of reproducing in the absence of male worms, grow a maximum length of about 1 mm, are highly transparent, and take 48-60 hours to reach adulthood when hatched. Male worms exhibit a similar morphology, except their tail is more fan-like in structure and lack the reproduction organs necessary for egg-laying. Photo by K. D. Schroeder, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=26958836

Lipid Profile of *C. elegans* Changes in Response to Glucose Demonstrated by Monomethyl Branched-Chain Fatty Acid Populations

Monomethyl branched-chain fatty acids (mmBCFAs) are lipids that account for 5.5% of the relative abundance of fatty acids in *C. elegans* and are critical for the activation of signaling pathways that facilitate metabolism (Kniazeva et al., 2004 and 2015). Studies in bacteria have shown that plasma membranes are composed of two classes of mmBCFAs, iso and anteiso, that influence the fluidity and packing of the membrane (Jones, 2009). In *C. elegans*, elongase enzymes produce C15ISO and C17ISO mmBCFAs that are essential to the development of the nematode (Kniazeva et al., 2004). Studies have shown that the ratio of iso/anteiso species is reduced in lower temperatures, but a lack of research has been conducted to see how these fatty acids respond to other conditions, such as the glucose-related oxidative stress (Rilfors, 1985).

Many organisms have regulation mechanisms that allow membrane composition to be maintained in oxidative environments caused by glucose stress. For example, *C. elegans* have the protein Progestin and AdipoQ Receptor 2 (PAQR- 2) which is essential for survival when membrane composition is affected by excess glucose. This protein works in conjunction with FAT-7 desaturase to provide unsaturated fatty acids to the membrane to enhance fluidity (Svensk et al., 2016). Vieira et al. expanded these studies to show the effects of low glucose concentration (15 mM and 45 mM) on the viability, life spans, and *C. elegans* lipid profiles. Vieira et al. (2021) found an active response to glucose in mmBCFAs populations using GC/MS, and after knocking down the production of these fatty acids, showed that nematodes did not survive subsequent glucose stress. Dietary supplementation of *Bacillus subtillis* rescued elongase protein mutant phenotypes after glucose exposure, suggesting that survival is specific to mmBCFA production. The study presented in this report seeks to expand upon the findings of Vieira et al. by utilizing

HPLC-MS/MS to study the intact phospholipid populations of *C. elegans* subjected to high glucose concentrations (100 mM) and metabolically inactive bacteria.

Oxidative Stress and Reactive Oxygen Species

As shown by Vieira et al., among others, glucose causes lipid compositional changes and cellular damage by increasing the oxidative stress within the cell. Broadly defined, oxidative stress is the imbalance of free radicals and antioxidants that leads to cellular damage (Betteridge, 2000). Free radicals of oxygen-containing molecules, known as reactive oxygen species (ROS), are created through oxidative phosphorylation as electrons are shuttled through the electron transport chain (Schulz et al., 2007). While an inevitable by-product of cellular respiration, too many ROS are detrimental to the cellular health, and thus ROS concentrations are controlled by antioxidants which can be generated from enzymes such as superoxide dismutase and glyoxalase-1 (Schlotterer et al., 2009). As more glucose is processed by the cell, the ROS concentrations increase, and cellular damage prevents the activation of the antioxidant enzymes. Continued ROS exposure reduces the fluidity of the plasma membrane, making conditions that are unfavorable for transmembrane glucose transport and O_2 diffusion (Desei and Miller, 2017).

Diseases such as Alzheimer's, Parkinson's, diabetes, some forms of cancer, and even aging all exhibit characteristic membrane alterations (Desai and Miller, 2017). As an example, let us consider obesity, a metabolic disease that can increase the risk for the development of type 2 diabetes. Obesity is a global health issue, with current trends showing that a third of the world can be classified as obese or overweight (Chooi et al., 2019). Significant research has been conducted to understand how an obese patient’s metabolism impacts the functioning of their cells. In addition to other metabolic stresses, studies have shown that ROS contribute to the overall development of obesity and insulin-resistance (Moreno-Arriola et al., 2014). ROS have

also been linked to the development of cancer, atherosclerosis, and general infection as well (Desai and Miller, 2017), so understanding how cellular membranes respond to oxidative stress is essential for developing novel treatments for these diseases. Due to the extensive amount of knowledge on their genetics and metabolic pathways, *C. elegans* provides researchers with the opportunity to model and study lipid and metabolic disorders at the intersection of metabolism and genetics.

The Diet of *C. elegans* – OP50 *E. coli*

C. elegans are bacteriophages that commonly consume the soil bacteria on rotting plant matter in the wild for nutrition and materials necessary for their growth and development (Corsi et al., 2015). The transparency of *C. elegans* was appealing to many of the founding worm researchers, so they utilized the OP50 *E. coli* bacterial strain to prevent the worms from being obscured. OP50 *E. coli*, or simply OP50, is an uracil auxotrophic bacterium, meaning uracil must be supplied for it to grow efficiently. Agar plates are limited in their free uracil, preventing the bacteria from overgrowing on the plate (Brenner, 1974). Also available is the HB101 *E. coli*, a hybrid strain that produces a thicker bacterial lawn (Brooks et al., 2009), but OP50 is more routinely used for lipidomic and metabolomic studies in *C. elegans*. Much like the *E. coli* in the human microbiome, OP50 resides in the intestines of *C. elegans* after ingestion (Kingsley et al., 2021). Through a commensal relationship, OP50 continues to metabolize products from food sources to derive other essential nutrients for the worm, like amino acids and various vitamins (Zečić et al., 2019).

Among the molecules processed by OP50, glucose is known to cause decreases in the lifespan and health in the worm (Lee et al., 2009). Previous studies have demonstrated that bacterial processing of glucose causes the development of glycosylated proteins, lipids, and other

macromolecules (collectively known as advanced glycosylated end products, or AGEs) that increase the oxidative stress within the cell (Kingsley et al., 2021). The effect of AGEs and oxidative stress has been studied broadly in *C. elegans* regarding the overall health of the worm, the fluctuations in gene transcription, and decreases in mitochondrial functioning (Alcántar-Fernández et al., 2018 and Schlotterer et al., 2009). Considerable research has also been conducted on how glucose influences the lipidome, but there is debate about the necessity of bacterial metabolism on the development of the plasma membrane. The Olsen lab has investigated this using genetically modified worms and 15 mM and 45 mM glucose concentrations. This study attempts to illuminate the role of OP50 in *C. elegans* plasma membrane maintenance under extreme glucose conditions (100mM), the highest amount the lab has used to date. As stated previously, *C. elegans* share similar intestinal, dietary, metabolic, and genetic mechanisms to those seen in humans. By understanding how glycolic oxidative stress impacts the formation, degradation, and replenishment of membrane lipids, we can continue to enrich our understanding of plasma membrane dynamics and how they relate to human health.

Materials and Methods:

C. elegans Strains and Maintenance

N2 (wild type, WT) strains were provided by the Caenorhabditis Genetics Center (CGC), which receives funding from the National Institutes of Health (NIH) Office of Research Infrastructure Program (P40 OD010440). The N2 strains were discovered in Bristol, England and are provided to the CGC by Oxford University in Oxford, England. Unless otherwise stated, *C. elegans* strains were grown and maintained on HG culture plates seeded with OP50 strain *E. coli* at 20ºC. Worms were synchronized for experimentation by bleaching gravid adults and leaving the eggs in M9 buffer to hatch overnight. The resulting L1 worms were plated with a density of 5,000 worms per 10 cm HG plate.

Heat-killing Bacteria

Concentrated OP50 *E. coli* stocks were generated from OP50 stock plates by inoculating 2-3 colonies in each of 2 flasks of 50 mL LB media. These flasks were left to shake overnight (8-10 hours) at 250 rpm and 37ºC. Each flask of 50 mL was put into an individual 50 mL centrifuge tube and spun at 3900 rpm for 10 minutes to pellet the bacteria. Both pellets were resuspended in M9 according to the pellet's mass at a concentration of Xg/mL. After heating a water bath to 60ºC, the resuspended pellets were heat-killed for 20 minutes. The pellets were centrifuged at 3900 rpm for 10 minutes again. One pellet was resuspended in M9, and the other was resuspended using a M9-100mM glucose solution. The M9 and M9-100mM glucose resuspensions were re-streaked on LB plates and left overnight in a 37ºC incubator to confirm death. Both centrifuge tubes were kept in a 4ºC refrigerator until needed for seeding plates. Before plates were seeded, the centrifuge tubes were placed in a 60ºC water bath for 20 minutes to ensure that the bacteria were entirely heat-killed.

Preparing Experimental Conditions

High growth (HG) plates were prepared by combining 3 grams of NaCl with 20 grams of peptone, 25 grams of agar, and 1000 mL of water. The mixture was then autoclaved for 40 minutes to ensure that any contamination was removed. For the glucose experiments, a 10mL solution of 100mM glucose was added to the HG mixture prior to pouring into 10 cm plates. Four experimental conditions were developed for these experiments. Worms were collected from HG plates after two days of growth and transferred to experimental plates. Control groups were created by plating 2 10 cm OP50-seeded HG plates with 5,000 worms each. This process was repeated on HG-100mM glucose plates and for the heat-killed conditions. For plates with heat-killed bacteria, 1mL of the prepared heat-killed bacteria was added to HG plates and left to dry. The same process was completed for bacteria suspended in M9-100mM glucose. For the first experiment the worms were left on these plates for 12 hours, collected, and frozen until lipid extraction. This entire process was repeated for the 24 hours experiments.

Development Analysis

After bleaching, 5,000 eggs from N2 *C. elegans* strains were plated on four experimental conditions: 10 cm HG plates seeded with OP50, 10 cm HG plates seeded with OP50 and 100mM glucose, 10 cm HG plates seeded with heat-killed (hk) OP50, and 10 cm HG plates seeded with hkOP50 and 100 mM glucose. After 24 hours, worms were resuspended using 50 µL of M9 and 10 µL of the 50 µL were collected for counting. The collected worms were then plated on 3 cm counting plates, and the numbers of worms at each developmental stage (L1, L2, L3, L4, and D1) was determined under a benchtop microscope and recorded. This process was repeated for 5-7 days. During the experimental period, days where eggs were laid or when the worms died were noted.

Lipid Extraction for HPLC-MS/MS Analysis

Total lipid fractions were prepared by adding 4 mL of 2:1 chloroform/methanol solution to frozen worm samples (approximately 5,000 animals). Internal lipid standards were added to the solution (20 µL of PC 11:0 and TAG 13:0, 0.05 mg/mL), and the solution was left to rotate at room temperature for 1.5 hours. Using 600 µL of a 0.9% NaCl solution, the carcasses were cleaned from the lipid layer, and the solution centrifuged for 2 minutes at 2000 rpm to completely separation the polar and lipid fractions. The lipid fraction was then isolated and dried under a continuous nitrogen stream. The dried lipids were then resuspended in 200 µL of acetonitrile/2-propanol/water (65:30:5 v/v/v) dilution buffer and stored in vials for analysis.

HPLC-MS/MS Protocol and Data Analysis

Whole lipid extracts were injected into the HPLC-MS/MS system in 10 µL increments using a negative mode. The model of the HPLC system was Dionex UHPLC Ultimate 3000, with a C_{18} Hypersil Gold 2.1 x 50 mm, 1.9 µm column and a 2.1 mm ID, 5 µm Drop-In guard cartridge. The lipids were analyzed by a Q Exactive Orbitrap mass spectrometer from Thermo Scientific with a heated electrospray ionization (HESI) source.

Analysis of HPLC-MS/MS data was completed using Lipid Data Analyzer (LDA). LDA uses mass, retention time, and isotope distributions from the negatively scan MS files to distinguish lipid abundance. RAW files were analyzed using a 0.1% relative peak cut-off value and against an LDA exact mass list for PC, PE, PS, PG, PI, P-PE, P-PC, O-PE, O-PC, LPC, LPE, and LPS lipid species. Subsequent analysis was performed on PC and PE lipid species due to their high abundance in most membranes, length, and high degrees of unsaturation. The

nomenclature used to describe specific lipid species was X: Y, where X is the number of carbons among both fatty acid tails and Y is the number of double bonds present. For example, a 35:5 PC lipid would have 35 carbons in total and 5 double bonds. Relative abundance of each phospholipid was determined by excluding lipid species containing less than 24 carbons and 0 double bonds or greater than 40 carbons and 10 double bonds. *C. elegans* usually have phospholipids within this range. After separating the three trials of each experimental condition, lipid species containing less than 1% relative abundance were excluded. The average value, standard deviation, and standard error were all calculated from the three trials for each lipid species.

For determining the degree of unsaturation in each phospholipid species, lipids containing 0, 1, 2-3, 4-5, 6-7, and 8+ double bonds were grouped together for each experimental condition. The relative abundance of each degree of unsaturation was determined. This process was completed for both the PC and PE lipids and both experimental times.

Graphical representations (in the form of bar graphs, heat maps, and volcano plots) and statistical analyses of the above processed data were generated using Microsoft Excel and GraphPad Prism 9 software.

Results and Discussion:

HPLC-MS/MS Analysis to Identify Changes in Phospholipid Profiles after 12-Hour Exposure to Glucose

Vieira et al. (2021) found that there was an upregulation of mmBCFA production that was necessary for *C. elegans* survival following glucose stress. After subjecting N2 nematodes to 12 hours of 15 mM glucose stress, stable isotope labeling showed a decrease in saturated fatty acids and increased incorporation of mmBCFAs into the plasma membrane. Similar trends were discovered when 12 hours of 45 mM glucose stress were induced. Vieira et al. further corroborated these findings by supplementing the standard OP50 diet with *Bacillus subtilis*, a bacterial species with naturally elevated levels of mmBCFAs. After eating *Bacillus subtilis*, nematode survivability was rescued back to wild-type levels in the presence of glucose (Vieira et al., 2021). These findings highlight the effects of oxidative stress caused by glucose on the lipid profiles and membrane composition on *C. elegans.* Due to the current debates in the field, it is important to clarify the exact relationship between bacterial metabolism and worm metabolism in response to glucose stress and to understand how glucose impacts the worm. This study seeks to maximize observed changes in lipid species by Vieira et al. by subjecting N2 *C. elegans* to acute oxidative stress with 12 hours of 100 mM glucose exposure, compared to the previous 12 hours of 15 mM or 45 mM glucose exposure.

To further define the effects of glucose stress on membrane composition, N2 worms were plated on HG media seeded with OP50 *E. coli* and grown to their adult stage (L4) for 48 hours. After development, the worms were plated on one of two experimental conditions: OP50 and OP50 + 100mM glucose. Lipids were extracted and analyzed from these nematodes using HPLC

and tandem MS/MS to determine total lipid profiles. Subsequent bioinformatics were performed using Lipid Data Analyzer (LDA) software. Of the 12 lipid classes characterized by LDA, the PC and PE lipid species were specifically chosen for analysis due to their high prevalence in plasma membranes, 32.3% and 54.5% of total membrane lipids, respectively (Satouchi, 1993). Relative abundance was calculated by dividing the raw abundance value from LDA by the sum of all abundances. Lipids with a relative abundance lower than 1% were excluded from further study, as we aimed to identify major changes within the membrane lipid populations (Supp. Figure 1 and Supp. Figure 2). Of the remaining lipid species, significance analysis using two-way analysis of variance (ANOVA) on GraphPad Prism 9 revealed species that changed significantly after glucose exposure, and the PC and PE lipids that did show significant changes were highlighted to observe trends in the plasma membrane's composition (Figure 3A and Figure 3B).

To determine if the PC population was altered by glucose-enriched diets, we compared the relative abundance of significant PC lipid species in *C. elegans* fed OP50 to those fed OP50 + 100 mM glucose. Compared to controls, only three PC species increased in relative abundance after glucose exposure: 36:5 (p-value = 0.0038), 36:6 (p-value < 0.0001), and 40:10 (p-value < 0.0001). Simultaneously, we observed a 2-asterisk decrease in the 36:2 lipid population. The increase in polyunsaturated fatty acids (PUFA)-containing lipids was unexpected, as glucose stress generates ROS that readily attack the electron-dense double bonds of PUFA-containing lipids via free radical chain reactions (Petersen et al., 2018). As mentioned previously however, PC lipids are enriched on the outer leaflets of plasma membranes thus more protected from the oxidative internal environment. The increase in PC PUFA-containing lipids may highlight a

compensation pathway for maintaining membrane fluidity while oxidative stress occurs within the cell.

Conversely, PE lipids exhibited many more significant changes in specific species following glucose exposure than those in PC lipids. When compared to control conditions, relative PE PUFA-containing lipids decreased, as shown by the p-value < 0.0001 decrease in the 37:5 and 38:5 lipids. Monounsaturated fatty acid (MUFA) 33:1 and minorly unsaturated fatty acid 34:2 increased in abundance. As PUFA species are used to promote membrane fluidity, growth, development, and signaling (Watts et al., 2002), their decreasing abundance in PE populations after glucose stress indicates possible cellular damage that can affect overall health and viability. Further analysis of PE lipids would provide a more telling description of the internal glucose metabolism within the nematode. Often localized on the internal leaflet of the plasma membrane and mitochondrial membranes, PE lipids would be more susceptible to the oxidative stress conditions produced by glucose. As a result, further analyses were completed using the total PE lipid profile.

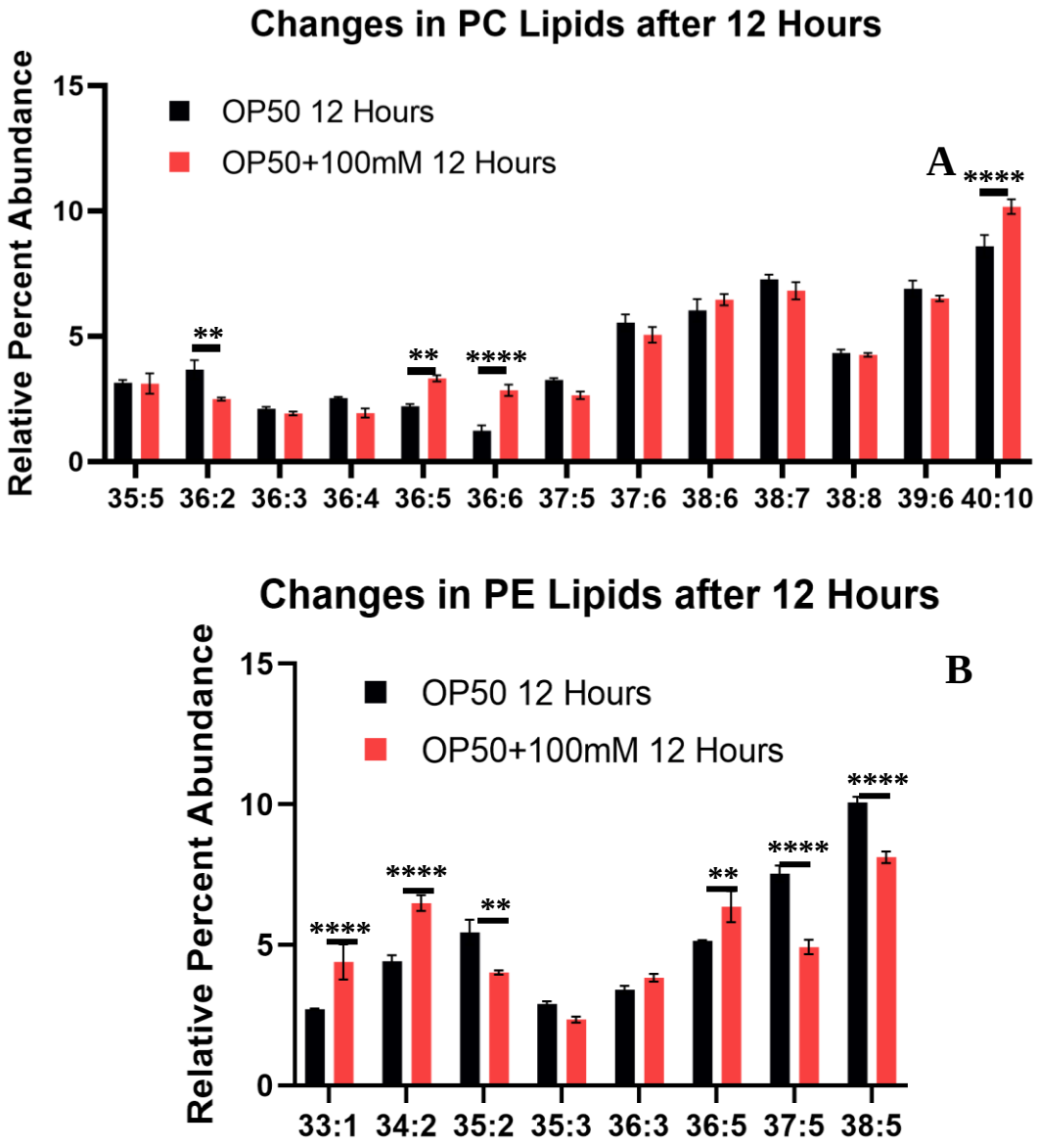


Figure 3: PC and PE Lipid Species with Significant Changes after 12 Hours of Glucose Exposure

(A) PC lipid species that showed a significant change in abundance post-glucose treatment and had a relative abundance greater than 1%. (B) PE lipid species that showed a significant change in abundance post-glucose treatment and had a relative abundance greater than 1%. graphs were plotted using the mean of three biological replicates. Abundance values are reflective of the total lipid population. Two-way ANOVA was completed to determine statistical differences from the control group. Significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Lipid species are written with X:Y nomenclature, where X is the number of combined carbons in the two fatty acid tails, and Y shows the number of double bonds present.

Overall Abundance of PUFA-Containing PE Lipids Decreases with Glucose Exposure, but Not Bacteria are Heat-killed

We observed that several PE lipid species show a significant decrease in PUFAs after glucose stress. To determine the overall impact on the fluidity of the membrane, the abundance values of all PE lipids were grouped based on the amount of double bonds present in each intact phospholipid in a process known as binning. Following HPLC-MS/MS quantification, PE lipid abundance values were separated into the following categories: 0, 1, 2-3, 4-5, 6-7, 8+ based on their number of double bonds. After excluding lipid species without abundance values, the relative percent abundance was quantified and grafted (Figure 4). As shown in Figure 4, there is a significant decrease in in 4-5 and 6-7 double bonded PE lipids after 12 hours of glucose stress. These findings are consistent with the observed decrease of 36:5 and 37:5 lipids shown in Figure 3B. Additionally, overall species with one MUFA increased over the 12-hour stress as previously observed.

Additionally, the role of bacterial process of glucose on the changes in PUFA-containing lipids was explored by subjecting worms to a diet of hk bacteria alongside the OP50 control and OP50 + 100 mM glucose condition. Heat-killing was conducted by placing a concentrated biomass of OP50 *E. coli* in a 60ºC water bath for 20 minutes. Once killed, a 100mM glucose solution was used to resuspend half of the prepared biomass. Before the experiments were conducted, the concentrated biomass was re-streaked and placed in another 60ºC water bath to ensure complete death of the bacteria.

We observed that the overall PE lipid population becomes more saturated during glucose stress. These decreases in 4-5 and 6-7 PUFA-containing lipids and increase in MUFA-containing lipids were not observed when the worms were fed hk bacteria, even in the presence of glucose.

Therefore, it was demonstrated that live bacterial metabolism is necessary to decrease the abundance of PUFAs in N2 worms when exposed to glucose. Two-way ANOVA did not show any significant changes in these species, however.

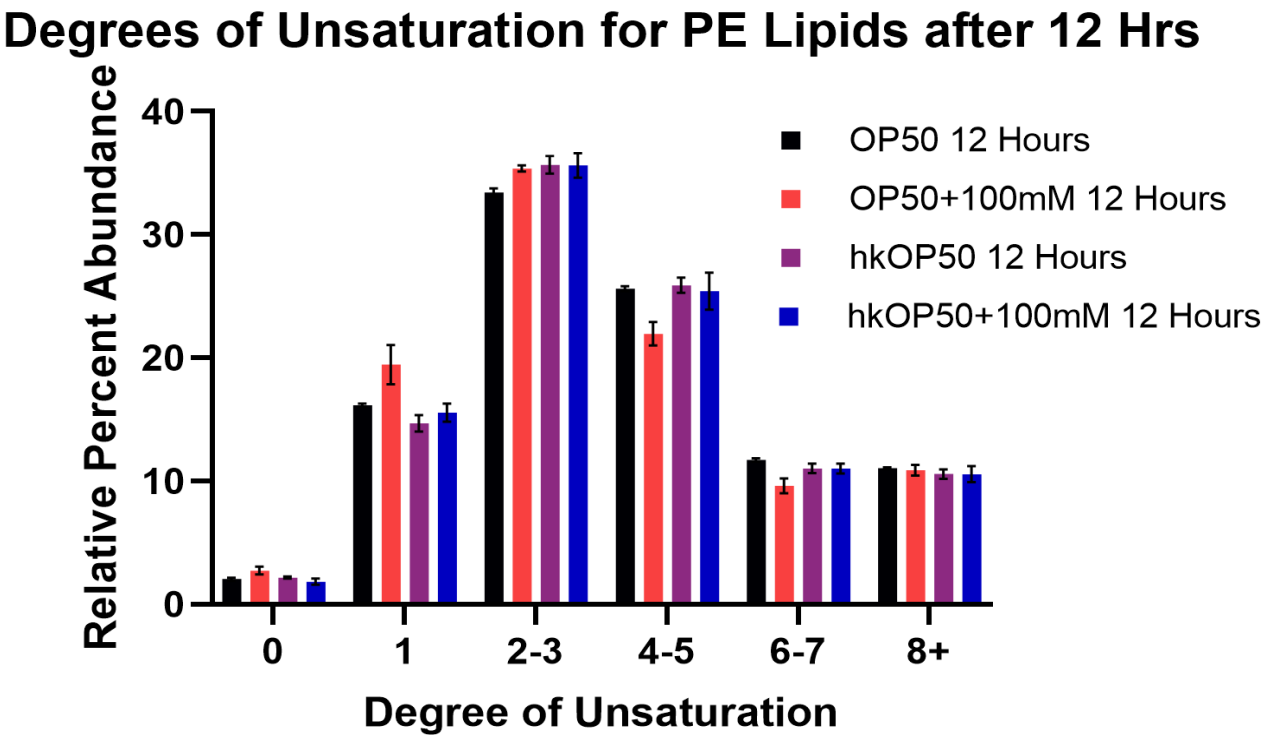


Figure 4: Relative Abundance of Double Bonds in PE Lipids after 12 Hours of Glucose Exposure

Abundance values from LDA were binned based on the number of double bonds present (the second number in lipid naming nomenclature). If a lipid species did not have and abundance at all three trials for a specific lipid composition, it was excluded from quantification. Error bars are plotted using ±SEM.

Lipid Populations are not Static, as Changes Continue Depending on Length of Glucose Exposure

As mentioned in the above section, plasma membranes are highly dynamic, as fluidity and composition are regulated by a variety of genetic and metabolic systems. Genes and metabolites cause variations in the types of lipids and fatty acids in the membrane to help control fluidity. Because of this, it is important to understand how prolonged exposure to stress and signals affect the composition of the plasma membrane. As 12 hours of glucose exposure may not be indicative of changes seen at longer time periods, we subjected *C. elegans* to the same glucose stress for longer time periods.

Worm populations were grown as described in the above section but left on the experimental conditions for 24 hours instead of 12 hours. After lipid extraction and quantification, abundance values of both PC and PE lipids were calculated, pruned, and graphed. Like the previous experiment, PE lipids were selected for double bond analysis.

As shown in Figure 5A, PC lipids demonstrated consistent change as 36:5 and 36:6 abundance increased after both lengths of glucose exposure. There was a decrease in 38:7 species during the 24-hour stress that was not observed during the 12-hour, and the increase in 40:10 species was also not observed. More sporadic changes can be seen in the PE population, as shown in Figure 5B. Consistent with the 12-hour stress, abundance in 35:2 and 37:5 lipids decreased with longer glucose exposure while 36:5 abundance increased. However, the decrease shown in 38:5 and increases in the MUFAs after 12-hour stress did not occur following 24-hour stress. Subsequent double bond grouping was needed to understand the overall PE lipid population (Figure 6). Interestingly, the decrease in 4-5 double bond abundance was not observed and had increased by more than 10% in both the control and glucose experiments

following 24 hours of stress. Similarly, there was no decrease in the abundance of 6-7 double bonds either. MUFA species also lost their significant increase after 24-hour stress. Finally, an overall decrease in abundance was observed among control groups for PUFAs with 8+ double bonds after 24-hour stress compared to 12-hour stress, but there was no significant decrease in these species following glucose exposure. Changes in PC species were determined as well, but due to focus on PE lipids in previous datasets, it was not included here (Supp. Figure 3). The drastic shift in some PE lipids following 24 hours of glucose stress indicates that potential pathways are initiated within the cell to adapt to a prolonged glycosylated environment. Follow-up studies using ^{13}C stable isotopic labeling could be conducted to further examine what and how these lipid precursors are being incorporated into the membrane during glucose stress.

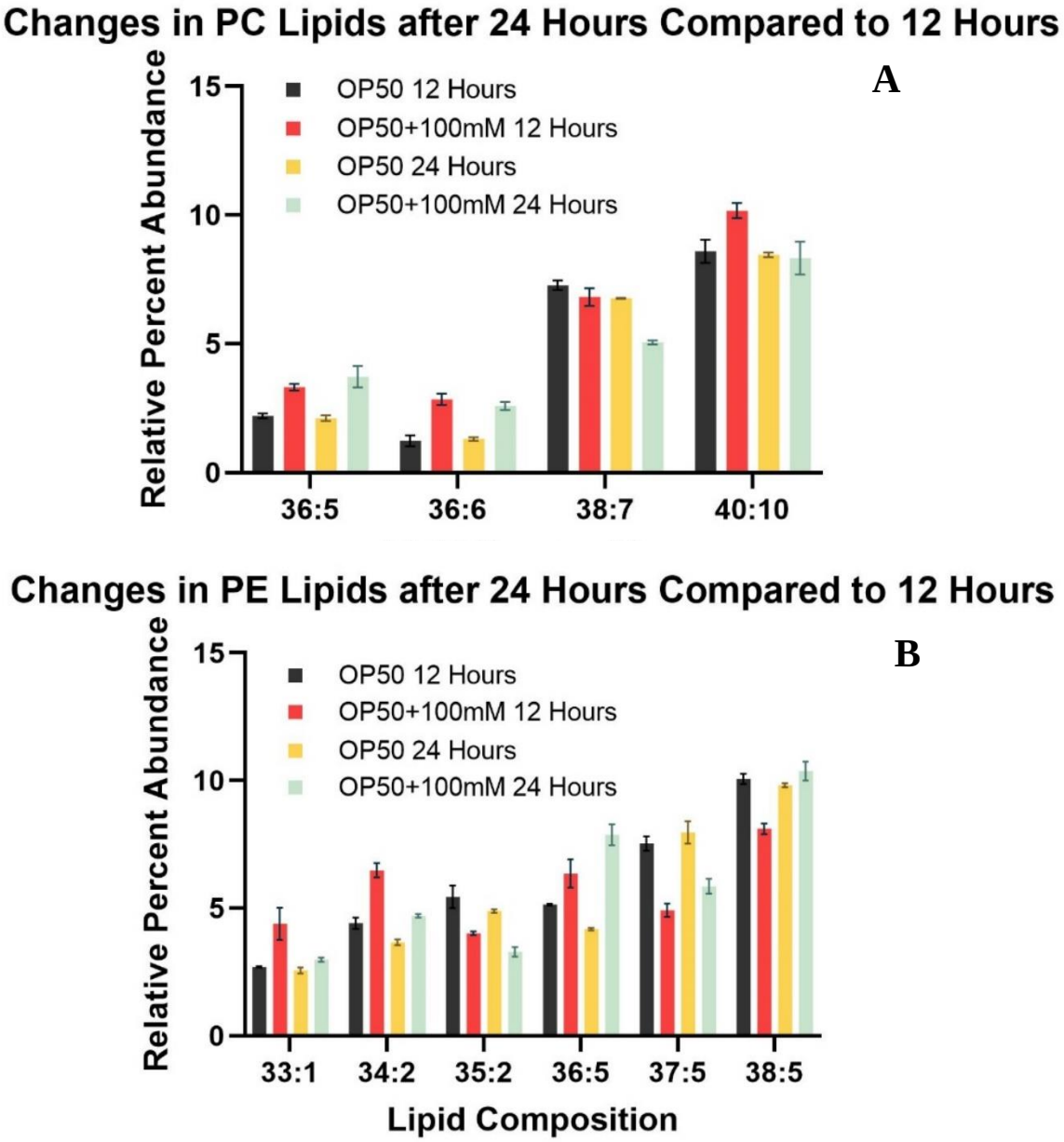


Figure 5: Lipid Profile of PC and PE Species after 24 Hours of Glucose Stress

(A) PC lipid species after 24 hours of glucose stress compared to species after 12 hours of glucose stress. Significant lipid species varied across the two time periods, so dataset was expanded so that no values were excluded. (B) PE lipid species after 24 hours of glucose stress compared to species after 12 hours of glucose stress. Dataset was expanded to prevent values from being excluded. Three replicates were completed for 24-hour trials and the average abundance was plotted with ±SEM.

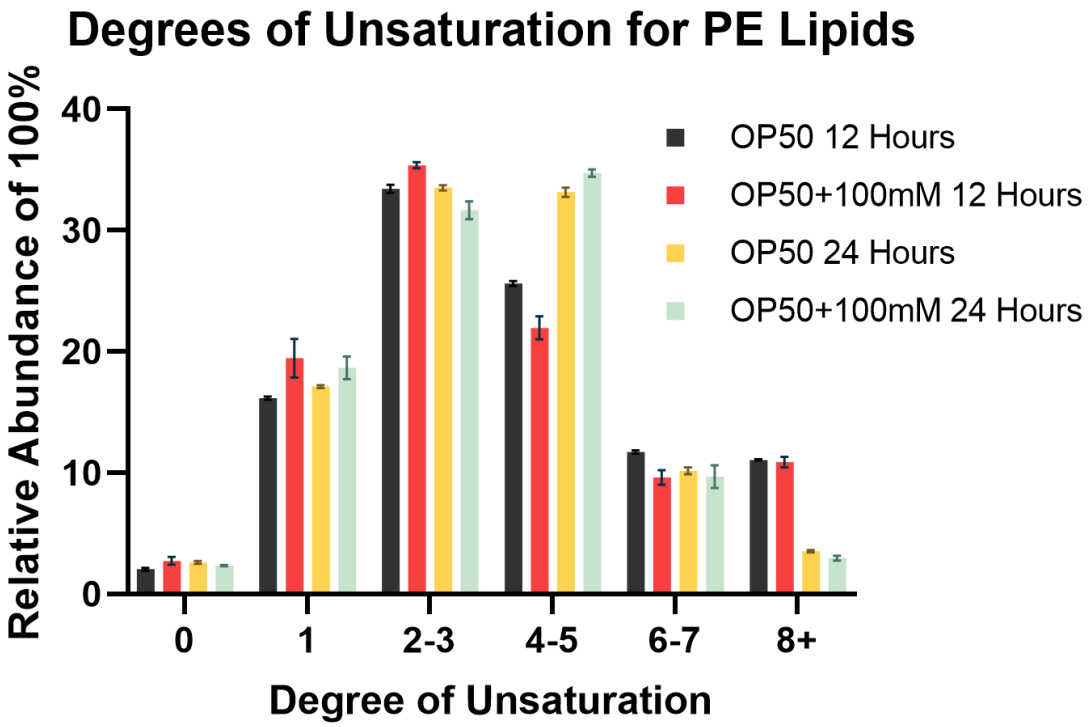


Figure 6: Relative Abundance of Saturated Lipid Species after 24 Hours of Glucose Exposure Compared to 12 Hours.

Abundance values from LDA were binned based on the number of double bonds present in the fatty acid chain. Lipids that were not shared among the 12- hour and 24-hour conditions, or lacked abundance values, were excluded from further analysis.

Development Assay of Worms Exposed to Glucose and Heat-Killed Bacteria

In addition to cellular protection and chemical signaling, the plasma membrane is necessary for the proper development of worms from their larval stage (L1) to adult stage (D1). Research into glucose metabolism and other oxidative stress mechanisms has shown a decrease in the lifespan and egg-laying potential of *C. elegans* that increase in severity with greater concentrations of glucose (Garcia et al., 2015). The previous sections show that bacterial metabolism is necessary to facilitate lipid profiles changes during glucose exposure. Therefore, we can infer that removing processed glucose from the diet by inhibiting bacterial metabolism should prevent glucose-related phenotypes from occurring. Here, we show the results of the phenotypic changes caused high concentrations of glucose stress and heat-killing the standard OP50 diet.

Unimpeded growth in *C. elegans* will cause the nematode to grow to ~1 mm in length and begin gestation after about 48-60 hours from hatching. Adult hermaphrodites have an average brood size of about 300 eggs and a mean lifespan of 18 days (Corsi et al., 2015). However, glucose exposure causes a 30% reduction in lifespan, lower brood sizes, and decreased locomotion. Likewise, glucose also decreases the worms' resistance to oxidative and heat-based stress (Kingsley et al., 2021).

To replicate these phenotypes, synchronized L1 worms were plated on one of four experimental conditions: OP50, OP50 + 100 mM glucose, hk OP50, and hk OP50 + 100 mM glucose. Once plated, worms were allowed to grow for 24 hours before collection. Worms were collected in 10 µL samples and counted under a benchtop microscope to determine their developmental stage. This process was repeated every 24 hours to determine the time necessary

for L1 worms to reach adulthood (marked in this experiment as the ability to lay many eggs). During one trial, photographs of worms were taken after 48 hours of development for each experimental condition (Figure 7). Worms fed OP50 grew as expected, as observations showed that worms reached full maturity within the known 48-60 window. Similarly, worms fed OP50 + 100mM glucose were observed to grow, although they displayed phenotypes consistent with those in previous studies. Conversely, observations on the worms fed hk bacteria showed extraordinarily little growth and development, regardless of how long they had been plated. Most worms remained arrested at the L1 stage throughout the observational period, and those that were not arrested did not develop into the D1 stage. There was no discernable difference in development when fed hk bacteria versus hk bacteria and 100mM glucose. Initially, the experiments described in previous sections of this report were to be completed using worms developed on these experimental plates. However due to the arrestment at the L1 stage in worms fed hk bacteria, the experiments were completed using L4 worms grown on OP50 plates. Attempts at quantifying the developmental stages of worms on experimental conditions and assessing their phenotypes was frequently confounded by contamination of an unknown bacteria on experimental plates, specifically those with hk bacteria (Supp. Figure 4). Worms preferentially ate this unknown bacterium and were able to grow as expected for control conditions.

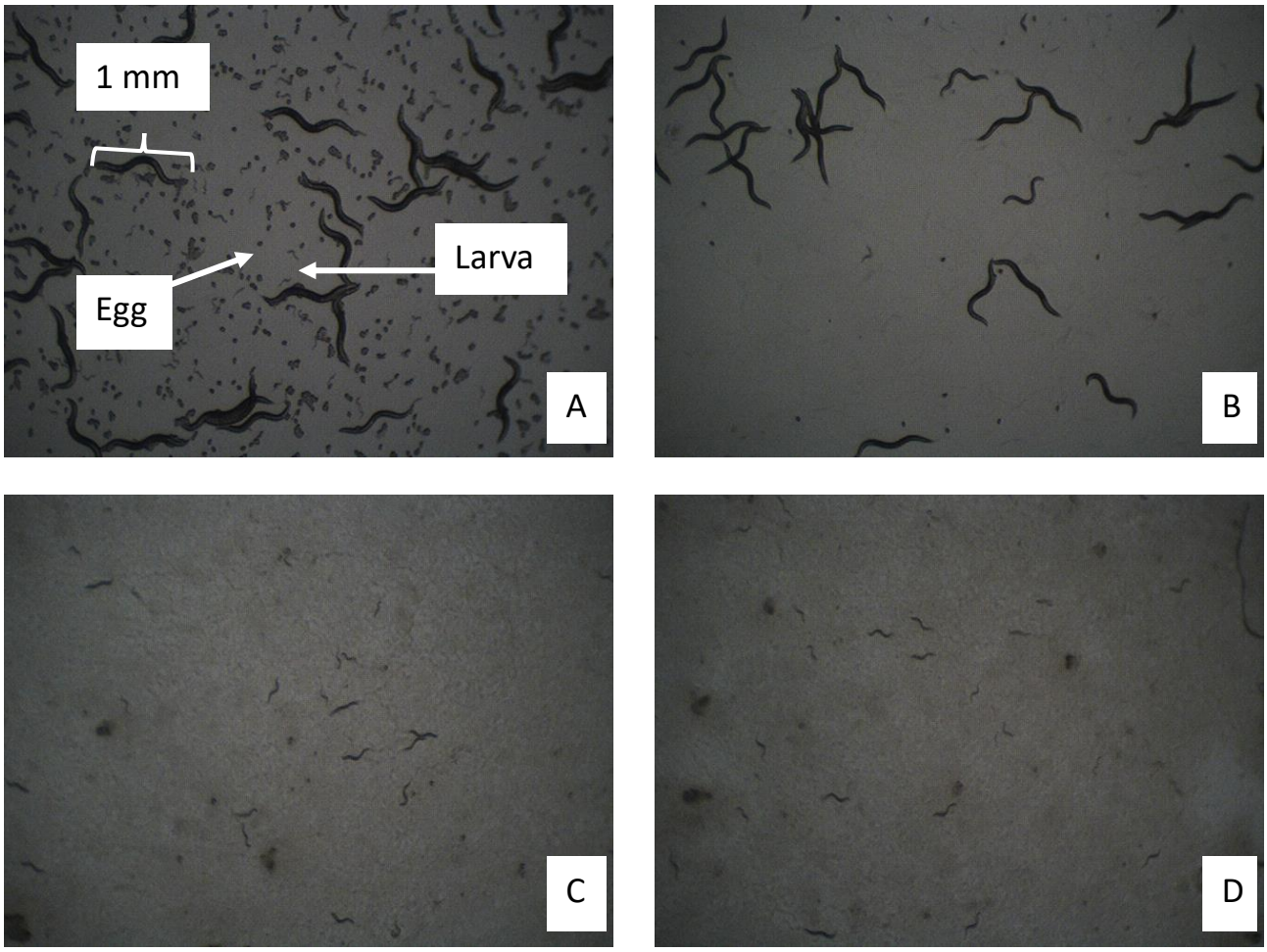


Figure 7: Developmental Characteristics of *C. elegans* after 48 Hours

(A) Control worms after 48 hours. These worms grew as expected: length was about 1 mm, eggs were laid, and several eggs already hatched into larva. (B) Worms on OP50 + 100mM glucose after 48 hours. These worms grew to 1 mm, but a majority were not ready to lay eggs. (C) Worms on hkOP50 after 48 hours. Little to no growth seen in these worms. (D) Worms on hkOP50 + 100mM glucose after 48. Like the hkOP50, there was little to no growth seen in these worms.

Fatty Acid Turnover Rate Data

Research into lipid dynamics has illuminated the role of mmBCFAs on the pathways related to *daf-2*, an insulin receptor orthologue, that controls food-sensing, post-embryonic growth, and development pathways (Kniazeva et al, 2008). Upon discovery of mmBCFAs and their regulation, more research has been conducted to understand their role in glucose responses. For example, supplementing the diet of the worm with mmBCFAs during glucose stress caused a rescue in the survival rate of mutant worms that cannot synthesize mmBCFAs themselves (Vieira et al., 2021). Current research has illuminated the metabolic pathway initiated by glucose and the subsequent cellular response with live bacteria. In conjunction with Andre Vieira of the Olsen lab, we report the mole excess percent, or the stable isotope concentration, of several mmBCFAs and their related molecules.

N2 worms were grown in control conditions, in the presence of 15 mM glucose, hk bacteria, and hk bacteria and 15 mM glucose. Worms were fed a labeling media consisting of a 1:1 ratio of carbon 12: carbon 13 isotope for six hours to measure the incorporation and distribution of mmBCFAs following analysis. Lipid extractions were separated by polarity, subjected to fatty acid methyl ester creation, and analyzed using GC coupled MS. The mole percent excess, or the enrichment of an isotope as a percentage of all isotopes present, was calculated as described in Dancy et al. (2015) and graphed (Figure 8).

The presence of glucose causes an increase in C17iso species and subsequent generation of mmBCFAs, as suggested by Vieira et al. (2021). When the bacteria were subjected to heat-killing, this increase was no longer found. However, decreases in other fatty acid species, like C18:0 and C16:0, because of heat killing highlight that the lack of bacterial metabolism causes a

unique stress on the lipid metabolism in the worm. This could impede the development of the worm into an adult.

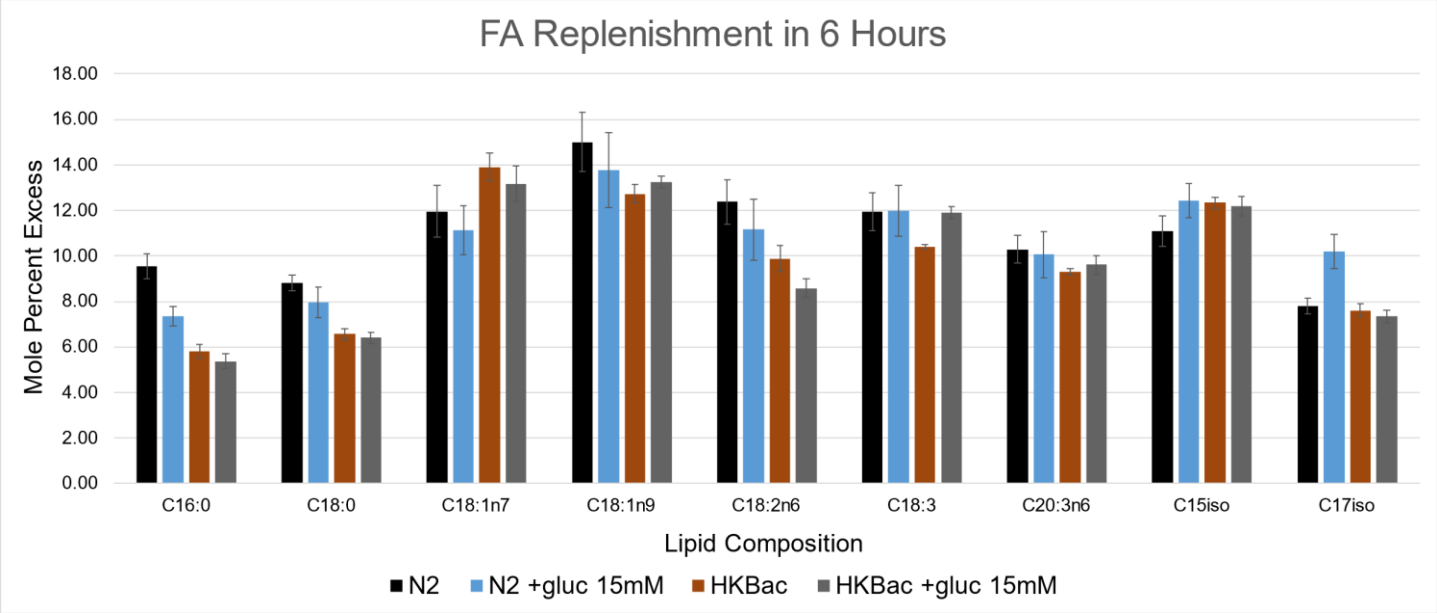


Figure 8: Replenishment of Fatty Acid Species after 6 Hour Isotopic Labeling

Mole percent excess for fatty acid species following isotopic labeling and subsequent GC-MS analysis. Values were calculated and plotted with ±SEM. Several species, like C16:1n7 were not present in *C. elegans* and were thus not reported.

Conclusions

Mass spectrometry analysis showed that glucose stress corresponds to an overall decrease in PUFA-containing lipids and increase in MUFA-containing lipids in PE populations immediately following glucose exposure. Due to the necessity of unsaturated fatty acid species to promote fluidity in the membrane, this downward shift in abundance indicates changes in the overall membrane structure, and thus membrane dynamics. As described previously, the plasma membrane in *C. elegans* is replaced by as much as 80% every 24 hours (Dancy et al, 2015). This allows for the dramatic restructuring of lipid species to compensate for external stresses that would change the fluidity of the membrane and impact survival. Understanding the saturation level of plasma membrane lipids reveals insights on how the cells respond to external stress and signals. For example, PUFAs can be cleaved from the membrane to act as short-range signaling molecules to regulate inflammation, immune responses, reproductive processes, and gene transcription (Watts, 2016). In terms of glucose stress, genes such as *paqr-2* and *iglr-2* encode fluidity sensors and induce the production of desaturase enzymes that increase PUFA synthesis when activated. This is necessary as increasing glucose stress has been shown to increase the presence of saturated fatty acids within the membrane, reducing its fluidity (Svensk et al., 2016). As a hypothetical compensation pathway, MUFA PE species are upregulated along with PC PUFA-containing lipids via desaturase enzymes to maintain membrane fluidity and an optimal saturated fat:unsaturated fat ratio necessary for survival.

A previous study by Devkota et al. (2017) suggests that the plasma membrane rigidifies in response to OP50 glucose metabolism. These findings are corroborated in this study, as PUFA-containing lipids, which are essential for promoting membrane fluidity, are decreased when exposed to glucose but not when the bacteria are metabolically inactive. Therefore, it is

important to understand how and why the metabolism of glucose causes reductions in membrane fluidity. OP50 digests glucose via glycolytic pathways, resulting in the formation of glyoxal groups (Kingsley, 2021). Before the OP50 is eaten by the worm, these glyoxal molecules attach themselves to proteins and other molecules within the *E. coli* cell. This results in the production of advanced glycosylation end products (AGEs) that the worm ingests (Kingsley, 2021). Cellular respiration within the worm already generates ROS that are regulated through various enzymes and products of the antioxidant system. Consumption of these AGEs increases the amount of oxidative stress experienced by the cell, generating an imbalance in the ROS/antioxidant system. In addition to increasing oxidative stress, AGEs have been shown to suppress the *gst-4* and *glod-4* genes, which encode the glutathione S-transferase 4 and glyoxalase 1, respectively. These proteins act to reduce concentrations of ROS and prevent the formation of more AGEs (Kingsley, 2021). When suppressed, ROS concentration rapidly increases and begins to damage cellular machinery via free radical chain reactions. Due to their considerable amounts of pi double bonds, which are especially susceptible to electrophilic attack, PUFA are prone to destruction by ROS attack. When hk, OP50 cannot metabolize glucose via its glycolytic pathways. Therefore, AGEs cannot be produced and ingested by the worm, preventing the additional formation of ROS, suppression of *gst-4* and *glod-4*, and decrease in PUFA-containing lipids. A graphical representation of this model can be found in Figure 9. This contrasts with two other studies (Lee et al., 2009 and Garcia et al., 2015) that demonstrated that direct contact with the worm and glucose causes toxicity.

After 24 hours of glucose exposure, we observed that the membrane composition shifts further in N2 nematodes, which may suggest that regulatory pathways are initiated by the upregulation of genes such as *paqr-2, iglr-2*, and *daf-2* attempt to prevent cellular damage

(Dancy et al. 2015). However, subsequent heat-killing of the OP50 bacteria highlighted the necessity of living bacterial metabolism to produce the shifts in fatty acid species observed. Hk bacteria lack the ability to process glucose efficiently, meaning worms will not be subjected to the same oxidative stress as those in the OP50 + 100 mM glucose experiments. Without the subsequent increase in ROS, membrane fluidity can be maintained. However, the oxidative load from the bacterial metabolism of glucose is necessary to provide *C. elegans* with the nutrients needed to properly grow and develop, as shown in the arrested L1 larva observed.

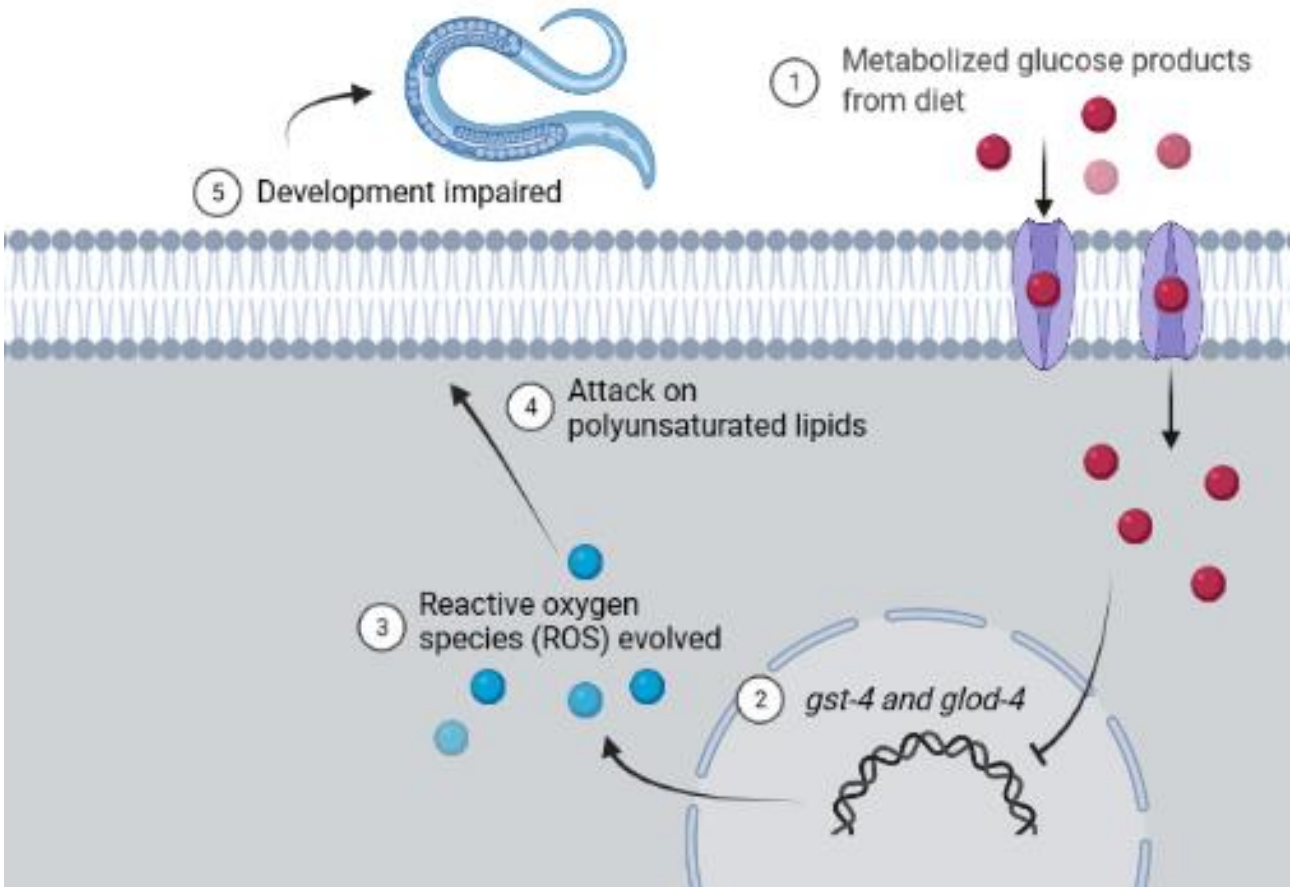


Figure 9: Potential Model of Decrease in PUFAs Following Ingestion of Glucose-fed Bacteria

(1) Bacteria digest glucose in addition to nutrients in the growth media. The glycolytic pathways in bacteria produce glyoxal groups that coordinate with other metabolic products that the worm then ingests. (2) These glyoxal products then induce oxidative stress on the worm and suppression the transcription of the gst-4 and glod-4 genes to relieve oxidative pressure. (3) Oxidative stress is increased as ROS are continual evolved. (4) The double bonds on PE PUFAs are especially vulnerable to electrophilic attack by ROS. However, PUFA sacrifice reduces oxidative stress by eliminating ROS and its associated free radicals. (5) Reduction in PUFAs rigidifies the membrane and additional cellular damage causes reduce lifespan and brood size in N2 *C. elegans.*

Future Directions

Stable Isotope Labeling

The exact nature of the composition pathways that regulate membrane fluidity via polyunsaturated and monounsaturated fatty acids in response to glucose stress can be assayed using stable isotope labeling. Developed by Perez and Van Gilst (2008), stable isotope labeling involves incorporating two isotopes of carbon into the diet of *C. elegans.* Specifically, half of the dietary OP50 is enriched with ^{13}C, and the other is grown using ^{12}C. Fats incorporated from the bacteria will be either labeled or unlabeled, whereas those synthesized *de novo* will contain a mixture of both isotopes (Perez and Van Gilst, 2008). Unlike other isotope labeling methods, C^{13} labeling in *C. elegans* allows for simultaneous observation of lipid absorption, elongation and desaturation, and synthesis throughout the entirety of development (Perez and Van Gilst, 2008). Therefore, the compensation of membrane lipids can be directly assayed after glucose stress to determine potential upregulations in synthase, desaturase, elongase, etc. genes and their associated proteins. Analyzing the other less abundance lipid classes such as PSs and sphingomyelins could also provide a more holistic understanding of the dynamics between metabolism and the lipid profile under high glucose stress environments.

Lipid Profiling with Different Sugar Diets

While glucose was the main contributor to oxidative stress in this study, various other sugars can be easily incorporated into the diet of *C. elegans*. Studies have also shown that *C. elegans* development reacts differently under sucrose and fructose stress compared to glucose stress. For example, nematodes exposed to sucrose stress had experienced prolonged lifespans in a limited concentration window (maximum of 50 mM) versus glucose stress (maximum of 500 mM) (Wang et al., 2020). By subjecting nematodes to further HPLC-MS/MS analysis under

different sugar conditions, the effects of diverse sugars on lipid metabolism and membrane composition can be explored. Similar methodologies to those employed in this study could investigate if oxidative stress from different sugars cause the same decrease in PE PUFA-containing lipids Finally, studying the effects of different sugars can also elucidate the functions of other catabolic pathways and their associated genes and enzymes necessary for *C. elegans* development for further research.

Killing Bacteria via Other Methods

Killing the OP50 bacteria by heat was used because of how quick it can prevent glucose processing with a simple procedure. While useful for this study, heat-killing bacteria destroys bacterial metabolism needed for *C. elegans* development (see Figure 7 on developmental analysis). Preventing the development of the worm confounds collected data on metabolism, so it is important to cause as minor change as possible to the OP50. As a result, subsequent experiments could make use of a different killing method for OP50. Feasible options include UV light exposure or paraformaldehyde (PFA) treatment. Like heat-killing, UV exposure is a simple and easy procedure that can quickly kill small colonies of bacteria. However, it is typically limited by the scale of the experiment, as larger studies make the procedure more laborious. It is also not guaranteed to kill all bacteria (Beydoun, 2021). Alternatively, future studies could make use of PFA treatment of OP50. PFA is a formaldehyde polymer that permeabilizes the cell without removing damaging the inner structures or plasma membrane (Beydoun, 2021). The lifespan and fecundity of the worm is also not impaired by PFA-treated bacteria, but researchers did observe a slight delay (about 4-5 hours) in overall development (Beydoun, 2021). Regardless, the worms were healthy, and PFA offers a better methodology for determining the role of living bacterial metabolism in *C. elegans*.

Bacteria Specific Heat-killed Stress on *C. elegans*

Once again, *C. elegans* fed hk OP50 were arrested at the L1 stage compared to those fed living OP50. Plasma membrane composition is dependent on the synthesis of fatty acids *de novo* and through dietary supplementation. This study shows the effects of glucose stress on the lipid composition, highlighting several abundance changes in PE and PC lipids, and how those changes in composition are nullified when the bacteria is heat-killed. A similar series of experiments could also be conducted with different strains of bacteria to determine the lipid composition changes in *C. elegans* subjected to hk bacteria stress. Such experiments would enhance our overall understanding of the commensal relationship between *C. elegans* and their bacterial diet (Figure 10) and how it relates to our own relationship with the human microbiome.

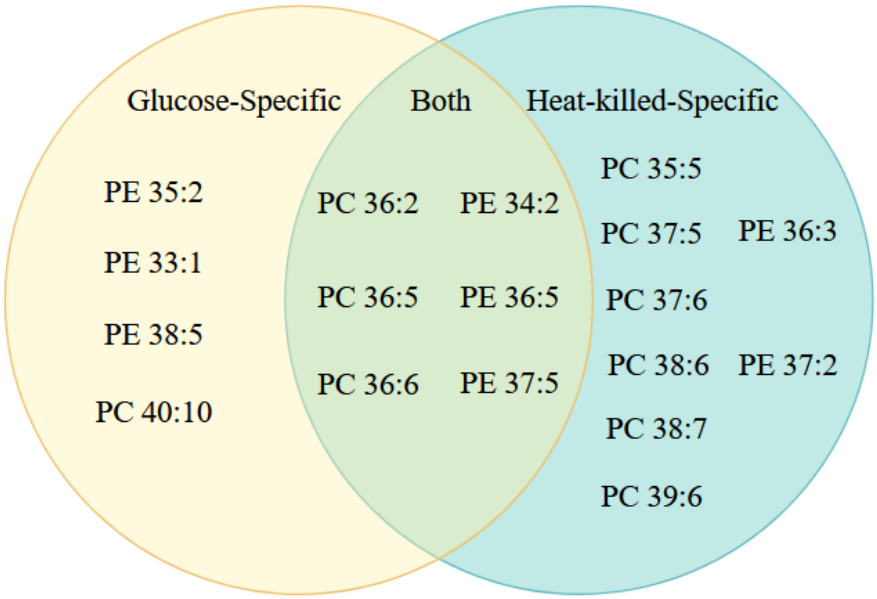
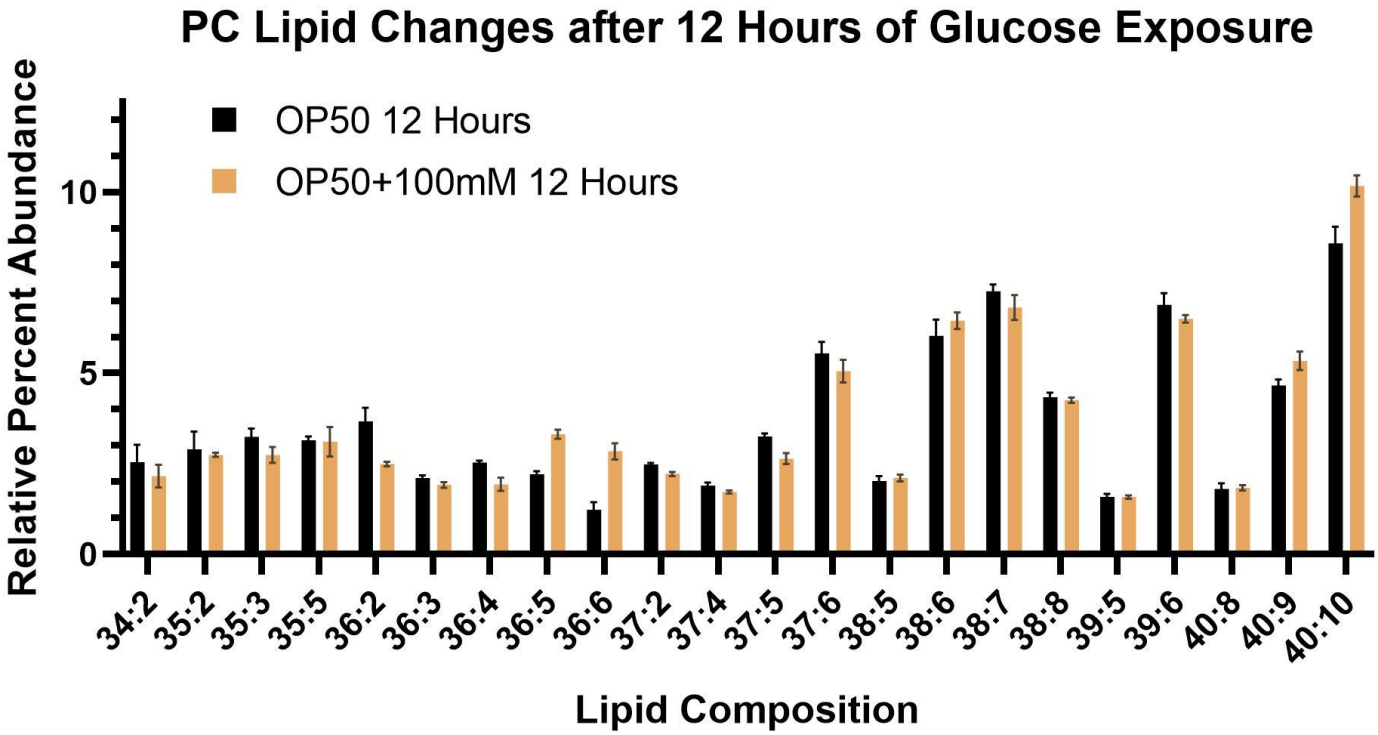


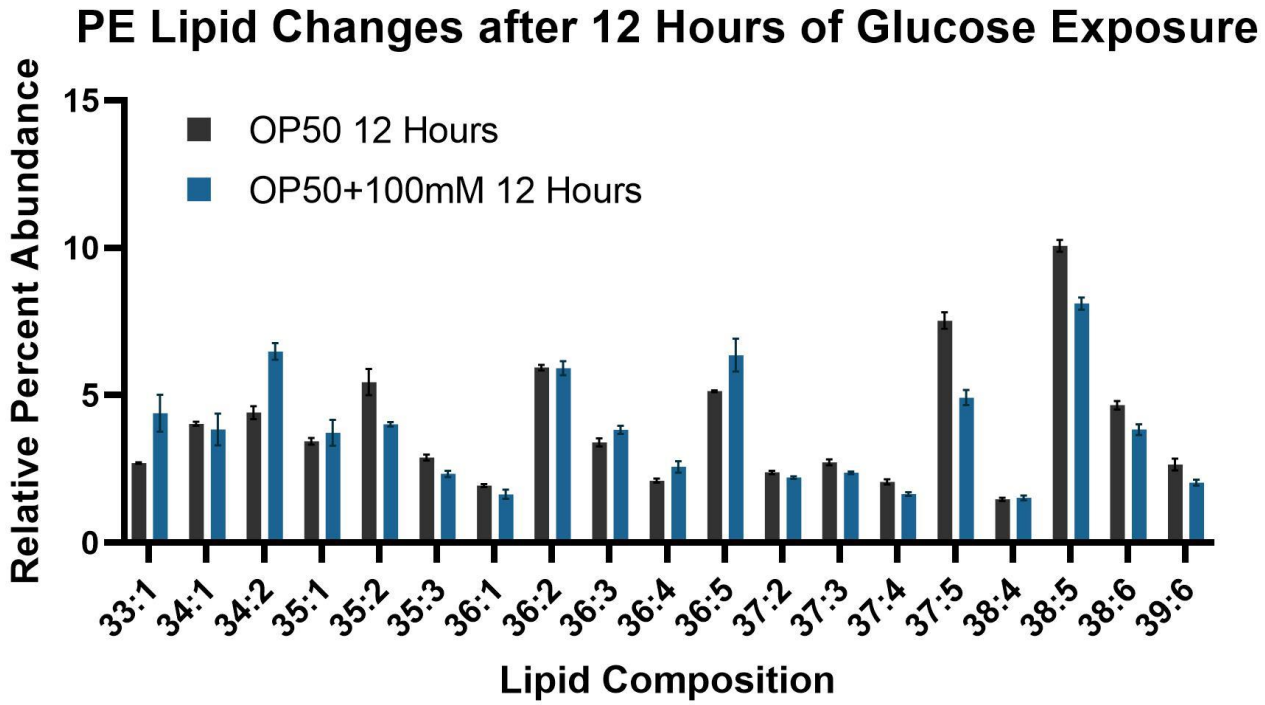
Figure 10: Changes in Specific Lipids due to Glucose and Heat-Killed Stress

Lipid species were determined to be significant by two-way ANOVA testing in GraphPad Prism 9. Glucose-specific stress was uniquely seen in 4 species. Conversely, heat-killed-specific stress was uniquely seen in 8 species. However, 6 species saw significant changes in both stress conditions.

Supplemental Figures

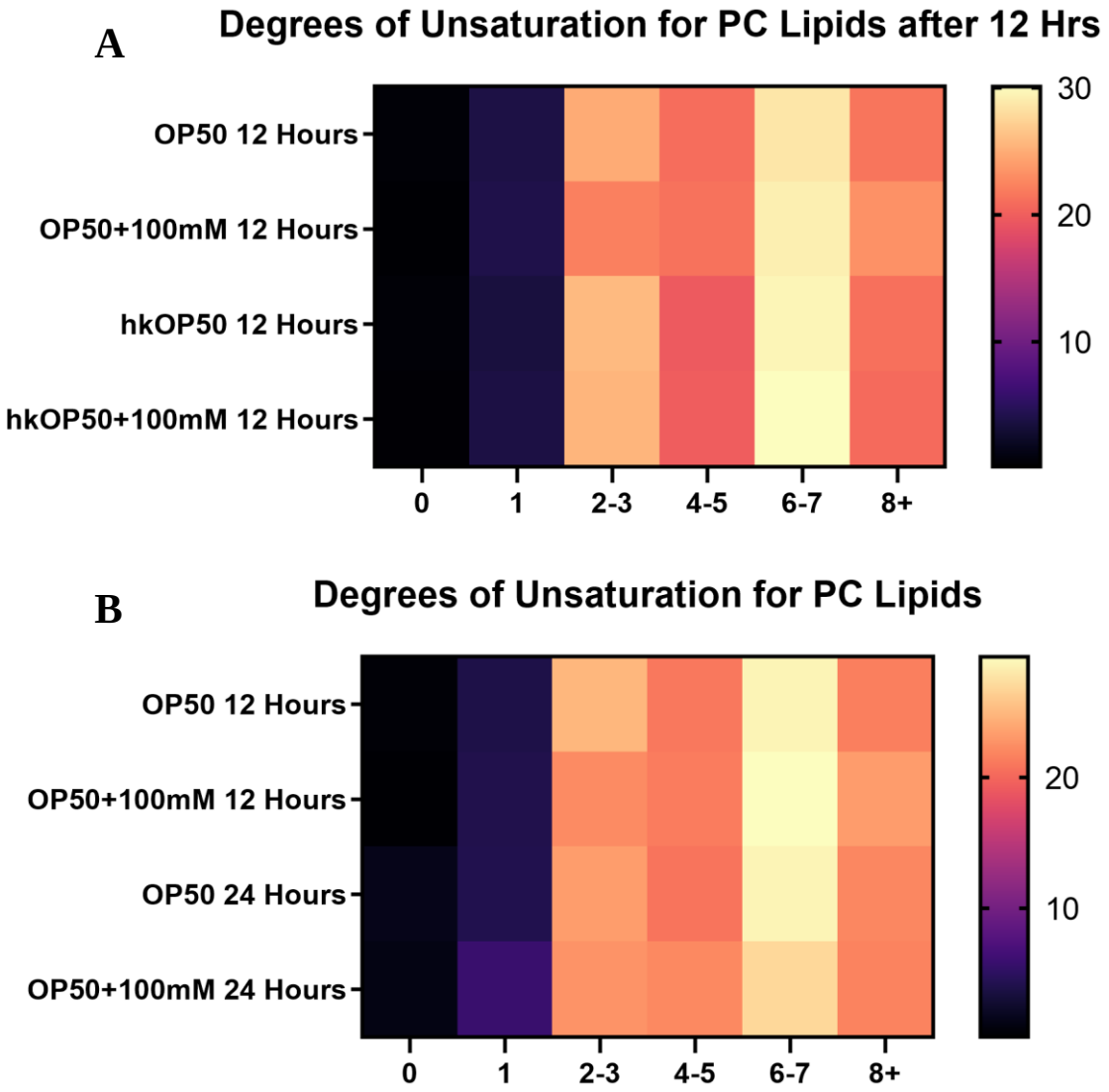


Supplemental Figure 1: PC Lipid Changes after 12 Hours of Glucose Exposure
Overall changes in PC phospholipid distribution for species with greater than 1% relative abundance. Control N2 worms were fed OP50 on HG plates. Experimental N2 worms were fed OP50 that had processed 100 mM glucose on the HG plate before the trial began. Lipid species are written with X:Y nomenclature, where X is the number of carbons in the fatty acid, and why shows the number of double bonds present. Error bars show the ±SEM among three replicates. Lipids that were determined to be insignificant by two-way ANOVA were excluded to create Figure 1.



Supplemental Figure 2: PE Lipid Changes after 12 Hours of Glucose Exposure

Overall changes in PE phospholipid distribution for species with greater than 1% relative abundance. Control N2 worms were fed OP50 on HG plates. Experimental N2 worms were fed OP50 that had processed 100 mM glucose on the HG plate before the trial began. Lipid species are written with X:Y nomenclature, where X is the number of carbons in the fatty acid, and why shows the number of double bonds present. Error bars show the ±SEM among three replicates. Lipids that were determined to be insignificant by two-way ANOVA were excluded to create Figure 1.



Supplement Figure 3: Binned Double Bond Analysis for PC Lipids for 12 Hours and 24 Hours of Glucose Stress

(A) The relative abundance of double bonds and PC lipids after binning RAW data from LDA after 12 hours of glucose stress. A higher relative abundance is denoted with a lighter color on the heat map, and a lower relative abundance is denoted with a darker color. (B) The relative abundance of double bonds for PC lipids at 24 hours compared to 12 hours. If a given lipid did not have an abundance at all three trials, it was excluded from further quantification. Error bars were plotted using plus ±SEM.

Supplemental Table 1: Quantification of *C. elegans* Larval Stages after Exposure to Glucose Stress and Heat-killed Bacteria

Trial 1									
N2	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	28	6	21.42857	22	78.57143	0	0	0	0
Day 2	64	1	1.5625	2	3.125	7	10.9375	54	84.375
Day 3	46 + eggs laid and hatched	0	0	0	0	1	2.173913	45	97.82609
hk N2	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	97	97	100	0	0	0	0	0	0
Day 2	35	28	80	7	20				
Day 3	Bacteria growth, development sped up								

Trial 2									
N2	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	32	8	25	24	75	0	0	0	0
Day 2	17	0	0	0	0	17	100	0	0
Day 3	Fungal Growth								
hk N2	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	27	27	100	0	0	0	0	0	0
Day 2	49	5	10.20408	22	44.89796	22	44.89796	0	0
Day 3	Fungal Growth								

Trial 3									
N2	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	6	6	100	0	0	0	0	0	0
Day 2	47	0	0	2	4.255319	45	95.74468	0	0
Day 3	25 + eggs laid/hatched	0	0	0	0	0	0	25	100
hk N2	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	63	63	100	0	0	0	0	0	0
Day 2	62	44	70.96774	16	25.80645	2	3.225806	0	0
Day 3	25 + eggs laid/hatched	0	0						

Trial 1									
N2 + 100 mM	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	91	5	5.494505	86	94.50549	0	0	0	0
Day 2	53	0	0	0	0	7	13.20755	46	86.79245
Day 3	24 + eggs beginning to be laid and hatch	0	0	0	0	1	4.166667	23	95.83333
hk N2 + 100 mM	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	73	73	100	0	0	0	0	0	0
Day 2	48	38	79.16667	10	20.83333				
Day 3	21	16	76.19048	5	23.80952				

Trial 2									
N2 + 100 mM	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	29	9	31.03448	20	68.96552	0	0	0	0
Day 2	63	6	9.52381	13	20.63492	44	69.84127	0	0
Day 3	Fungal Growth								
hk N2 + 100 mM	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	44	44	100	0	0	0	0	0	0
Day 2	47	9	19.14894	19	40.42553	19	40.42553	0	0
Day 3	Fungal Growth								

Trail 3									
N2 + 100 mM	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	44	44	100	0	0	0	0	0	0
Day 2	55	0	0	7	12.72727	48	87.27273	0	0
Day 3	22	0	0	0	0	5	22.72727	17	77.27273
hk N2 + 100 mM	Amount transferred	# L1	% L1	# L2	% L2	# L3	% L3	# L4	% L4
Day 1	89	89	100	0	0	0	0	0	0
Day 2	15	12	80	3	20	0	0	0	0
Day 3	34 + eggs laid/hatched	0	0	6	17.64706	5	14.70588	23	67.64706

Abbreviations

AGEs: Advanced glycosylation end products; *C. elegans*: *Caenorhabditis elegans*; GC: Gas chromatography; HG: High growth media; hk; Heat-killed; HPLC: high performance liquid chromatography; mmBCFAs: monomethyl branched-chain fatty acids; MS; mass spectrometry; MS/MS: tandem mass spectrometry; MUFA: monounsaturated fatty acids; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PFA: Paraformaldehyde; PS: Phosphatidylserine; PUFA: Polyunsaturated fatty acid; RNAi: RNA interference; ROS: Reactive oxygen species

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