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THE ROLE OF POLYUNSATURATED FATTY ACIDS IN BACTERIAL PATHOGENESIS
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Dissertation completed on the University of Southern Denmark, under the mobility program Erasmus, to fulfill the requirements for the degree of Master in Molecular Biotechnology on the University of Aveiro, held under the scientific guidance of Dr. Nils J. Færgeman, Professor of the Department of Biochemistry and Molecular Biology, University of Southern Denmark and co-supervision of Dr. Jakob Møller Jensen, Associate Professor of the Department of Biochemistry and Molecular Biology, University of Southern Denmark.
I dedicate this work to my parents for the endless love and support.
**Acknowledgements**

First of all I would like to express my gratitude to my advisor, Nils J. Færgeman for his guidance and support. I honestly thank him for giving me the opportunity to work in his group, providing me a great atmosphere for doing research. I would also like to thank my co-supervisor Jakob Møller Jensen for the great ideas he introduced in this project.

I must thank to all the members of the Lipid Group for their support, for being so nice and friendly and for helping me always with a smile. A special thanks goes to Sandra and Eva for all the help in the lab and for proof-reading of this thesis.

I would like to thank my dearly beloved Portuguese friends and colleagues that were always there for me and that followed my academic journey since the beginning.

To all the friends that I met in this amazing adventure and that helped me mainly in those dark, cold and snowy days in Denmark. A special thanks goes to my amazing flatmates that always provided such nice get-togethers.

I want to deeply thank Rui, for being always there for me. Besides being over 2,000 km far apart I want to thank him for being my rock, my best friend and my love.

Last but not least, I want to thank all my family, especially my parents, for supporting all my studies and for giving me the chance to go abroad to study and work.
Polyunsaturated fatty acids (PUFAs) comprise a class of essential micronutrients, which are essential for normal development, cardiovascular health, and immunity. The role of lipids, including long-chain fatty acids, in the immune response is increasingly being recognized as beneficial regulators of the immune systems. However, the mechanisms by which PUFAs modulate innate immunity are yet to be fully clarified. *C. elegans* has been used in several recent studies as a simple animal model for the study of host-pathogen interactions, generating important insights into both bacterial pathogenesis and host innate immunity. Many of the virulence mechanisms used by bacterial pathogens to cause disease in mammalian hosts have also been shown to be important for pathogenesis in *C. elegans* and, similarly, important features of the host innate immunity have been evolutionarily conserved between *C. elegans* and mammals.

This project is focused on addressing the role of polyunsaturated fatty acids in bacterial pathogenesis using *C. elegans* as model system. We find that knockdown of some elongase genes increase the worms’ susceptibility towards infection with the adherent-invasive *Escherichia Coli* LF82, isolated from a patient suffering from Crohn’s disease. Moreover, dietary supplementation with the fatty acid γ-linolenic acid rescued the enhanced pathogen susceptibility of *C. elegans* lacking a Δ6 desaturase. The fatty acid profile of the nematode is altered upon infection with pathogenic LF82. qRT-PCR analysis allowed to determine that stress and autophagy genes are induced in *C. elegans* infected with this particular type of *E. coli*. Autophagy was found to be increased on *C. elegans* challenged with LF82, as determined by fluorescence microscopy. Collectively these results suggest an important role for PUFAS in the innate immune response and indicate that autophagy may have a contribution for *C. elegans* response towards the pathogen *E. coli* LF82.
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List of Abbreviations

AA Arachidonic Acid
AIEC Adherent invasive Escherichia coli
akt-1,2 Serine/threonine kinase Akt/PKB
AMPs Antimicrobial peptides
AP-1 Activating protein-1
B. thuringiensis Bacillus thuringiensis
BHI Brain-heart infusion
C. elegans Caenorhabditis elegans
CD Crohn's disease
CEACAM6 Carcinoembryonic antigen-related cell adhesion molecule 6
CGC Caenorhabditis Genetics Center
COX Cyclooxygenase
D. coniospora Drechmeria coniospora
DAF Dauer formation abnormal
DAG Diacylglycerol
DAPK-1 Death-associated protein kinase 1
Dar Deformed anal region
DGLA Di-homo-γ-linoleic acid
DHA Docosahexaenoic acid
DR Dietary restriction
dsRNA Double stranded RNA
E. coli Escherichia coli
E. faecalis Enterococcus faecalis
EPA Eicosapentanoic acid
EPEC Enteropathogenic Escherichia coli
FAME Fatty acid methyl ester
FOXO Forkhead transcription factor class O
FuDR 5-Fluoro-2′-deoxyuridine
GC Gas chromatography
GFP Green fluorescent protein
GLA γ-linolenic acid
GST Glutathione S-transferase
IBD Inflammatory bowel disease
IEC Intestinal epithelial cell
IGF Insulin/insulin-like growth factor
IPTG Isopropyl β-D-1-thiogalactopyranoside
IRAK Interleukin-1 receptor-associated kinase
IxB Inhibitor of NF-κB
LA Lysogeny broth agar
LB Lysogeny broth
LKB1 Liver kinase 1
LOX Lipoxygenase
LTB4 Leukotriene B4
MAPK Mitogen-activated protein kinase
mmBCFA Monomethyl branched-chain fatty acid
mTOR Mammalian target of rapamycin
MUFA Monounsaturated fatty acid
Myd88 Myeloid differentiation primary response 88
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NGM Nematode growth medium
NLPs Neuropeptide-like peptides
<table>
<thead>
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<tr>
<td>P. aeruginosa</td>
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</tr>
<tr>
<td>P. luminescens</td>
<td>Photorhabdus luminescens</td>
</tr>
<tr>
<td>pdk-1</td>
<td>3-phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PHA-4</td>
<td>Transcription factor FOXA.</td>
</tr>
<tr>
<td>PPR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>S. enterica</td>
<td>Salmonella enterica</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearoyl-CoA desaturases</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming-growth factor β</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAF1</td>
<td>TNFR-associated factor 1</td>
</tr>
<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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1. **Introduction**

Lipids play a central role in biological systems as signaling molecules, energy storage and structural components of membranes. They emerge as a class of molecules that affects behavior, growth and survival, not only because of their energetic value, but also because of their structural and signaling functions.\(^1\)

Lipid composition is very important to the balance between health and disease, especially if we think on diseases related with lipid metabolism, such as obesity and diabetes, emergent problems in the modern world. Lipids also have a key role in the immune system, where they can act as immune precursors, messengers or signaling molecules.\(^2\) Numerous studies point out the importance of having a balanced lipid composition, and even suggest using fatty acid supplements to improve responses of the immune system.

In this work, our interest was to study the role of Polyunsaturated Fatty Acids (PUFAs) in the innate immune response, using the nematode *Caenorhabditis elegans* as a model system. We were able to identify different responses to infection with the pathogenic strain of *Escherichia coli* LF82 by using RNAi, a powerful and rather simple technique to knockdown the expression of key genes on PUFA synthesis pathway.

1.1. **Caenorhabditis elegans as a biological model**

*C. elegans* was first introduced as a biological model in the 1970s by Sydney Brenner. In 1974, he published a groundbreaking paper describing methods for isolating and mapping disease-related genes in the mutagenized free-living nematode, *C. elegans*.\(^3\) In 2002 he shared The Nobel Prize in Physiology or Medicine for the work developed on the genetics of organ development and programmed cell death using *C. elegans*. Since then, the worm has been widely used as a model in research laboratories. The research using *C. elegans* already granted two other groups with a Nobel Prize, namely a Nobel Prize in Physiology or Medicine and one Nobel Prize in Chemistry in 2006 and 2008, respectively.

*C. elegans*, referred to several times as roundworm, is a small nematode that is about 1 mm in length when reaching the adulthood. It is a soil nematode, which has been isolated from anthropogenic habitats as compost, garden soil and rotting fruits. However, its natural habitat remains unknown.\(^4\) Wild type *C. elegans* (referred to as N2) has a lifespan of about 2-3 weeks at 20°C and reaches adulthood within 3 days after hatching (lifespan is discussed in more detail below). The animals are multicellular organisms comprising only about 1000 somatic cells, yet they have specified tissues and an organ-based physiology.
There are many advantages of using *C. elegans* as a biological model; such as its small size and rapid life cycle, which makes the manipulation in the laboratory simple. *C. elegans* exists either as a hermaphrodite or a male. The hermaphrodites are capable of self-fertilization, which makes easy to isolate recessive mutants on all chromosomes. The predominant sex form in *C. elegans* is the hermaphrodite, capable of producing both sperm and oocytes, meaning they do not depend on mating for propagation, which allows to produce stocks from homozygous mutants with severely defective phenotypes. Additionally, it was the first multicellular organism to have its complete genome sequenced, and approximately 65% of the genes associated with human disease are conserved in the nematode. Also, the fact that the nematode can be cryopreserved at -80°C allows the maintenance and security of the different strains in a laboratory.

Furthermore, the worm allows the use of powerful methods in the laboratory in order to access different biological processes. Because *C. elegans* is transparent in every stage of their lifecycle, visualization of the cells by differential interference contrast (DIC) and fluorescence microscopy can be performed. The possibility of performing forward and reverse genetics in the worm unraveled many genotype-phenotype relations. Forward genetics starts out from a phenotype to a mutated gene. After a mutant is phenotypically identified, the mutated gene is revealed through standard molecular techniques. In reverse genetics, the opposite happens: First a gene which sequence is known is perturbed, and then the effect on the development or behavior of the animal is analyzed. In the present study we start out with a mutated gene to a phenotype. One way of doing reverse genetics in the nematode is performing RNA-mediated interference (RNAi). The ability to perform knockdown of genes using RNAi allowed the rapid assessment of gene function in the nematode (reviewed by Zhuang *et al.*, 2012).

The worm has a simple nervous system which is composed of 302 neurons in the hermaphrodite and 391 neurons in the male. Despite the simplicity, worms share cellular and molecular pathways with higher organisms. Thus, *C. elegans* is a great model to study neuronal diseases, especially as its entire neuronal layout has been mapped. Moreover, neurotransmitters, receptors, and neuronal processes, are highly conserved between *C. elegans* and vertebrates.

The connection of the nervous system with epithelial immunity has also been studied using *C. elegans* as a model, as reviewed by Kawli *et al.*, 2010. The nematode’s simple and well defined nervous system allows us to increase our understanding on how the neuroendocrine system affects immunity at the level of the all organism; studies that are rather difficult in humans due to the complexity of our nervous system.

Altogether, *C. elegans* is a perfect model to use in a research lab, because it requires simple growth conditions which translates into cost and space efficient conditions. *C. elegans* has already been used as a model for diverse studies such as insulin signaling, response to dietary glucose, host-pathogen interactions, influence of serotonin on obesity, feeding and hypoxia-associated illnesses.
In the laboratory *C. elegans* standard food source is *E. coli* OP50, since it is considered as non-pathogenic for the worms. The OP50 strain is a uracil auxotroph that lacks the O-antigen component of its outer membrane, therefore this strain does not have the pathogenic characteristics that *E. coli* usually displays.\(^{13}\) However, there are some evidences that this strain can be sensed as pathogenic by *C. elegans*.\(^{14}\) It has also been demonstrated that *C. elegans* has an extended lifespan when fed with dead OP50 bacteria, than when fed with live bacteria.\(^{15}\) Also, the growing conditions of OP50 are important, since it has been shown that when propagated on BHI medium, the *E. coli* OP50 caused significant killing of *C. elegans*, suggesting that this rich medium induced the expression of virulent factors that are lethal to the worm.\(^{16}\)

Despite these facts, *E. coli* OP50 remains to be the common food source used in all studies with *C. elegans*, including the present study, where OP50 was used as a control in the majority of the assays, if not otherwise stated.

The pathogenicity in *C. elegans*, as performed in this study, can be evaluated by simple replacing the food source with the pathogen of interest, and then monitor the health and survival of nematodes over time.

### 1.2. Lifecycle

**Figure 1: Lifecycle of *C. elegans* hermaphrodite at 22°C.** *C. elegans* progresses from embryo to adult passing through 4 different larval stages (L1-L4). In blue along the arrows is represented the length of time the worm spends at a determined stage. The length of the animal (µm) is shown in parentheses. If population density is high or if food source is sparse, the L1 larvae can enter the alternative dauer stage (shown in red), until the conditions improve. Image from WormAtlas\(^{17}\).
Figure 1 illustrates *C. elegans* lifecycle from embryo through the four larval stages (L1-L4) to an adult worm, capable of egg laying. *C. elegans* has a relatively short life cycle, since the worm can complete its development from a fertilized egg into fertile adult in 3 days at 20°C. The temperature can influence the worm lifespan, with worms grown at 25°C having a much faster development than worms at 15°C. When feeding on *E. coli* OP50, *C. elegans* has a lifespan of approximately two weeks at room temperature.

A population of *C. elegans* is composed by both existing sexes: hermaphrodites (XX) and males (XO). However, males arise in a much smaller proportion (0.1%). The hermaphrodite has 959 somatic cells and is capable of self-fertilization, since it produces both sperm and oocytes. The male, which is relatively smaller compared to the hermaphrodite, has 1031 somatic cells and possess genitalia that make him capable to mate. Regardless of the origin of the sperm that fertilizes the oocyte, the embryo begins developing inside the hermaphrodite uterus. When the embryo comprises 28-30 cells, it is laid through the vulva opening; therefore the rest of the embryogenesis, which takes approximately 13 hours more, is completed outside the hermaphrodite. After hatching, the L1 larva starts to feed and develop through the four larval stages, until reaching adulthood. Mature hermaphrodites are capable of laying eggs during the course of 3-5 days. Throughout its reproductive period it can lay around 300 eggs.

1.2.1. **Dauer stage**

During times in which the conditions are unfavorable for growth like for example when the food is limited, in presence of high temperatures, or if there is overcrowding on the plates, the L1 and L2 larvae can enter the dauer stage as an alternative to continuing the development. Dauer animals are characterized by having large amounts of fat, a thin body and they do not age, which allows them to survive adverse conditions for several months. When the conditions are again favorable for growth, the worms return their normal life cycle.

As dauer larvae do not feed, they must use the lipid stores that were synthetized during the first larval stage in order to get energy. Unlike mammals, they possess the necessary enzymes for the glyoxylate pathway. Therefore, they can use the acetyl-CoA produced by β-oxidation of fatty acids as a substrate for gluconeogenesis to produce carbohydrates and amino acids.

The lipid metabolism during dauer has been reported as an important factor for survival through that phase. It has been shown that impaired signaling LKB1/AMPK affects the hydrolysis of stored fat, which in turn affects the lipid metabolism during periods of energetic stress and influence lifespan.
1.3. Immunity in *C. elegans*

The ability to recognize and defend itself from a pathogenic attack is essential to all organisms. Therefore, the presence of an efficient immune system is vital to survival. *C. elegans* feeds on microbes including bacteria and fungi; therefore the worm is constantly exposed to a large array of bacterial pathogens and has developed an innate immune system for defense. The fact that the worm lacks an adaptive immune system makes the innate immunity of the worm important since it is the only response that *C. elegans* possess against pathogen attack.

*C. elegans* was first used as a model system for study of bacterial pathogenesis in 1999, when the Ausubel lab demonstrated that *Pseudomonas aeruginosa* strain PA14 kills the worm, and that the bacteria used conserved virulence factors to do so. In this manner they were the first group establishing the worm as a viable model system for microbial studies. Since then, *C. elegans* has been widely used as a model in the study of host innate immunity and to identify bacterial virulence factors.

As a first line of defense against pathogens, *C. elegans* possess mechanisms such as behavioral avoidance and physical barriers (reviewed by Schulenburg et al., 2004). *C. elegans* is capable of distinguishing between different bacteria and of avoiding noxious substances. The nematode exhibits two types of behavioral responses when confronted with potential pathogens: pathogen evasion and reduced food ingestion. The physical barriers that *C. elegans* possesses include a hard extracellular cuticle secreted from the hypodermis, which covers the outer layer and the pharynx of the worm, and is impermeable to pathogens. At the end of the pharynx, *C. elegans* has a grinder that physically disrupts bacteria, preventing intact bacteria from reaching the intestinal lumen of the worm. When these barriers fail, *C. elegans* uses its inducible innate immune response utilizing conserved signaling pathways, which will be further discussed in chapter 1.4. Furthermore, it is capable of producing antimicrobial molecules.

Among these immune effectors are a variety of antimicrobial peptides (AMPs) and proteins (reviewed by Ewbank et al., 2011). These include caenopores, lysozymes, lectins, ABF peptides, caenacins and neuropeptide-like peptides (NLPs).

Caenopores comprise a class of saposin-like proteins in *C. elegans*, that contain the saposin domain, similar to the mammalian peptides NK-lysin and granulysin, and to the protozoan amoebapores. More than a decade ago, *spp-1* and *spp-5* were the first caenopores found to have a bactericidal function. It was later found that *spp*-gene expression can be modulated by the presence of different bacteria, and that *spp-5* is constitutively expressed in the intestine, being important for the worm to cope with its regular food source, *E. coli*. Caenopores, encoded by *spp-5*, kill bacteria by permeabilizing their cytoplasmic membrane and display pore-forming activity. *Spp-1* also demonstrated to have a role on the infection of the nematode with the pathogens *Salmonella*...
typhimurium\textsuperscript{30} and Pseudomonas aeruginosa.\textsuperscript{31} Recently, \textit{sbp-12}, exclusively expressed on the pharynx of the worm, was found to have a protective role against infection with \textit{Bacillus thuringiensis}, but intriguingly, \textit{sbp-1} mutants challenged with the same pathogen had extended lifespan when compared to wild-type.\textsuperscript{32} One explanation for this event is that when an antimicrobial effector is knocked down, other immune effectors will be overexpressed; however this is not completely elucidated.\textsuperscript{32}

Lysozymes are found in many species and they are known lytic enzymes that are primarily involved in the disruption of the peptidoglycans present in the cell wall of bacteria. In \textit{C. elegans}, these are localized in the intestine, where the site of bacterial infection is more frequently taking place.\textsuperscript{33} The \textit{C. elegans} genome encodes ten lysozyme genes of the protist-type (\textit{lys-1} to \textit{lys-10}) and five invertebrate-type lysosomes (\textit{ilys-1} to \textit{ilys-5}).\textsuperscript{34} Overexpression of \textit{lys-1} caused enhanced resistance upon infection with \textit{Serratia marcescens}.\textsuperscript{35} \textit{lys-7} was found to be up regulated upon infection with \textit{M. nematophilum}\textsuperscript{36}, \textit{S. marcescens}\textsuperscript{35}, and \textit{Salmonella Typhimurium}\textsuperscript{31}, and knockdown of this gene caused enhanced susceptibility towards infection with \textit{E. coli LF82},\textsuperscript{37} \textit{P. aeruginosa},\textsuperscript{31} and \textit{Cryptococcus neoformans},\textsuperscript{38} suggesting a protective role for \textit{lys-7} against these pathogens.

C-type lectins have been proposed to act as pathogen-recognition molecules, and they function in the intestine of \textit{C. elegans} as specific pathogen recognition molecules that differ on their expression upon different pathogen infections.\textsuperscript{36} C-type lectins have a calcium-dependent carbohydrate binding C-type lectin domain (CTLD), are mainly sugar binding proteins and \textit{C. elegans} possess a vast array of these, including more than two hundred proteins.\textsuperscript{39} The expression of these lectins is up-regulated by several pathogens such as \textit{M. nematophilum},\textsuperscript{36} \textit{S. marcescens}, \textit{E. faecalis} and \textit{P. luminescens},\textsuperscript{40} \textit{P. aeruginosa} and \textit{S. aureus},\textsuperscript{41} among many others (reviewed by Schulenburg \textit{et al}., 2008).\textsuperscript{42} Galexclins comprise another class of lectins, they are specifically β-galactoside-binding proteins, and the \textit{C. elegans} genome contains about 26 predicted genes encoding them.\textsuperscript{43} The function of many of these galelectins is still unknown and RNAi knockdown or deletion mutants of these genes did not reveal obvious abnormalities.\textsuperscript{44, 45} Using fluorescently labeled recombinant galexclins it was possible to identify to which proteins they bound and where they are located in diverse tissues, including the intestine, pharynx, and the rectal valve.\textsuperscript{45} Until now, only LEC-8 was found to be relevant for the host defense against infection with \textit{B. thuringiensis}. LEC-8 prevented the crystal toxin Cry5B of \textit{B. thuringiensis} from binding to the glycolipids receptors on the surface of the worm, acting as a competitor for these.\textsuperscript{46} Mallo \textit{et al}. have previously reported up-regulation of \textit{lec-8} in \textit{C. elegans} infected with \textit{Serratia marcescens},\textsuperscript{33} strengthening the hypothesis that this may be a common defense
mechanism against this kind of bacterial infection. Recently, the importance of lec-1 to C. elegans response against oxidative stress was pointed out, as deletion mutants demonstrated to be more sensitive towards H$_2$O$_2$ and paraquat, which are both oxidative stress-inducing agents. This lectin is mainly localized in the pharynx of the nematode, so it would be interesting to find out if it can also have an important role for C. elegans defense against pathogens, especially those who invade the worm through that route.

ABF peptides, or antibacterial factors, are antimicrobial peptides found in C. elegans. ABF-2 is mainly produced in the pharynx and was the first one described to have antimicrobial activity. Furthermore, it is thought to contribute to the digestion of ingested bacteria. To this date, six kinds of ABF were found in C. elegans (ABF-1 to ABF-6). From this set of genes, abf-1 and abf-2 are the best characterized, and they were both found up-regulated upon infection with the fungus Cryptococcus neoformans. Expression of abf-1 was induced upon infection with the natural fungal pathogens Drechmeria coniospora and Harposporium sp. whereas abf-2 is up-regulated by Candida albicans. It is still not clear what factors influence their differential expression, but the different modes of pathogen infection and the distinct spectra of antimicrobial activities that abf encompasses are the speculated reasons (reviewed by Pujol et al., 2012).

Another set of AMP genes found in C. elegans are the members of the nlp (neuropeptide-like protein) and cnc (caenacin) families. These were found up regulated after infection with the fungus D. coniospora. These genes are mainly expressed in the epidermis, which can explain why they are overexpressed upon D. coniospora infection, since this fungus forms spores which attach to the worm cuticle, that later germinate into C. elegans epidermis (reviewed by Pujol et al., 2012). Two caenacin genes, cnc-4 and cnc-7, were also found up regulated in C. elegans infected with the yeast C. albicans and this response is most likely under the control of the p38 MAPK pathway. More in depth reviews about these antimicrobial peptides written by Bogaerts et al. and DEK Tarr constitute interesting readings for those who are further interested in this subject.

On top of these defense molecules, C. elegans also encompasses six different coelomocytes. They seem to be responsible for the uptake and absorption of some materials such as India ink, rhodamine-dextran, GFP and others, thus a role similar to macrophages has been suggested. In opposition to similar cell in other organisms such as Drosophila, these cells are not specific and do not seem able to phagocyte bacteria. Moreover, they are not able to migrate so their displacement relies only in movements of the worm and the body cavity fluid for accessing foreign material.
Figure 2: C. elegans pathogens and their route of infection. The majority of known pathogens of C. elegans establish an infection in the intestinal lumen, through the accumulation of bacteria. Other pathogens such as P. aeruginosa and Burkholderia pseudomallei produce toxins that can kill the nematode. The fungus D. coniospora and the bacteria M. nematophilum adhere to the cuticle of the worm and cause infection via the epidermis. Nematocida parisii invades C. elegans intestinal cells, but contrasting with other pathogens, it is found intracellularly. Figure adapted from 59

In its natural environment, C. elegans encounters many pathogens and it is capable of mounting an effective immune response in order to resist infection. Four natural pathogens have been explored in more detail: Drechmeria coniospora, Nematocida parisii, Microbacterium nematophilum, and most recently a virus.

Drechmeria coniospora forms spores that can attach to the head of the nematode and extend hyphae into the body of the worm. 60 This induces an immune response in C. elegans, which includes the induction of NLPs and caenacins. 52

Nematocida parisii, establishes an intracellular infection within the intestinal epithelium of the nematode, which later results in its death. This microsporidial pathogen seems to be a common parasite of C. elegans in its natural environment. 61

Microbacterium nematophilum, a Gram-negative bacterium, adheres to the post anal cuticle and colonizes the rectal opening of C. elegans, leading to a chronic, non-lethal infection. This infection results in swelling of the post-anal region and the Dar phenotype (deformed anal region), which seemed like a simple morphologic mutation when it was first discovered. 62

Interestingly, naturally-occurring viruses infecting the worm were only found very recently. 63 Until then, the many attempts to study virus in C. elegans had failed. 64 A recent study found viruses that infected the nematode. Through sequencing, these identified viruses infecting the worm, showed 40% similarity with Nodaviridae virus family and electron microscopy revealed that the intestine is the most affected organ, suggesting that the virus may spread through the oral/fecal route. 63

Pseudomonas aeruginosa PA14 was the first human pathogen used to infect C. elegans, establishing the nematode as a host model, as it demonstrated to effectively kill the worm. 25
P. aeruginosa infection leads to death of the worm within 2–3 days, and several bacterial virulence factors used by the bacteria, are also required for pathogenesis in mammalian hosts. Depending on the experimental conditions, the PA14 strain can kill the nematode in a period of days (“slow killing”) or hours (“fast killing”). The fast killing is mediated by diffusible toxins, such as phenazines, which generate ROS. For another strain of P. aeruginosa, specifically the PAO1 strain, a third killing mechanism was identified which consists of a rapid and lethal paralysis mediated by a toxin, later identified as being cyanide.

After that, C. elegans was already used as a model host for a variety of pathogens, ranging from Gram-negative bacteria such as Burkholderia, Pseudomonas, Salmonella, Serratia, and Yersinia; Gram-positive bacteria such as Enterococcus, Staphylococcus, and Streptococcus; and the fungus Cryptococcus neoformans (reviewed by Zhang et al., 2013 and in Wormbook, 2005).

Unfortunately, the nematode has a limitation when it comes to study microbial virulence factors. As many pathogens grow optimally at 37ºC or produce virulent factors at this temperature, and as this temperature is lethal to C. elegans over extended periods of time, assays are usually carried out at lower temperatures, which can eliminate the identification of some bacterial factors.

As the majority of pathogens affect the intestine of the worm, due to the fact that C. elegans eats bacteria and microbes, a closer look into C. elegans intestine anatomy and characteristics is given below.

Figure 3: The intestine of C. elegans is a large organ that carries out multiple functions. It is positioned on the left side of the body anteriorly to the vulva and on the right side of the body posterior to it, ventral view. Image adapted from

The intestine of C. elegans consists of 20 intestinal epithelial cells (IECs) that are not renewable in opposition to the mammalian IECs. However, both have a similar morphology, which makes the nematode a good model to study the interactions of microbial pathogens with epithelial cells. Among other features that make C. elegans a good model to investigate those interactions are the transparency of the worm and its intestine, which allows seeing the colonization of the intestinal
cells as well as making possible to follow the translocation of fluorescent labeled proteins over the course of infection. Moreover, the intestine makes about one third of the total somatic mass of the worm,\textsuperscript{72} and both extracellular and intracellular infections of the intestinal tract have been described.\textsuperscript{73, 74}

The relatively benign food source \textit{E. coli} OP50 is rarely found intact on the intestinal lumen of \textit{C. elegans}, because very few bacteria resist the mechanical disruption caused by the grinder during feeding. For those who make it through, defecation ends up expelling them, functioning as a protective mechanism for the worm. Feeding \textit{C. elegans} with GFP-labeled \textit{E. coli} and GFP-labeled \textit{S. aureus} (Figure 4) shows that when feeding on pathogenic bacteria, the digestive tract becomes distended.

\textbf{Figure 4: Bacterial species colonize the \textit{C. elegans} intestinal tract.} (A) \textit{C. elegans} feeding on the common food source \textit{E. coli} does not accumulate intact bacteria and the intestinal lumen remains narrow. (B) \textit{C. elegans} feeding on \textit{S. aureus}, a pathogenic Gram-positive coccus, accumulate a large number of bacteria on the intestinal track, causing distension of the lumen. Pictures adapted from\textsuperscript{75}

\textit{C. elegans} has also been used as a model for Enteropathogenic \textit{E. coli} (EPEC) infection.\textsuperscript{76} In the nematode, killing from EPEC infection happens due to colonization and accumulation of bacteria on the intestinal lumen, over several days.\textsuperscript{76} Similar to \textit{P. aeruginosa}, EPEC also exhibits a “fast killing” mediated by secreted toxins, when grown in a rich medium supplemented with tryptophan.\textsuperscript{77}

1.4. Defense against pathogens – signaling

\textit{Caenorhabditis elegans} and other nematodes have reduced genomes with a simple immune system; still the roundworm can mount an inducible response to various bacterial and fungal pathogens. Although \textit{C. elegans} has one gene encoding a toll-like receptor (TLR) that plays a role in defense against some bacteria,\textsuperscript{78} it lacks Myd88, NF-\textit{kB} and several other components of the
canonical Toll pathway. It also lacks immune cells, such as macrophages and neutrophils. However, *C. elegans* detects bacterial infection and elicits pathogen-specific host defense responses, which makes the nematode a good model to study the innate immunity and host-pathogen interactions (reviewed by Irazoki et al., 2010).⁷⁰

There are some pathways involved in the innate response of *C. elegans* which are already elucidated, at least partially, that are going to be briefly discussed below.

1.4.1. **Toll-like receptors**

In mammalians, the discrimination between distinct pathogens in the defense against infection is thought to occur primarily by differential ligation of Pattern Recognition Receptors (PPRs), such as Toll-like Receptor (TLR), that activate downstream kinase cascades that control transcription factor like NF-κB and AP-1 family transcription factors. TLR pathways are highly conserved between species, indicating that they arose early during evolution.⁷⁹ Despite this, they are not required for host defense in all multicellular animals, suggesting that they might be involved in other undefined pathways.

Analysis of the *C. elegans* genome identified only one gene encoding a TLR (*tol-1*) and other genes encoding components related to the insect and mammalian TLR pathways, such as *trf-1* (related to TRAF1 [TNFR-associated factor 1]), *pik-1* (related to Pelle and IL-1R-associated kinases [IRAks]) and *ikb-1* (related to inhibitor of NF-κB [IkB]).⁷⁸

However, in *C. elegans* TOL-1 plays a limited role in host defense against infection, in contrast to mammalian systems.⁸⁰ TOL-1 has been proposed to be important in the recognition of a specific bacterial component, resulting in a change of behavior of *C. elegans*, making the worm able to avoid potentially harmful bacteria.⁷⁸ It has been also shown that a loss of function in *tol-1* caused enhanced susceptibility to infection by *Salmonella enterica*, suggesting that this Toll-like receptor is important for *C. elegans* innate immunity; however, studies infesting the nematode carrying the same mutation with other pathogens did not exhibited the same results.⁸⁰ This implies that TOL-1 is important somehow, but not a central component to the innate immune response of the worm, as it is in flies and mammals.

In addition to TOL-1, *C. elegans* possess another protein with a Toll/IL-1R (TIR) domain which is the TIR-1 protein. Toll and the vertebrate TLRs consist of an extracellular domain containing multiple leucine-rich repeats, involved in ligand binding, and an intracellular domain involved in signaling. TIR domains are evolutionary ancient protein modules found in defense-related proteins in plants and animals.⁸¹ Mutation or knockdown of *tir-1* results in increased susceptibility to several pathogens like the fungus *D. coniospora* and the bacterial species *E. faecalis* and *P. aeruginosa*.⁸¹,⁸²
Figure 5: Toll-like receptors in *C. elegans*. A single TLR homolog has been identified in *C. elegans*, TOL-1, which is involved in behavioral avoidance of some pathogens, but does not seem to play a role in resistance against others. In addition, the worm possesses the scaffold protein TIR-1 (that contains the Toll/ILR-1 domain), which acts independently from *tol-1* and that acts upstream of NSY-1 in the p38 MAPK cascade. The receptor that acts upstream TIR-1 remains unknown. Image from 70

1.4.2. **p38 mitogen-activated protein kinase pathways**

The p38 mitogen-activated protein kinase (MAPK) cascade is an important signaling pathway that acts downstream of TLRs in insects and mammals. It is one of the most ancient and evolutionarily conserved signaling pathways, and is essential for many processes in immune responses.83

In the nematode, this pathway involves the kinases NSY-1, SEK-1 and the p38 MAPK called PMK-1 (Figure 5). The p38 MAPK pathway was found to induce the expression of immunity related genes including C-type lectins, lysosymes and antimicrobial peptides.84

*C. elegans* infected with *Pseudomonas aeruginosa* was used as a host model to study the mechanisms of innate immune responses. A genetic screen for mutants with enhanced susceptibility to *P. aeruginosa* infection identified the NSY-1/SEK-1/PMK-1 cassette as being an evolutionary module required for defense against this pathogen.85 RNAi against the mammalian p38 homolog PMK-1 caused less resistance to infection and biochemical analysis shown that PMK-1 acts as the downstream kinase required for pathogen defense.85
TIR-1 (already mentioned above) functions upstream of the cascade. The upstream regulation of the PMK-1 cassette in *C. elegans* differs, whether the tissue is the intestine or the epidermis (Figure 6). In the intestine, the protein kinase Cδ (PKCδ) TPA-1 activates the protein kinase D (PKD) DKF-2, which accumulates mainly in the intestine, upstream of the PMK-1 cassette. The mechanism of action of these upstream components is still not clear, but it is suggested that phosphorylation events are involved. The upstream signals that control TPA-1 activity also remain unknown, diacylglycerol (DAG) being a likely candidate. In the epidermis, wounding triggers a p38 MAPK/PMK-1 cascade that activates transcription of antimicrobial peptide genes. During wounding, DAPK-1 (death-associated protein kinase 1) functions as an upstream negative regulator of the PMK-1 cassette, but the exact point of input is unknown. The upstream components regulating this cassette also differ between wounding and infection.

1.4.3. **Transforming-growth factor β (TGF-β)**

The TGF-β pathway is conserved throughout the Metazoa kingdom and it is important for diverse physiological processes in a wide variety of animals. DBL-1, the *C. elegans* TGF-β homolog, binds to the heterodimeric receptor DAF-4/SMA-6 and acts through the SMA-2/SMA-3/SMA-4 SMAD complex to control gene expression. During *D. coniospora* infection genes producing caenacins (a family of peptides similar to the neuropeptide like proteins) are activated. Infection of *C. elegans* by *S. marcescens* unveiled some genes activated by the DBL-1/TGF-β pathway including genes encoding lectins and lysozymes, and also demonstrated that *dbl-1* mutants had increased susceptibility to infection.

1.4.4. **DAF-2–DAF-16 Insulin-Like Signaling**

The DAF-2/insulin-like receptor (ILR) pathway is also important for the immune response in *C. elegans*. This pathway is known to regulate lifespan, dauer formation and stress resistance, but it is also involved in resistance to pathogens. The *daf-2* and *age-1* mutants are more resistance to a variety of bacterial pathogens in *C. elegans*. These mutants have been extensively studied, since more than 20 years ago scientists discovered that silencing these genes resulted in worms that live twice as long as wild-type worms. *daf-2* encodes a receptor tyrosine kinase, that is the only insulin/IGF-1
receptor expressed in worms, and *age-1* encodes an ortholog of the catalytic subunit of the downstream phosphoinositide 3-kinase (PI3K).

The long lived phenotype of *daf-2* mutants is dependent of DAF-16, a downstream forkhead transcription factor. DAF-2 negatively regulates DAF-16, through phosphorilation of the phosphoinositide 3-kinase AGE-1, and subsequent activation of four threonine kinases AKT-1, AKT-2, PDK-1 and SGK-1. The AKT-1/AKT-2/SGK-1 complex phosphorylates DAF-16 sequestering it in the cytoplasm, thus preventing DAF-16 from modulating the transcription of its target genes in the nucleus (reviewed by Landis *et al.*, 2010). These kinases appear to have distinct roles in *C. elegans*, and they regulate lifespan and pathogen resistance differentially. While *sgk-1* and *pdk-1* mutants shown little difference in resistance to pathogens when compared to wild-type, *akt-1* and *akt-2* mutants display enhanced resistance, suggesting that these processes are controled by a different subset of serine threonine kinases downstream of AGE-1.

Upon nuclear translocation, DAF-16 regulates the expression of a large number of genes. These target genes mainly fall into two categories, being the first comprised by stress-responsive and detoxifying genes, including genes that encode enzymes responsible for clearance of ROS such as superoxide dismutase (*sod-3*), catalases (*ctl-1* and *ctl-2*), glutathione-S-transferase (*gst-4*) and heat shock proteins, and the second is mainly comprised by genes encoding antimicrobial effector molecules such as lysozymes (*lys-7* and *lys-8*), saposin-like genes (*spp-1*), antimicrobial peptides (*nlp-31, abf-2*) and the pathogenesis-related proteins called thaumatins (*thn-2*).

In *daf-2* mutants, where DAF-16 is constitutively active, or in mutant strains where DAF-16 is overexpressed, there is enhanced pathogen resistance to different pathogens such as enteropathogenic *E. coli, E. faecalis, S. aureus, P. aeruginosa, S. enterica, B. thuringiensis*, among others.

### 1.4.5. β-catecin

A study using a reversed genetic approach showed that *bar-1*, a component of the *C. elegans* canonical WNT signaling pathway, is important to induce genes in response to *S. aureus* infection. In the canonical pathway, Wnt binding leads to stabilization of the transcription factor β-catecin which enters the nucleus to regulate its target genes. The *C. elegans* genome encodes three different β-catecin proteins (*bar-1, wrm-1* and *hmp-2*).

In humans, the β-catecin signaling pathway targets defensins, antimicrobial peptides produced in the intestinal epithelium. Defensins are important elements of the innate immune system of plants and animals. They are a class of peptides that have a broad-spectrum antimicrobial activity against bacteria, fungi and viruses. Interestingly, increased susceptibility to Crohn’s disease has been associated with a lack of β-defensin induction.
Figure 6: Signaling pathways involved in the immune response on C. elegans. The TGF-β pathway is present on the epidermis (shown on the left). The PMK-1/p38 MAPK pathway functions in both epidermis and intestine, but the upstream regulation of the PMK-1 cassette differs according to the site of action (middle of the picture). The DAF-2/insulin like pathway is present on the intestine of the worm (shown on the right). Image from 70

1.4.6. Unfolded Protein Response (UPR)

The unfolded protein response is a signaling pathway activated by the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER).

Genes involved in this pathway are required for S. enterica infection where they are regulated by the apoptotic regulator CED-1.108 CED-1 is required for engulfment of apoptotic corpses downstream of the programmed cell death pathway.109 C. elegans ced-1 mutants are more susceptible to Salmonella enterica and Candida-mediated killing.50, 108 Microarray analysis of ced-1 mutants identified a cluster of pgn/apu UPR genes that were significantly down-regulated. RNAi mediated knockdown of these genes showed that these animals were sensitive to S. enterica infection. In the same study, it was demonstrated that overexpression of these genes protected C. elegans from
pharyngeal destruction and invasion, suggesting a mechanism involving UPR that protects the animal against bacterial infections.\textsuperscript{108}

XBP-1-mediated UPR was also pointed as having an essential role during \textit{C. elegans} larval development by protecting against the activation of innate immunity. Activation of the p38 MAPK kinase PMK-1-mediated response to infection with \textit{Pseudomonas aeruginosa}, inducing the XBP-1-dependent unfolded protein response, in order to handle the accumulation of unfolded proteins in the ER.\textsuperscript{110} In addition, exposure of \textit{C. elegans} to the pore-forming-toxin Cry5B on an RNAi screen revealed that the p38 MAP kinase PMK-1 and c-Jun N-Terminal Kinase (JNK) form a signaling network which regulates the UPR response following exposure to the toxin.\textsuperscript{111}

Although the described pathways have been studied in \textit{C. elegans}, there is still a lot to learn about innate immunity in the nematode, since the molecular mechanisms on how the defense is mounted and how the interaction with the pathogens occurs, are not yet elucidated. Also, the interactions of the mentioned pathways with other pathways remain still to be discovered. Thus, in the future it should be interesting to study how the signaling pathways work in the context of the whole organism, how the worm distinguishes the different pathogens and how a pathogen-tailored response is mounted.

1.5. Lipid metabolism

\textit{C. elegans} is an important model for the study of fat metabolism, because the worm possesses the entire collection of fatty acid desaturases activities found both in plants (Δ12 and Δ3 desaturases) and animals (Δ5, Δ6 and Δ9 desaturases).\textsuperscript{112} On the contrary, mammals lack Δ3 and Δ12 desaturases, which prevents them from producing polyunsaturated fatty acids endogenously, creating the necessity for their intake through the diet.\textsuperscript{113} These essential PUFAs include linoleic acid (LA) and α-linolenic acid (ALA) and the conditionally essential PUFAs docosahexanoic acid (DHA), eicosapentanoic acid (EPA) and arachidonic acid (AA) all of which are very important for normal nerve activity. The conditionally essential PUFAs, also called Long-chain polyunsaturated fatty acids (LC-PUFAS) can be converted from their precursors LA and ALA by human metabolism, but since the conversion rate is low, it is also advisable their consumption through diet.\textsuperscript{114,115}
Figure 7: Fatty acid desaturation and elongation in *C. elegans*. Depicted in orange - fatty acids: PA, Palmitic acid; SA, Stearic acid; PLA, Palmitoleic acid; VA, Vaccenic acid; OA, Oleic acid; LA, Linoleic acid; ALA, Alpha-linolenic acid; GLA, Gamma-linolenic acid; DGLA, Di-homo-gamma-linolenic acid; SDA, Stearidonic acid; AA, Arachidonic acid; ETA, Eicosatetraenoic acid; EPA, Eicosapentaenoic acid. Depicted in blue and yellow boxes - gene activities: FAT-5, FAT-6 and FAT-7, Δ9 desaturases; FAT-2, Δ12 desaturase; FAT-1, omega-3 desaturase; FAT-3, Δ6 desaturase; FAT-4, Δ5 desaturase; ELO, fatty acid elongase; LET-767, 3-ketoacyl-CoA reductase. Image adapted from 116

*Figure 7: Fatty acid desaturation and elongation in *C. elegans*. Depicted in orange - fatty acids: PA, Palmitic acid; SA, Stearic acid; PLA, Palmitoleic acid; VA, Vaccenic acid; OA, Oleic acid; LA, Linoleic acid; ALA, Alpha-linolenic acid; GLA, Gamma-linolenic acid; DGLA, Di-homo-gamma-linolenic acid; SDA, Stearidonic acid; AA, Arachidonic acid; ETA, Eicosatetraenoic acid; EPA, Eicosapentaenoic acid. Depicted in blue and yellow boxes - gene activities: FAT-5, FAT-6 and FAT-7, Δ9 desaturases; FAT-2, Δ12 desaturase; FAT-1, omega-3 desaturase; FAT-3, Δ6 desaturase; FAT-4, Δ5 desaturase; ELO, fatty acid elongase; LET-767, 3-ketoacyl-CoA reductase. Image adapted from 116*

*C. elegans* obtains fatty acids through its bacterial diet and also synthesizes *de novo* fatty acids from acetyl-CoA. The first step for the *de novo* synthesis consists of carboxylation of acetyl-CoA to malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACC). Afterwards, fatty acid synthase (FAS) synthetizes palmitic acid (PA) through a series of reactions catalyzing the conversion of acetyl-CoA and malonyl-CoA to palmitate (16:0). This series of reactions consist of incorporating a two carbon unit (derived from malonyl-CoA) each time, into the growing fatty acid chain at the carboxyl end. After, the Δ9-desaturase FAT-5 can act on PA to produce the MUFA 16:1 Δ9 (palmitoleic acid), or PA can further be elongated by elo-1 to produce 18:0 (stearic acid). Palmitoleic acid is elongated to 18:1Δ11 (vaccenic acid), whereas stearic acid is desaturated to 18:1Δ9 (oleic acid) by FAT-6 or FAT-7. Oleic acid is then the substrate for the Δ12 desaturase FAT-2, which yields 18:2ω6 (linoleic acid). Linoleic acid can be converted to 18:3 fatty acids, by action of FAT-1 or FAT-3. FAT-3 acts on linoleic acid introducing a Δ6 desaturation, which is required for later elongation of 18-carbon PUFAs to 20-carbon PUFAs by ELO-1 and ELO-2 (Figure 7).
C. elegans has three Δ9-desaturases originated from the genes fat-5 which encodes a palmitoyl-CoA desaturase, and the genes fat-6 and fat-7 which in turn encode stearoyl-CoA desaturases (SCDs). Inhibition of any of these genes is associated with reduced fat levels. Interestingly, while knockout of one of these desaturases alone shows only slight phenotypical differences, the triple knockout is embryonically lethal.

fat-6 and fat-7 were also found up-regulated in daf-2 mutants, and RNAi against these genes shortened the lifespan of the long-lived daf-2 mutants, suggesting that the synthesis of MUFAS and PUFAS is important for lifespan of the nematode.

The Δ9-desaturases are responsible for formation of monounsaturated fatty acids (MUFAs) thus their activity is responsible for maintaining the appropriate ratio between MUFAs and saturated fatty acids.

1.5.1. Monomethyl branched-chain fatty acids

Monomethyl branched-chain fatty acids (mmBCFA) are a particular class of fatty acids, present in a wide variety of organisms, which are associated with disease states in humans, but still little is known about their synthesis or function in eukaryotes.

In the nematode, the mmBCFAs C15iso and C17iso are exclusively derived from de novo synthesis, since these fatty acids are not present in the E. coli diet and cannot be produced from modification of bacterial fatty acids.

1.5.2. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are long chain fatty acids with two or more double bonds on the chain. Depending on the position of the first double bond appearing closest to the methyl end group on the fatty acid chain, PUFAS can either be classified as omega-3 (ω-3) or omega-6 (ω-6). In mammals, these fatty acids derive from α-linolenic acid (ω-3 PUFAS) or from linoleic acid (ω-6 PUFAS), which cannot be synthetized de novo in the mammalian cells, therefore they are classified as essential fatty acids, since they have to be acquired through diet. They cannot be interconverted, so both are essential nutrients. On the other hand, as C. elegans possess Δ3 and Δ12 desaturases, as mentioned before, the worm is capable of generate a variety of PUFAS.

A study revealed that upon fasting, C. elegans increased their relative levels of C20 PUFAS. This fact is attributed to the enhanced expression of fatty acid desaturases that occur in fasted worms; however the reason for this response is still not clear.
It was also shown that PUFAS are important in *C. elegans* reproduction as they act as signaling precursors controlling sperm recruitment. Abnormalities in their synthesis cause nonautonomous sperm mobility defects, such as defects in sperm direction and velocity. Subsequent studies revealed that the insulin/IGF signaling pathways promote the conversion of oocyte polyunsaturated fatty acids into prostaglandins that attract the sperm to the fertilization site.

Synthesis of PUFAS in *C. elegans* is catalyzed by elongase and desaturase enzymes, which in the nematode are encoded by *elo* and *fat* genes, respectively.

The first step in the PUFA synthesis is catalyzed by the Δ12 desaturase FAT-2. This consists on the desaturation of oleic acid (18:1 cis-9) to produce linoleic acid (18:2 cis,cis-9,12). *fat-2* mutants accumulate oleic acid and lack most of the PUFAS, and display severe developmental, movement and reproductive defects. Interestingly, when expressed in yeast, FAT-2 desaturase from *C. elegans* had not only Δ12 desaturase activity, but Δ15 as well.

FAT-3 is a Δ6 desaturase that acts on linoleic acid (18:2ω6) and alpha-linolenic acid (18:3ω3) and catalyzes the formation of gamma-linolenic acid (18:3ω6) and stearidonic acid (18:4 3 ω3), respectively. This step is required before elongation of 18-carbon PUFAs to 20-carbon PUFAs. Mutations in *fat-3* leads to worms which fail to produce C20 PUFAs and with neuromuscular defects, cuticle abnormalities, reduced fertility, and altered biological rhythms. Other PUFAS are formed by FAT-1 and FAT-4 which modify the 20-carbon PUFAs to generate eicosapentaenoic acid 20:5ω3.

FAT-1 is required *in vivo* for n-3 polyunsaturated fatty acid (PUFA) synthesis. Interesting studies arose using this desaturase from *C. elegans*. Rat cortical neurons overexpressing FAT-1 desaturase were able to decrease in the ratio of n-6/n-3 PUFAs which in turn inhibit apoptotic cell death in neurons. Another study, in which the FAT-1 from *C. elegans* was expressed in a mouse model, the transgenic mice were able to convert omega-6 to omega-3 fatty acids. Later the FAT-1 gene was also used to generate transgenic pigs rich in omega-3 fatty acids. The authors produced an n-3 fatty acid-enriched pork without further diet supplementation, which they claim to be economically advantageous since, at the present, the only way to achieve that is by feeding the animals with flaxseed, fish or other marine products, which is not in the best interest of the food industry. However this finding came with big controversy.

*Elo-1* and *elo-2* are necessary for fatty acid elongation beyond C16:0, so their knockdown may be sufficient to block or reduce PUFA formation. On the other hand, *elo-5* and *elo-6* elongate MUFAS producing C15:0-iso and C17:0-iso. These fatty acids are exclusively produced by *de novo* synthesis and do not arise from ingested bacteria, in contrast to some fatty acids containing a cyclopropane ring.
Elo-1 mutants display increased levels of 18:3n6 and 18:4n3, but reduced levels of C20 PUFAs, however the mutation does not eliminate completely these species. When elo-2 was suppressed in elo-1 mutants, C20 PUFAs were eliminated, suggesting that both genes act together to elongate C18n6 and C18n3 PUFAs. RNAi against ELO-2 causes an accumulation of palmitate and a decrease in the PUFA fraction in triacylglycerides and phospholipid classes. RNAi against elo-5 is not expected to affect longevity of the worm, since ELO-5 extends only monomethyl branched-chain fatty acids involved in developmental signaling. However, wild-type eggs subjected to elo-5 (RNAi) have progeny that arrest at the L1 stage. When the parental animals were subjected to elo-5 RNAi at later larval stages, their progeny developed until adulthood but the worms became progressively sick. This arrest could be reversed when 17-carbon mmBCFA was added as supplement.

1.5.3. Sterols

C. elegans is a cholesterol auxotroph, which means that dietary supplementation of this sterol is required. Due to the small amount that the worm needs for viability, it is proposed that cholesterol functions as a precursor for sterol-derived hormones or signaling, rather than playing a structural role in membrane composition and fluidity.

1.5.4. Transcriptional Regulation of Lipid Metabolism

The accumulation, biosynthesis and metabolism in C. elegans are processes controlled by transcription factors which are also regulatory factors of mammalian lipid metabolism. This suggests that lipid metabolism is a highly conserved process in C. elegans and mammals.

1.5.4.1. Daf-16

As discussed previously, daf-16, a homolog of the mammalian forkhead transcription factor class O (FoxO), is also involved in lipid accumulation in C. elegans. This transcription factor is under the control of the daf-2/insulin like signaling pathway, a homolog of the mammalian insulin/IGF-1 (insulin growth factor 1) signal pathway. daf-2 insulin-like signaling mutants show increased fat stores when stained with Nile Red. This effect was suppressed by mutations in the forkhead transcription factor daf-16, showing that daf-16 is negatively regulated by daf-2 signaling. DAF-16 is also known to regulate Δ9 desaturase expression levels and fat-6 has been identified as a direct target of DAF-16.
1.5.4.2. C/EBP (CCAAT enhancer binding protein)

RNAi against a gene homologous to mammalian C/EBP transcription factor resulted in worms displaying a “lipid-depleted” phenotype, recognized as pale, skinny worms lacking fat stores. Knockdown of this gene, identified as lpd-2, also affected the expression of several lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, acyl-CoA synthetases, and glycerol 3-phosphate acyltransferase.\textsuperscript{139}

1.5.4.3. Sterol Regulatory Element-Binding Protein (SREBP)

Sbp-1 is the \textit{C. elegans} homologue for the mammalian SREBP. RNAi or mutations against this gene resulted in worms with low fat stores, high saturated fatty acid percentage, impaired growth and reduced expression of the lipogenic enzymes \textit{fat-5} and \textit{fat-7}.\textsuperscript{119, 140, 141}

1.5.4.4. NHR

Nuclear hormone receptors (NHRs) are DNA-binding proteins responsible for regulating the transcription levels on several processes such as metabolism, reproduction and homeostasis. The genome sequence of \textit{C. elegans} revealed the presence of 284 genes encoding NHRs, which is a far larger number than the genes found in the human genome (48 genes).\textsuperscript{142} It is believed that this amount of receptors arose due to gene duplication, but why the worm possess so many is not yet clear.\textsuperscript{143}

NHR-49 regulates 13 genes involved in fatty-acid metabolism in \textit{C. elegans}, including genes of the fatty acid beta-oxidation and desaturation pathways. Mutant worms for this nuclear hormone receptor, exhibited shortened lifespan and high fat content.\textsuperscript{144} Two other receptors, NHR-66 and NHR-80, were shown to directly bind and interact with NHR-49 and modulate separate pathways, namely sphingolipid processing and lipid remodeling genes, when it is partnered with NHR-66; and fatty-acid desaturation when it partners with NHR-80.\textsuperscript{145}

Well known targets of the nuclear receptor factor NHR-80 are also the Δ9 desaturases: \textit{fat-5}, \textit{fat-6} and \textit{fat-7}. Moreover, NHR-80 has been identified as having a necessary role for lifespan extension in germline-less mutants.\textsuperscript{146}

NHR-64 is also an important regulator of lipid homeostasis. RNAi against NHR-64 improved the growth and brood size on \textit{fat-6;fat-7} double mutants and on \textit{sbp-1} mutants, along with an increase in the fat stores.\textsuperscript{147}

Recently it was proposed that NHRs are implicated in the lifespan extension induced by dietary restriction (DR). It was found that \textit{nhr-62} is required for the lifespan extension, increased autophagy and reduction of triglyceride levels in \textit{C. elegans} on a DR regimen.\textsuperscript{148}
1.5.4.5. **MDT-15**

The Mediator Subunit MDT-15 was identified as an NHR-49 coactivator. Additionally, MDT-15 is necessary for expression of NHR-49 target genes fat-5 and fat-7 and it is required for maintenance of normal fat-6 mRNA levels. RNAi against *mdt-15* resulted in worms lacking MUFA and PUFA.\textsuperscript{149} Other study shows that MDT-15 regulates fatty acid desaturases along with *sbp-1*, and illustrates the importance of both in fatty acid homeostasis.\textsuperscript{140}

1.6. **C. elegans** and Crohn’s disease

Crohn’s disease (CD) was first described in 1932 and named after Burril B. Crohn, an American physician who made the first report relating 14 cases of the disease.\textsuperscript{150} Crohn’s disease is a chronic inflammatory bowel disease that may appear in any part of the gastrointestinal tract, but it is more frequently found on the terminal ileum and colon. It is categorized under a broader group of illnesses known as inflammatory bowel diseases (IBD).\textsuperscript{151} The pathogenesis of CD is complex and includes several factors such as genetic susceptibility factors, altered microflora and immune-mediated tissue injury. Major symptoms of the disease include abdominal pain, diarrhea, and weight loss. Nonetheless, making a definitive diagnosis of this disease can be difficult because the symptoms are overlapping and can be mistaken with other gastrointestinal disorders.\textsuperscript{152}

1.6.1. **Adherent-invasive E. coli strain LF82**

LF82 was first isolated from the ileal mucosa of a biopsy originating from a patient with Crohn’s disease.\textsuperscript{153} Several studies on the adherence properties of *E. coli* in Crohn’s disease revealed that *E. coli* strains are able to adhere to various human cells or cell lines. The invasive ability of *E. coli* strain LF82 isolated from a patient was characterized has a true invasive pathogen and put into a new potentially pathogenic category of invasive *E. coli*, which was designated AIEC for adherent-invasive *E. coli*.\textsuperscript{154}

LF82 invades efficiently intestinal epithelial cells\textsuperscript{155,155} (Figure 8). A study using primary ileal epithelial cells isolated from patients with CD and controls revealed that LF82 bacteria adhered preferentially to the brush border of the enterocytes isolated from diseased patients.\textsuperscript{156} This study also illustrates the importance of the type 1 pili present in the bacterial surface and its interaction with the adhesion molecules (CEACAM6) on the apical surface of ileal epithelial cells.
Patients with CD were found to have an increased expression in the ileal mucosa of the CEACAM6 molecule, which acts as a receptor for type 1 pilus bacterial adhesin. This fact leads to an abnormal colonization of the ileal mucosa, being that AEIC strains adhere to the brush border of ileal enterocytes isolated from CD patients but not to control patients without any inflammatory bowel disease.

Altogether, LF82 is considered an invasive pathogen due to the different cells lines to which has proven to efficiently adhere and penetrate. However, in *C. elegans*, LF82 proved to colonize and kill the worm by a distinct mechanism from those described before. It appears that the infection is restricted to the intestinal lumen of the worm, not causing any changes on the luminal surface of the intestine even 5 days after infection (unpublished data, acquired on a previous study done at the Lipid group). As the worm lacks CEACAM6-like structures, to which type 1 pili of LF82 adhere, colonization of the worms intestine has to rely on different mechanisms. To this point, only the Hfq RNA chaperone has been proven to be strictly important to the virulence of LF82 towards *C. elegans*.

1.7. Polyunsaturated fatty acids and health

The innate immune system acts as the first line of defense against invading pathogens. The downstream signaling pathways often lead to the production of anti-inflammatory compounds such as cytokines. A common response for a pathogen attack is therefore inflammation. Inflammation is considered a mechanism of innate immunity. Crohn's disease, discussed above in detail, is itself a chronic inflammatory bowel disease thought to result from impaired innate immunity.

A lot of research has been made around the role of fatty acids on inflammatory process, mainly focusing on the opposing roles that ω-3 and ω-6 polyunsaturated fatty acids may have.
Eicosanoids are important mediators and regulators of the inflammatory process and are derived from LC-PUFAs. Eicosanoids include prostaglandins, thromboxanes, leukotrienes, and other oxidized derivatives and they all share the same precursor, arachidonic acid, an omega-6 (ω-6) fatty acid. The amount of arachidonic acid in inflammatory cells may be influenced by dietary intake of its precursor, linoleic acid. Consequently, dietary n−6 PUFAs (linoleic or arachidonic acid) influence the inflammatory processes.160

Dietary intake of omega-3 (ω-3) PUFAs competes with omega-6 (ω-6) PUFAs because they displace arachidonic acid that is incorporated in cell membranes. Additionally, ω-3 PUFAs compete for the enzymes that convert ω-6 PUFAs onto eicosanoids. Consumption of the long-chain ω-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decreases the eicosanoid production. Acting as a substrate for the Cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes, it gives rise to a different form of eicosanoids which are less potent than the ones originated from arachidonic acid. Moreover, they originate a group of mediators called E- and D-resolvins which act as anti-inflammatory and inflammation-resolving species, respectively.161

Altogether, supplementation with long-chain ω-3 fatty acids has been described on a variety of studies as having benefits like anti-inflammatory agents, which may profit patients at risk for acute and chronic inflammation.162

The importance of dietary supplementation of n-3 polyunsaturated fatty acids on patients with inflammatory bowel diseases has been described. Studies conducted in Greenland, concluded that the Eskimo diet was richer in polyunsaturated fatty acids, namely fatty acids of the linolenic class (n-3), and that fact may be responsible for the lower prevalence of IBD among them.163, 164 Children suffering from ulcerative colitis, supplemented with EPA (eicosapentaenoic acid), show a decrease in production of eicosanoids such as leukotriene B4 (LTB4) and thromboxane A2 (TXA2), which are thought to play an important role in the pathophysiology of UC in the colonic mucosa.165 Remarkably, IBD biopsies show increased tissue synthesis of the leukotriene B4.166 Studies in mice show that IL-10 knockout mice (mutant mice that spontaneously develop a chronic inflammatory bowel disease) display reduced inflammation when fed with diets enriched with fish oil containing EPA and/or DHA.167

It is clear that n-3 PUFA alleviate the progression of IBD, however n-6 PUFA have been implicated in the origin of IBD. It is thus suggested that a decrease in n-6 PUFA intake and an increase in n-3 PUFA ingestion may be necessary to maintain IBD remission. A recent study suggests that in order to keep this remission, an n-3/n-6 ratio of 0.65 or higher should be kept.168

A recent study shows that n-3 polyunsaturated fatty acids can also affect lipid rafts composition.169 Membrane rafts are heterogeneous, small, highly dynamic domains rich in sterol and sphingolipids that compartmentalize cellular processes.170 This study relates the importance of n-3 PUFAs in prevention and treatment of colon cancer and the role that lipid rafts play in colonic...
tumorigenesis. Altogether, n-3 PUFAs increase the size of the lipid rafts, which impairs its functional activity, as well as it alters its composition, and also perturbs the lipid raft mediated cell signaling.\textsuperscript{169}

Some studies in \emph{C. elegans} also highlight the importance of dietary supplementation with certain polyunsaturated fatty acids.

In \emph{C. elegans}, the polyunsaturated fatty acids GLA and SDA, were shown to be required for the PMK-1/p38 pathway that influences resistance against \emph{P. aeruginosa}.\textsuperscript{171} A \textit{fat-3} mutant displays increased susceptibility to this pathogen infection, which can be explained by the decrease in the basal expression of immunity genes such as \textit{spp-1}, \textit{lys-7}, and \textit{lys-2}, and disrupted activity of the p38 MAP kinase. Dietary supplementation of the fatty acids GLA and SDA fully restored these defects, indicating that these lipids are required for a functional immune system in \emph{C. elegans}. Interestingly, supplementation with AA or EPA did not have the same effects.\textsuperscript{171}

A recent study shows that \(\omega\)-6 PUFAS are important in starved \emph{C. elegans}, since they are enriched upon starving; due to over expression of the lipase \textit{lipl-4}.\textsuperscript{172} Supplementation of these fatty acids (specially the \(\omega\)-6 PUFAS AA and DGLA) increased the nematode lifespan in well fed animals, through the activation of autophagy. Supplementation of human epithelial cells with these \(\omega\)-6 PUFAS also activated autophagy even when nutrients were available. Autophagy (discussed below) provides nutrients when external sources of energy are not available and mediates the turnover of damaged organelles, and long-lived proteins. Hence, this study indicates that \(\omega\)-6 PUFAS promote survival by activating autophagy in both conditions of food deprivation and food abundance.\textsuperscript{172}

\section*{1.8. Autophagy}

Autophagy is a pathway by which cytoplasmic constituents, including intracellular pathogens, are degraded, by means of their delivery to lysosomes.

The study of this cellular process was first possible using \emph{S. cerevisiae}, where genetic screens allowed the identification of the genes involved in autophagy (ATG genes). Orthologs to these genes were later found in higher eukaryotes, including \emph{C. elegans}, \emph{Drosophila}, and mammals.\textsuperscript{173} Autophagy is evolutionarily conserved in eukaryotes from yeast to mammals and has important roles in various cellular functions (reviewed by Yorimitsu \textit{et al.}, 2005).\textsuperscript{174} In \emph{C. elegans}, numerous functions of autophagy were documented, such as: adaptation to stress, aging, normal reproductive growth, cell death, cell growth control, neural synaptic clustering, and the degradation of aggregate-prone proteins.\textsuperscript{145}

There are three different types of autophagy; microautophagy, chaperone mediated autophagy and macroautophagy. Among these, macroautophagy (broadly referred merely as autophagy) is the one who has received more attention. In macroautophagy, cytoplasmic double-membrane vesicles called autophagosomes are formed. Autophagosomes can engulf large portions of cytoplasm
including organelles. These autophagosomes fuse with a late endosome or a lysosome and form a structure called autophagolysosome, where cellular components are degraded and their constituents recycled (Figure 9).\textsuperscript{145}

![Figure 9: Schematic diagram of the steps of autophagy.](image)

Autophagy begins with the formation of the autophagosome which can engulf portions of cytoplasm, including organelles. The autophagosome can later fuse with a lysosome, forming an autophagolysosome. Finally, the sequestered material is degraded inside the autophagolysosome and recycled. Image from\textsuperscript{145}.

The key signaling mechanisms for autophagy regulation appear to be also conserved in \textit{C. elegans}. A major regulator of autophagy is the mammalian target of rapamycin (mTOR) kinase. When nutrients are available, TOR kinase is active and autophagy is inhibited, whereas under nutrient-deficient conditions TOR is inactivated and autophagic activity is enhanced. In \textit{C. elegans}, the TOR ortholog, \textit{let-363}, encodes two proteins orthologous to \textit{S. cerevisiae} Tor1 and Tor2, and to the human TOR.\textsuperscript{145,175} Altogether, 15 \textit{C. elegans} orthologs of 12 yeast autophagy genes (\textit{atg} genes) have been identified.\textsuperscript{176}

Autophagy in \textit{C. elegans} has received a lot of attention regarding its implications in lifespan extension and ageing. TOR is itself a key modulator of metabolism, autophagy and aging.\textsuperscript{177} Autophagy has proven to be required for longevity on dietary restricted worms and that lifespan extension requires the PHA-4 transcription factor.\textsuperscript{178} In general, all studies have shown that \textit{C. elegans} with reduced TOR or insulin/IGF-1 receptor \textit{daf}-2 activity as well as dietary-restricted \textit{eat}-2 worms have increased levels of autophagy, and that the autophagy-related genes are required for the long-lived phenotype.\textsuperscript{179-181}

Recent studies highlight numerous associations between autophagy and lipid metabolism. The five main classes of lipids (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids and sterol lipids) have at some level shown an implication in autophagy; however their mechanisms of action are not yet elucidated (reviewed in by Dall’Armi et al., 2013).\textsuperscript{182}
The lifespan extension of germline-less animals, glp-1 mutants, as well as daf-2 mutants was dependent on increased expression of a triglyceride lipase, lipl-4.\textsuperscript{183} Constitutive expression of this lipase in fat storage tissue generates lean and long-lived animals.\textsuperscript{155} Moreover, the negative regulator of, TOR, was found to be down regulated in glp-1 mutants.\textsuperscript{156} Animals over expressing lipl-4 have increased autophagic activity, as well as autophagy genes and pha-4 mRNA levels; and pha-4 is required for the elevated lipase activity, which was observed in glp-1 mutants. lipl-4 over expressing animals are long-lived and both pha-4 and autophagy genes are required for this extended lifespan.\textsuperscript{184}

On the other hand, some autophagy genes, including bec-1, are required for lipid storage in normally developing C. elegans. Additionally, daf-2 and glp-1 mutants failed to increase lipid levels during development when autophagy was impaired, reinforcing the critical role that autophagy plays in lipid storage.\textsuperscript{185}

The sphingolipids ceramide and the sphingosine-1-phosphate are also both known activators of autophagy, and ceramide levels were found up regulated when autophagy is induced (reviewed by Dall’Armi et al., 2013).\textsuperscript{182}

Although many studies have been carried out to understand the molecular pathways that regulate autophagy and its connection with lipid metabolism, a lot of work is still needed to fully understand it.

Autophagy has also been identified as an important process in immunity and inflammation, since autophagy can also be responsible for degradation of intracellular pathogens in a selective form of autophagy, called xenophagy (reviewed by Levine et al., 2011).\textsuperscript{186} There are several evidences that autophagy and/or autophagy proteins have a crucial role in resistance to bacterial, viral and protozoan infection in metazoan organisms, however in C. elegans those studies are still limited.

In the nematode, autophagy appears to be protective against infection with S. typhimurium. When two autophagy genes, namely bec-1 and lgg-1, were silenced, C. elegans had a decrease in the lifespan and shown increased susceptibility to this pathogen.\textsuperscript{187} These autophagy defective mutants accumulated intact Salmonella in the intestinal lumen and were not able to clear the infection. Interestingly, the atg knockdown was sufficient to suppress the pathogen resistance conferred by the daf-2/insulin receptor mutation and of a strain that over expresses the daf-16 transcription factor. This suggests that increased autophagic activity in intestinal cells have a role in the resistance to pathogens that daf-2 mutants display.\textsuperscript{187} As Salmonella typhimurium is an intracellular pathogen, it would be interesting to find if these autophagy genes also contribute to the resistance of C. elegans towards extracellular pathogens.

A recent study shows that dietary supplementation with ω-6 PUFAS, in particular AA (arachidonic acid) and its precursor DGLA (di-homo-γ-linoleic acid), resulted in activation of autophagy which lead to an increase in C. elegans lifespan in both conditions of food deprivation and
food abundance. Subjecting a worm over expressing *lipl-4*, which is more resistant to starvation, to FAME analysis revealed an increase in AA and EPA (eicosapentanoic acid), 20-carbon ω-6 and ω-3 fatty acids, which indicates that somehow ω-3/ω-6 trigger a response that promotes resistant to nutritional deprivation.\(^\text{188}\)

Interestingly, a connection between autophagy and genetic susceptibility to Crohn’s disease was established. In humans, a polymorphism in the *atg* gene ATG16L1 (autophagy-related 16-like 1) is pointed out as a genetic risk factor for Crohn’s disease, leading to the speculation that mutations on the autophagy pathway may alter the normal gut response to enteric bacterial pathogens.\(^\text{189}\) Atg16L1 transgenic mice presented some features seen on the pathology of humans Crohn’s disease,\(^\text{190}\) and expression of the Atg16L1 T300A variant in human epithelial cells dramatically impaired the clearance of bacteria by autophagy.\(^\text{191}\) Following these evidences, it would be interesting to study the autophagy contribution on impaired intestinal immunity to bacterial pathogens.

1.9. RNAi Treatment

RNA interference (RNAi) is a powerful technique that allows the knockdown of gene expression by the introduction of double-stranded RNA (dsRNA) into a biological system, which targets a specific mRNA sequence. This phenomenon was first discovered in *C. elegans*, when injecting dsRNA into the germline produced an interference effect throughout the worm, showing the ability of the dsRNA to cross cell boundaries.\(^\text{192}\) Later on, it has been shown that feeding the worms with *E. coli* expressing target-gene dsRNA also produced the same systemic RNAi effect.\(^\text{193}\) Since RNAi does not usually result in a 100% knockout of gene expression, and also because some genes are more sensitive to RNAi than others, RNAi is referred as a knockdown approach, instead of knockout. On the other hand, experiments in *C. elegans* revealed that the RNAi effect can be transmitted for multiple generations.\(^\text{194}\)

![Figure 10: L4440 double-T7 vector inside HT115 RNase-deficient E. coli. Image from\(^\text{195}\)](image-url)
In feeding RNAi, the HT115 (DE3) strain of *E. coli* is commonly used. On this *E. coli*, the RNaseIII gene, a double-strand-specific RNase, is replaced with a cassette containing a T7 RNA polymerase whose expression is IPTG-inducible. This strain also contains a plasmid (L4440) with the gene of interest, flanked by two T7 promoters on an inverted orientation (Figure 10). Therefore, when the bacterium is exposed to IPTG, it produces T7 polymerase, which in turn will transcribe sense and antisense single-stranded RNA that spontaneously anneal to produce dsRNA. RNAi treatments not always result in a complete loss of function. An RNAi library was constructed on the Ahringer lab, which consists of bacterial clones expressing dsRNA that cover approximately 90% of the 19,427 predicted genes of *C. elegans*. That library is available and is widely used by scientists, providing an important tool for research, especially for genome-wide screens in *C. elegans*.

When dsRNA is introduced into the cytoplasm of cells it is cut into short interfering RNAs (siRNAs) that target homologous mRNA for degradation. In *C. elegans*, the effect spreads to tissues far away from the site of application, allowing knockdown of the selected gene throughout the organism, although this effect is limited in certain tissues, including the nervous system.

The RNAi mechanism is complex, and is still not completely understood, but a model to identify how the silencing is exerted has been traced (Figure 11). Briefly, the dsRNA strands are cut by DICER (an RNase III), into Small Interfering RNA (siRNAs) which consists of 21-23 nucleotide sequences. The strands are unwound in an ATP-dependent process and attach themselves to the RNA-induced silencing complex (RISC), where they bind to target mRNA through base-pairing interactions. When RISC binds to the mRNA, the complex degrades the mRNA, preventing its translation (reviewed by Agrawal *et al.*, 2003).
Figure 11: RNAi interference mechanism. dsRNA is incorporated into the cytoplasm, and cut in siRNAs by the endonuclease DICER. The small pieces of interference RNA are loaded to the RISC complex and bound to the target mRNA. The mRNA strand is cut and further degraded. Image from 200.

2. Motivation

With this project we can acquire some insights on the use of *C. elegans* as a model organism for pathogen infection, relationship between the genotype and phenotype when silencing genes involved on PUFA synthesis, importance of fatty acid species on the innate immunity of the worm, stress responses in *C. elegans* upon infection, and contribution of autophagy on the pathogenicity caused by the *E. coli* strain LF82.

A lot of information can be obtained from *C. elegans* when evaluating the pathogenicity of an organism. To monitor pathogenesis in this study, the following practices were carried out: Quantification of nematode survival, observation of morphological and behavioral changes, measuring of gene expression by qRT-PCR, quantification of lipid levels by GC-FAME, autophagy levels, and fluorescent microscopy with reporter constructs in transgenic worms.
3. Materials and Methods

3.1. Nematode Strains and Culture Conditions

*Caenorhabditis elegans* strains were cultured at 15°C or 20°C on NGM (Nematode Growth Medium) plates (17 g/L agar, 2.9 g/L NaCl, 2.5 g/L peptone, 1 mM CaCl₂, 5µg/mL cholesterol, 25 mM KH₂PO₄ and 1 mM MgSO₄) seeded with *E. coli* strain OP50 as previously described.³ Growth and manipulation of *C. elegans* was performed as described.²⁰¹

Bristol N2, used as the wild-type strain, and fat-3 mutant strain BX30 [fat-3(wa22)] were obtained from the Caenorhabditis Genetics Center (CGC), Minnesota, USA.

Transgenic strains TJ356: zls356 [Pdaf-16::daf-16a/b-gfp; rol-6] and SJ4005 (zclIs4[phsp-4::gfp]), were also obtained from CGC.

Transgenic strains DA2123: adIS2122 [lgg-1p::GFP::lgg-1 + rol-6(su1006)] and MAH-34 eat-2 (ad1116)II, adIs2122[lgg-1::gfp+rol-6] were kindly gifted from Malene Hansen, Sanford-Burnham Medical Research Institute.

3.2. Bacterial Strains and Culture

Bacterial strains used in this study:

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP50</td>
<td>Uracil auxotrophic <em>E. coli</em> B</td>
</tr>
<tr>
<td>HT115 (DE3)/pEGFPk</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT115 (L4440)</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>LF82</td>
<td>Prototypical AIEC isolate; serotype O83:H1</td>
</tr>
<tr>
<td>LF82*</td>
<td>Isogenic LF82; *ampC</td>
</tr>
<tr>
<td>LF82*/pEGFPk</td>
<td>Isogenic LF82 expressing EGFP. Kan&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All the *E. coli* strains were routinely cultured in LB-medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C with shaking and aeration.

The *E. coli* strain LF82 was kindly provided by Professor Arlette Darfeuille-Michaud (Université d’Auvergne, France).
3.3. General Methods

3.3.1. Nematode Synchronization

Adult worms were washed off NGM plates with sterile water, and transferred to 15 mL falcon tubes. To synchronize the adult nematodes, 3.5 mL of the worm suspension was incubated with 0.5 mL 5M NaOH and 1 mL 5% solution of NaOCl for at least 5 minutes. The eggs were collected by spinning at 1300×g for 1 min. After washing twice with sterile water, the eggs were cultured overnight at 20°C until hatching in S-basal buffer (5.85 g/L NaCl, 1 g/L K₂HPO₄, 6 g/L KH₂PO₄) to which 5 µg/mL cholesterol was added. L1 larvae synchronized by hypochlorite treatment were pelleted by spinning at 2000 rpm for 5 min and placed onto OP50-seeded NGM plates and grown at 20°C for 44-46 hours until reaching the L4 stage. When RNAi treatment was used, NGM plates containing 25 µg/mL carbenicillin and 1 mM IPTG were used instead. The bacteria used also differed, depending on which genes were to be silenced. See RNA interference section on methods for further details.

3.3.2. Lifespan Assay

L4 synchronized worms were transferred to seeded 6 cm NGM plates. 15 nematodes were transferred to each plate. The animals were then kept at 25°C, and scored every second day. Worms were gently poked with a platinum wire to test for live or dead animals. After scoring, animals were moved into freshly seeded plates to have enough fresh food and to separate them from their progeny. Worms that died of bursting vulva, bagging or crawled off the agar were defined as censored animals. A total of 120 worms were used per strain per experiment.

When using some RNAi treated strains that resulted in small worms phenotypes, hard to distinguish from the progeny, NGM seeded plates containing 0.1 mg/mL of 5-fluorodeoxyuridine (FUDR) were used, to prevent progeny from hatching.

Survival data was compiled to produce Kaplan-Meier survival curves. Statistical analyses were performed according to the log-rank test (Mantel-Cox) in the GraphPad Prism version 5.00 (GraphPad Prism Software, Inc). A p-value of <0.0001 was considered statistically significant.

Seeding of the plates was performed as follows. Briefly, an overnight culture of LF82*/pEGFPk or HT115/pEGFPk containing 50 µg/mL kanamycin was resuspended to an OD₆₀₀ of 10. NGM plates containing 50 µg/mL kanamycin were seeded with 100 µL of the bacterial suspension and incubated overnight at 37°C. E. coli HT115 (DE3)/pEGFPk was used as a negative control.
3.3.3. **RNA interference**

RNAi gene knockdown was performed by feeding the worms RNAi bacteria. The RNAi feeding strain was *E. coli* HT115 transformed with either empty vector (L4440) for controls or with vectors containing dsRNA-producing constructs. Worms fed with bacteria carrying the *dpy-13* vector were used as a visual control, in order to determine if the RNAi treatment was effective. Experiments and plates were prepared as described previously in Kamal *et al.* 195

RNAi bacteria were cultured overnight at 37°C in LB medium containing 150 µg/mL ampicillin and seeded in RNAi plates (NGM agar plates containing 25 µg/mL carbenicillin and 1 mM IPTG). The plates were dried overnight at room temperature to induce the dsRNA production. A population of synchronous L1 worms was placed on the plates, and was fed RNAi bacteria until they reached the L4 stage.

For RNAi from young adult, i.e. after development, the synchronized eggs were allowed to grow to young adult on normal NGM plates seeded with OP50 and then transferred to RNAi plates for feeding with dsRNA expressing bacteria for the period of 48h.

3.3.4. **Fatty Acid Supplementation**

GLA was obtained from Sigma-Aldrich Co. The fatty acid was dissolved in 96% ethanol, and added to a final concentration of 4mM to an overnight culture of *E. coli* OP50. The bacteria were then seeded onto NGM plates. After seeding, synchronized L1 larvae were immediately transferred to the plates in order to perform the experiment before oxidation of the supplemented fatty acid occurred. Animals without the addition of the fatty acid were used as a control.

The worms were allowed to grow on the supplemented plates at 20°C until they reached L4, and then transferred to the killing plates to proceed with the lifespan assay.

3.3.5. **Gas Chromatography analysis on *C. elegans***

To determine the fatty acid composition of the nematodes, worms were washed off the plates with 0.9% NaCl after 4 days of infection. After spinning down 1000 rpm for 1min, the worms were washed with sterile water and placed into glass screw-capped tubes. After centrifugation, water was removed and replaced with 1 mL of 2.5 % H$_2$SO$_4$ dissolved in water-free methanol to extract fatty acids and transmethylate them. BHT was added to a final concentration of 10 µg/mL to prevent the samples from being oxidized. The samples were capped and incubated at 80°C on a sand bath for 5 hours. After the addition of 0.5 mL of hexane and 1.5 mL of water, the methyl esters were extracted into the hexane layer by vortexing and centrifugation at 1500 rpm for 2 min. Samples were dried.
under a stream of N$_2$. Prior to GC analysis, samples were dissolved in an appropriate volume of hexane (±20 µL). 2 µL of the organic phase was injected in the GC, and FAMES were analyzed on a Chrompack CP 9002 instrument equipped with a DB-WAX, Agilent column. FAMES were identified by comparing to standards purchased from Larodan (Malmo, Sweden). The temperature of the oven had an increase of 10ºC per minute, starting at 140ºC and finishing at 240ºC. Statistics and comparisons were performed by using the unpaired two-tailed t-test in the GraphPad Prism version 5.00 (GraphPad Prism Software, Inc). A p-value of <0.0001 was considered statistically significant.

3.3.6. RNA purification and Quantitative Real-Time PCR

N2 synchronized animals were placed on NGM plates seeded with OP50 bacteria and grown at 20ºC until they reached the L4 stage. L4 or young adults were washed with M9 buffer and placed onto fresh plates seeded either with control bacteria (L4440) or pathogenic bacteria (LF82). The plates were kept at 25ºC. A total of 15 plates were seeded per strain and per condition. Biological triplicates of this experiment were carried at the same time. Samples were harvested 24h and 72h after their transfer to the LF82 or L4440 plates. Animals were washed off the plates with M9 buffer and were incubated in M9 buffer for approximately 20 min rotating, in order to empty their intestines. Next, samples were flash-frozen in liquid N$_2$ and kept at -80ºC until RNA purification. Total RNA was extracted using Isol-RNA lysis reagent (5 PRIME). RNA concentration was accessed using NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, USA). Electrophorese on a 1% agarose gel was performed to analyze the quality of the purified RNA. The intact RNA samples were further used for cDNA synthesis. cDNA was synthetized from 1 µg of total RNA. To prevent DNA contaminations, samples were treated with DNase, in a total volume of 10 µL comprising the RNA on a concentration of 1µg/7µL, 2 µL DNase buffer (100 µL 5x First strand buffer (Invitrogen), 50 µL sterilized milliQ H$_2$O), and 1 µL of 10 units/µL DNase I (Roche). Following incubation at 37ºC for 15 min, 3 µg of random hexamers 1 µg/µL (Roche) were added and the samples were vortexed and spun down. Samples were incubated 5 minutes at 85ºC and placed on ice afterwards. Next, samples were spun down and added 11 µL of reverse transcriptase premix (5 µL 5x First strand buffer (Invitrogen), 2.5 µL DTT (100 mM), 1 µL DEPC water, 2.5 µL dNTP mix (10mM)). 1 µL reverse transcriptase M-MLV was added, and the samples were incubated 10 minutes at room temperature, followed by incubation for 1 hour at 37ºC. After the incubation, DNA was diluted on 287 µL of milliQ H$_2$O and stored at -20ºC.

qRT-PCR was performed on a Stratagene MXPro 3000 instrument (Agilent Technologies) using 2× SYBR Green JumpStart™ Taq ReadyMix™ and Sigma Reference Dye (Sigma-Aldrich) as described by the manufactures. PCR reactions were performed in a 96 well plate, with each well containing 20 µL reaction comprising 5 µL of diluted cDNA, 10 µL SYBR Green Jumpstart™ Taq
ReadyMix™ (Sigma-Aldrich), 0.04 µL reference dye (Sigma-Aldrich), 0.6 µL forward primer (10pmol/µL), 0.6 µL reverse primer (10pmol/µL) and 3.76 milliQ H2O. Reactions were performed at 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 seconds, 60ºC for 45 seconds and 72ºC for 45 seconds. All reactions were performed in technical duplicate and normalized to the levels of the thp-1 gene (encoding a TATA-binding protein, the C. elegans orthologue of the human TATA-box-binding protein). The primer sequences of the used primers are listed on the supplementary table S1).

3.3.7. **Autophagy Assay**

The level of autophagy was accessed using a LGG-1::GFP translational reporter as previously described. The animals were kept at 25ºC and the second generation of worms was used. Adult laying-eggs worms were placed in plates seeded with non-fluorescent L4440 or LF82 bacteria. After laying eggs, these worms were removed from the plates and their progeny was allowed to grow until reach the L4 stage, when they were collected to microscopic observation. At this stage, 10-15 worms were placed on 2% agarose pads on glass slides on a drop (10 µL) of 10 mM Levamisole, overlaid with a cover slip. GFP positive puncta per seam cell were counted in L4 transgenic worms using a total magnification of 1600.00 × on a Leica DMI 6000 B microscope using a GFP filter. The number of autophagosomes per seam cell was averaged and this average was used for calculate the mean number of LGG-1::GFP containing puncta per seam cell. Statistical analyses were performed by using the unpaired two-tailed t-test in the GraphPad Prism version 5.00 (GraphPad Prism Software, Inc). A p-value of <0.0001 was considered statistically significant.

3.3.8. **Nuclear DAF-16::GFP localization**

To study the localization of DAF-16, a transgenic strain TJ356 expressing a DAF-16::GFP fusion protein was used. L4 animals were picked and placed onto plates seeded with L4440 or LF82 bacteria. The plates were maintained at 25ºC. After 4h, 10-15 worms were placed on 2% agarose pads on glass slides on a drop (10 µL) of 10 mM Levamisole, overlaid with a cover slip. Animals were observed using a total magnification of 80.00 × and 160.00 × on a Leica DMI 6000 B microscope using a GFP filter with an exposure of 100.000 ms. Two independent experiments were performed. Animals incubated at 30ºC for 4h were used as a positive control.

3.3.9. **Unfolded protein response analysis**

To evaluate the involvement of the UPR pathway as response to pathogenic *E. coli* LF82, worms of the strain SJ4005, which harbor the hsp-4::gfp gene were used. For this experiment two different
approaches were used. On the first approach, the second generation of worms was used. Adult laying-eggs worms were placed in plates seeded with non-fluorescent L4440 or LF82 bacteria. After laying eggs, these worms were removed from the plates and their progeny was allowed to grow until reach the L4 stage, when they were collected to microscopic observation. At this stage, 10-15 worms were placed on 2% agarose pads on glass slides on a drop (10 µL) of 10 mM Levamisole, overlaid with a cover slip and the ends were sealed with nail polish. Animals were observed using a total magnification of 160.00 × on a Leica DMI 6000 B microscope using a GFP filter with an exposure of 250.000 ms. As for the second approach, L4 worms previously feeding on control bacteria, were picked and placed onto plates seeded with L4440 or LF82 bacteria. The plates were maintained at 25°C. After 24h post-infection, 10-15 worms were placed on 2% agarose pads on glass slides on a drop (10 µL) of 10 mM Levamisole, overlaid with a cover slip and the ends were sealed with nail polish. Animals were observed using a total magnification of 80.00 × on a Leica DMI 6000 B microscope using a GFP filter with an exposure of 350.000 ms.
4. Results

4.1. Observed phenotypes

Using a microscope, simple observation of phenotypes can be performed. Several parameters are easily assessed like: health, movement, body shape, brood size, among others. Phenotypes exhibit by the deletion strains used in this study are listed:

Table 1: Phenotypes of lipid metabolic genes inactivated by RNAi

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4440</td>
<td>Normal</td>
</tr>
<tr>
<td>dpy-13</td>
<td>Small, round, dumpy movements</td>
</tr>
<tr>
<td>elo-1</td>
<td>Apparently Normal</td>
</tr>
<tr>
<td>elo-2</td>
<td>Smaller size, less progeny</td>
</tr>
<tr>
<td>elo-3</td>
<td>Lethal when RNAi treatment is applied at L1/ normal size if RNAi at L4</td>
</tr>
<tr>
<td>elo-4</td>
<td>Apparently Normal</td>
</tr>
<tr>
<td>elo-5</td>
<td>Smaller size, Less progeny</td>
</tr>
<tr>
<td>elo-6</td>
<td>Smaller size, Less progeny</td>
</tr>
<tr>
<td>elo-7</td>
<td>Reduced brood size</td>
</tr>
<tr>
<td>fat-3</td>
<td>Slow growth, reduced brood size, defects in movement</td>
</tr>
<tr>
<td>atg-12</td>
<td>Apparently Normal (RNAi treatment at L4)</td>
</tr>
</tbody>
</table>

It is also interesting to note that generally, through the course of all experiments, the worms fed with pathogenic bacteria *E. coli* LF82 had a different behavior in the plates, when compared to worms fed on *E. coli* HT115. *C. elegans* fed on HT115 remained on the center of the petri dish, around the seeded bacterial lawn; whereas *C. elegans* fed on LF82 would be disperse on the plate, often crawling the plate walls and resulting on a censored worm. The change in behavior in *C. elegans* has already been reported, and it is considered to have an important role as the first line of defense against pathogens, including evasion and possibly reduced ingestion of parasites.

A major part of this thesis relied on the assessment of the importance of fatty-acid elongation (*elo*) genes on the lifespan of the worm. This was achieved by conducting Survival Assays, also called Lifespans, which will be further presented. In nematodes, lifespan is typically defined as the number of days an animal remains responsive to external stimuli. Animals were scored as dead, when there was no movement, in response to light touch with a worm picker. Worms dying from internal hatching of progeny (bagging) or that crawled up the sides of the plates, were censored during this experiment.
4.2. Survival assays

To determine if the pathogenic bacteria *E. coli* strain LF82 can kill *C. elegans*, N2 worms previously fed with HT115 (L4440) RNAi bacteria from L1 to L4, were then placed and fed with lawns of LF82 at 25°C. As shown in Fig. 11, larval stage 4 (L4) worms show decreased lifespan when feeding in *E. coli* LF82 when compared to worms fed on *E. coli* strain HT115, a control food for *C. elegans* in the laboratory. These results are consistent with previous studies which demonstrate that LF82 establishes a persistent infection in the intestinal lumen of the worm, diminishing the lifespan significantly.157

![Figure 11: Adherent-invasive E. coli LF82 shortens C. elegans lifespan.](image)

Results from three independent experiments show consistent results and reveal that *C. elegans* feeding on LF82 displayed 50% of mortality in 10 days after infection, while worms fed on HT115 had a median lifespan of 12 days. As previously described,157 LF82 infection exerts the “slow killing” mechanism with worms dying due to bacterial accumulation. This study also showed that LF82 is able to establish a persistent infection on the nematode intestine, since LF82 was able to persist after *C. elegans* being feed on the pathogenic bacteria only for 24 hours and transferred to control bacteria. After 6 days of infection, LF82 not only had persevere but actually had also increased in number, showing her capacity to resist and multiply within the worms’ intestine.
4.2.1. RNAi against *C. elegans* elongation genes elo-1 and elo-2

Mutants which were lacking key enzymes for the proper fat metabolism on *C. elegans* were next analyzed. RNAi was first used to knockdown the elongase genes elo-1 and elo-2. Both proteins are involved in the elongation of ω6 and ω3 C18 PUFAS. As discussed previously, both genes seem to act together to elongate PUFAs, and single mutation of any of these genes did not completely eliminate the level of C20 PUFAs. After feeding N2 worms with HT115 (L4440), elo-1 or elo-2 RNAi bacteria from L1 to L4, worms were placed on lawns of pathogenic bacteria *E. coli* strain LF82 at 25°C and survival was accessed.

![Figure 12: RNAi against elo-1 and elo-2 shortened lifespan of *C. elegans* and increased susceptibility towards infection with *E. coli* LF82.](image)

(A) Survival curves of wild-type nematodes (L4440 control vector RNAi) and elo-1 RNAi nematodes fed on *E. coli* HT115 (DE3)/pEGFPk and *E. coli* LF82*/ pEGFPk at 25°C. (B) Survival curves of wild-type nematodes (L4440 control vector RNAi) and elo-2 RNAi nematodes fed on *E. coli* HT115 (DE3)/pEGFPk and *E. coli* LF82*/ pEGFPk at 25°C. L4 synchronized nematodes were placed onto the killing plates and scored for survival every second day. *C. elegans* lifespan was significantly reduced in worms fed with dsRNA to the elongation genes elo-1 and elo-2, when compared to control worms (*, p=0.0005). Kaplan-Meir method was used for the statistical analysis.
Table 2: Median lifespan of N2 worms treated with different RNAi, respectively with L444O (control), elo-1 and elo-3, on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk. Data shown is based in one experiment. P values were calculated with Log-rank (Mantel-Cox) test using GraphPad Prism version 5.00.

<table>
<thead>
<tr>
<th>Strain</th>
<th>RNAi treatment</th>
<th>Bacteria</th>
<th>Median lifespan (days)</th>
<th>Lifespan change (%)</th>
<th>p-value vs. control</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>L4440</td>
<td>HT115</td>
<td>12</td>
<td>0</td>
<td></td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>10</td>
<td>-16.67</td>
<td>&lt;0.0001</td>
<td>*** 117</td>
</tr>
<tr>
<td></td>
<td>elo-1</td>
<td>HT115</td>
<td>12</td>
<td>0</td>
<td>0.0077</td>
<td>** 116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>10</td>
<td>0</td>
<td>0.0971</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>elo-2</td>
<td>HT115</td>
<td>8</td>
<td>-33.34</td>
<td>&lt;0.0001</td>
<td>*** 118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>6</td>
<td>-40.00</td>
<td>&lt;0.0001</td>
<td>*** 91</td>
</tr>
</tbody>
</table>

As shown in Figure 12, RNAi against elo-1 and elo-2 decreased the lifespan of the nematode in relation to control RNAi, being this effect more prominent on elo-2 mutants. Nematodes lacking elo-2 displayed a 33% reduction in their mean survival (Table 2). Elo-2 had a 40% decrease in median lifespan when infected with pathogenic LF82, suggesting a role for this gene on susceptibility to infection.

4.2.2. RNAi against C. elegans elongation genes elo-5 and elo-6

Next, the elongase genes elo-5 and elo-6 were silenced by feeding the worms with dsRNA producing bacteria. Elo-5 and elo-6 are responsible for elongation of monounsaturated fatty acids (MUFAs), producing C15:0-iso and C17:0-iso.

The procedure to access their importance on C. elegans immunity was the same as described above, with an alteration on the NGM plates used for the killing assay. In the first attempt performing the lifespan assay with elo-5 and elo-6 mutants, at day 8 after infection it was impossible to distinguish the progeny from the original worms, since knockdown of these genes resulted in rather smaller worms. Although the worms were moved to freshly seeded plates every two day, if some eggs were left on the plates, after two days at 25ºC these worms would be around L2/L3 larval stage, which is this case was about the same size of elo-5 and elo-6 mutants.

For that reason, the progeny arresting substance FUDR was added to the NGM plates, in order to prevent the worms of laying eggs and having progeny.
Figure 13: RNAi against elo-5 shortened lifespan of C. elegans and increased susceptibility towards infection with E. coli LF82. RNAi to elo-6 had less dramatic changes on lifespan of C. elegans. (A) Survival curves of wild-type nematodes (L4440 control vector RNAi) and elo-5 RNAi nematodes fed on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk at 25°C. (B) Survival curves of wild-type nematodes (L4440 control vector RNAi) and elo-6 RNAi nematodes fed on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk at 25°C. L4 synchronized nematodes were placed onto the killing plates and scored for survival every second day. C. elegans lifespan was reduced in worms fed with dsRNA to the elongation genes elo-5 when compared to control worms (*, p=0.0005). Kaplan-Meir method was used for the statistical analysis.
Table 3: Median lifespan of N2 worms treated with different RNAi, respectively with L444O (control), elo-5 and elo-6, on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk. Data shown is based in one experiment. P values were calculated with Log-rank (Mantel-Cox) test using GraphPad Prism version 5.00.

<table>
<thead>
<tr>
<th>Strain</th>
<th>RNAi treatment</th>
<th>Bacteria</th>
<th>Median lifespan (days)</th>
<th>Lifespan change (%)</th>
<th>p-value vs. control</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>L444O</td>
<td>HT115</td>
<td>12</td>
<td>0</td>
<td></td>
<td>104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>12</td>
<td>0</td>
<td>0.0873</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>elo-5</td>
<td>HT115</td>
<td>12</td>
<td>0</td>
<td>&lt;0.0001 ***</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>10</td>
<td>-16.67</td>
<td>&lt;0.0001 ***</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>elo-6</td>
<td>HT115</td>
<td>12</td>
<td>0</td>
<td>0.0003 ***</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>12</td>
<td>0</td>
<td>0.0015 **</td>
<td>119</td>
</tr>
</tbody>
</table>

In opposition to the results seen upon knockdown of the genes elo-1 and elo-2, knockdown of elo-5 and elo-6 only elicited small changes on the lifespan of C. elegans (Figure 13). Moreover elo-5 and elo-6 worms did not show increased susceptibility to infection with LF82, apart from elo-5 mutants who displayed a mean lifespan 20% shorter than wild-type, when fed with pathogenic bacteria (Table 3). This effect is however more subtle, than the effect instigated by silencing the elo-1 and elo-2 genes. The result obtained point out to that C20 PUFAs and not so much MUFAS are required for proper protection against infection.

4.2.3. RNAi against C. elegans elongation genes elo-4 and elo-7

Following the same line of though, two more elongase genes were silenced. This time elo-4 and elo-7 dsRNA producing bacteria was used. Investigation of these genes in C. elegans is fairly limited, and there is not much information available concerning them. WormBase states that these genes encode a paralog of the elo-1 and elo-2 genes, and that they have no known function in-vivo. Nevertheless, as the RNAi bacteria was available on the library used, and to increase the knowledge about these genes, they were the next genes analyzed in order to access if their knockdown was important for survival of the nematode and to access if they had any significant role on immunity.
Figure 14: RNAi against elo-4 and elo-7 shortened lifespan of *C. elegans* and increased susceptibility towards infection with *E. coli* LF82. (A) Survival curves of wild-type nematodes (L4440 control vector RNAi) and elo-4 RNAi nematodes fed on *E. coli* HT115 (DE3)/pEGFPk and *E. coli* LF82*/pEGFPk at 25°C. (B) Survival curves of wild-type nematodes (L4440 control vector RNAi) and elo-7 RNAi nematodes fed on *E. coli* HT115 (DE3)/pEGFPk and *E. coli* LF82*/pEGFPk at 25°C. L4 synchronized nematodes were placed onto the killing plates and scored for survival every second day. *C. elegans* lifespan was significantly reduced in worms fed with dsRNA to the elongation genes elo-4 and elo-7, when compared to control worms (*, p=0.0005). Kaplan-Meir method was used for the statistical analysis.
Table 4: Median lifespan of N2 worms treated with different RNAi, respectively with L444O (control), elo-4 and elo-7, on *E. coli* HT115 (DE3)/pEGFPk and *E. coli* LF82*/pEGFPk. Data shown is based on one experiment. *P* values were calculated with Log-rank (Mantel-Cox) test using GraphPad Prism version 5.00.

<table>
<thead>
<tr>
<th>Strain</th>
<th>RNAi treatment</th>
<th>Bacteria</th>
<th>Median lifespan (days)</th>
<th>Lifespan change (%)</th>
<th>p-value vs. control</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>L4440</td>
<td>HT115</td>
<td>12</td>
<td>0</td>
<td>&lt;0.0001 ***</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>8</td>
<td>0</td>
<td>&lt;0.0001 ***</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>elo-4</td>
<td>HT115</td>
<td>8</td>
<td>-33.34</td>
<td>&lt;0.0001 ***</td>
<td>118</td>
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<td></td>
<td></td>
<td>LF82</td>
<td>8</td>
<td>0</td>
<td>&lt;0.0001 ***</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>elo-7</td>
<td>HT115</td>
<td>8</td>
<td>-33.34</td>
<td>&lt;0.0001 ***</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LF82</td>
<td>8</td>
<td>0</td>
<td>0.0039 **</td>
<td>118</td>
</tr>
</tbody>
</table>

Both knockdown of *elo-4* and *elo-7* genes produced similar effects on lifespan of *C. elegans* (Figure 14). On worms fed with control bacteria (HT115) during the killing assay, knockdown of these genes resulted in a reduction of the mean lifespan of about 33% (Table 4). However these mutants did not show to be more susceptible to infection when fed on LF82 bacteria.

4.2.4. **Supplementation with GLA rescued fat-3 pathogen susceptibility**

*Fat-3* mutants lack the Δ6 desaturase activity, which prevents the worm from producing C20 PUFAs. These mutants exhibit reduced brood size, movement defects, slower growth and produce fewer progeny than wild-type nematodes. When supplemented with the missing C20 PUFAs, *fat-3* mutants phenotype was restored. Other study with *fat-3* mutants shown that dietary supplementation with specific PUFAs alleviated the selective behavior defects that the adult mutants exhibited. Dietary supplementation with polyunsaturated fatty acids can induce major changes in the fatty acid composition of the membrane. PUFAs accumulate in total lipid fractions and the amount of dietary PUFA can be up to 50% of fatty acids in the total amount of lipids.

To access the importance of γ-Linolenic acid (GLA), a long chain PUFA, in *C. elegans* defense against the pathogen *E. coli* LF82, synchronized worms were allowed to develop from L1 to L4 stage on OP50 bacterial lawns supplemented with 4 mM of GLA. L4 worms were subsequently transferred to the killing plates, with *E. coli* LF82*/pEGFPk, and scored for survival every second day.
Figure 15: Rescue of fat-3 pathogen susceptibility by dietary supplementation with GLA. Survival curves of wild-type nematodes, of fat-3 mutants and of wild-type and fat-3 worms supplemented with 4mM GLA. Supplemented nematodes were grown from L1 to L4 in the presence of 4mM GLA. L4 worms were placed on the killing plates with E. coli LF82*/pEGFPk at 25°C and scored for survival every second day. C. elegans lifespan was significantly rescued on fat-3 mutants supplemented with GLA (*, p=0.0005). Kaplan-Meir method was used for the statistical analysis.

N2 animals supplemented with the GLA PUFA, had no significant changes on lifespan, and did not show enhanced pathogen survival, when compared to untreated wild-type animals. On the other hand, supplementation of fat-3 mutants with GLA rescued pathogen susceptibility (Figure 15). GLA supplementation recovered the 25% median lifespan that was lost in fat-3 mutants (Table 5). This result implicates the requirement for GLA on C. elegans immunity.

Table 5: Median lifespan of N2 and fat-3 mutants on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk. Data shown is based in one experiment. *P values were calculated with Log-rank (Mantel-Cox) test using GraphPad Prism version 5.00. (GLA: γ-linolenic acid)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Supplemented PUFA</th>
<th>Median lifespan (days)</th>
<th>Lifespan change (%)</th>
<th>p-value vs. control</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>-</td>
<td>8</td>
<td>0</td>
<td>0.8087</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>GLA</td>
<td>8</td>
<td>0</td>
<td>0.8087</td>
<td>105</td>
</tr>
<tr>
<td>fat-3</td>
<td>-</td>
<td>6</td>
<td>-25.00</td>
<td>&lt;0.0001 ***</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>GLA</td>
<td>8</td>
<td>0</td>
<td>0.5287</td>
<td>84</td>
</tr>
</tbody>
</table>

This study shows that supplementation with gamma-linolenic acid on a concentration of 4mM, rescues the pathogenicity induced by LF82. Interestingly, treatment with 1mM only rescued partially the lifespan of the worm, as shown on a previous study in the Lipid group, suggesting a dose dependent effect of supplementation with gamma-linolenic acid in C. elegans.
4.3. Gene expression changes upon infection with *E. coli* LF82

Alterations in gene expression can be monitored using quantitative reverse transcriptase PCR (qRT-PCR). For this purpose, worms were harvested after 24 and 72 hours after being transferred to plates seeded either with control bacteria HT115(L4440) or pathogenic bacteria *E. coli* LF82. RNA was isolated and purified, and cDNA was synthetized. Unfortunately, when the RNA extracted from the worms at a post-infection period of 72 hours was tested by gel electrophoresis, the bands appeared smeared on the gel, meaning the RNA was degraded. For that reason, only the 24 hours post-infection samples were used. The repetition of this experiment for the 72 hours period would be interesting, since many genes may only be expressed latter, as infection progresses. However the temperature sensitive strain *glp-4* (which does not produce progeny at 25ºC) or FUdR has to be used, since the presence of progeny may have been the cause for the degraded RNA.

### 4.3.1. Oxidative stress related genes

![Figure 16: qRT-PCR analysis of mRNA expression of oxidative stress related genes in response to *E. coli* LF82 infection.](image)

*Figure 16: qRT-PCR analysis of mRNA expression of oxidative stress related genes in response to *E. coli* LF82 infection.* Total RNA was harvested from adult wild-type worms fed either with HT115 or LF82 bacteria for 24 hours, and the mRNA level of the genes indicated was determined by qRT-PCR. Data from three independent experiments is depicted as mean ± sem. Expression levels are normalized to *tbp-1* mRNA levels.

The *C. elegans* genome encodes five superoxide dismutase genes (*sod-1; sod-2; sod-3; sod-4* and *sod-5*). SOD (superoxide dismutase) is a major enzyme that protects against oxidative stress by catalyzing the removal of *O$_2^-$*, a central ROS involved in the generation of various toxic ROS.
Table 6: Genes coding superoxide dismutases (SODs) in *C. elegans*, the type of SODs and their locations. Data collected from WormBase.206

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>sod-1</td>
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<td>cytoplasmic</td>
</tr>
<tr>
<td>sod-2</td>
<td>Fe/Mn</td>
<td>mitochondrial</td>
</tr>
<tr>
<td>sod-3</td>
<td>Fe/Mn</td>
<td>mitochondrial</td>
</tr>
<tr>
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<td>Cu/Zn</td>
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</tr>
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<td>cytoplasmic</td>
</tr>
</tbody>
</table>

*sod-1* and *sod-2* encode the major cytosolic Cu/ZnSOD and MnSOD isoforms, respectively, whereas *sod-5* and *sod-3* are minor cytosolic Cu/ZnSOD and MnSOD isoforms being these last two up-regulated in dauer worms.207 *sod-4* encodes two extracellular CuZnSODs.208

The mRNA levels of both of the superoxide dismutases genes, *sod-1* and *sod-2* were increased on *C. elegans* fed with pathogenic LF82 for 24 hours (Figure 16). Among all the *sod* genes tested, *sod-1* and *sod-2* were the superoxide dismutases found to have higher expression levels on *C. elegans*; independently of the bacteria worms were fed.

The levels of *sod-3*, a target gene of DAF-16 were also up regulated in this experiment (Figure 16), suggesting that DAF-16 may be activated upon infection with *E. coli* LF82, resulting in increased expression of this gene.

*Sod-4*, an extracellular Cu/Zn superoxide dismutase, was also found to have higher levels of expression as a response to *E. coli* LF82 infection (Figure 16).

Glutathione S-transferases (GSTs) are enzymes involved in phase II detoxification that catalyze the conjugation of glutathione to electrophiles. They play a major role in the protection against xenobiotics and endobiotic compounds.209 *C. elegans* genome contains 52 genes encoding this enzymes.210

In this experiment, the mRNA levels of *gst-4* and *gst-5* were accessed in *C. elegans* fed with control and with pathogenic bacteria. Similarly to the *sod* genes, the *gst* genes were also found to have enhanced expression in LF82-infected worms (Figure 16).

Despite being not statistically significant, the results show that all stress genes tested are up regulated in *C. elegans* upon infection with *E. coli* LF82.
4.3.2. Autophagy related genes

Next, qRT-PCR was used to determine mRNA levels of autophagic genes on wild-type worms challenged with pathogenic *E. coli* LF82. Autophagy function has been documented for genes acting in autophagy induction (*unc-51/ATG1*), vesicle nucleation (*bec-1/ATG6, vps-34/VPS34*), the protein conjugation systems (*atg-7/M7.5/ATG7, lgg-1/ATG8, lgg-3/ATG12*), retrieval and vesicle recycling (*atg-18/F41E6.13/ATG18*).\(^\text{211}\) For this study the mRNA levels of *atg-12* (also known as lgg-3), *atg-18* and *lgg-1* were measured.

All of the autophagy related genes tested were found up regulated on *C. elegans* fed with *E. coli* LF82, when compared to wild-type worms fed on HT115 (Figure 17). However, the results were not statistically significant. Nevertheless, the data acquired indicates that the autophagic pathway is induced or activated in worms infected with *E. coli* LF82. This result is later confirmed by the use of a strain containing the reporter GFP::LGG-1, which indicates formation of the autophagosome (Figure 26).

![Figure 17: qRT-PCR analysis of mRNA expression of autophagy related genes in response to *E. coli* LF82 infection.](image)

*Figure 17: qRT-PCR analysis of mRNA expression of autophagy related genes in response to *E. coli* LF82 infection.* Total RNA was harvested from adult wild-type worms fed either with HT115 or LF82 bacteria for 24 hours, and the mRNA level of the genes indicated was determined by qRT-PCR. Data from three independent experiments is depicted as mean ± sem. Expression levels are normalized to *tbp-1* mRNA levels.
4.3.3. Dauer formation and pharyngeal pumping related genes

The mRNA levels of dauer formation genes such as *daf-2*, *daf-16* and *daf-22* as well as the genes related to pharyngeal pumping in *C. elegans*, *eat-2* and *pha-4* were also determined.

![Diagram showing mRNA expression levels of dauer formation and pharyngeal pumping related genes in response to *E. coli* LF82 infection.

As discussed previously, the insulin/insulin-like growth factor 1 receptor (I/IGF-1R) homolog DAF-2 is one of the principal components affecting lifespan in *C. elegans*. The insulin/IGF-1 signaling (IIS) also regulates reproduction and lipid metabolism, as well as entry into a state of developmental diapause, called the dauer larva. The activation of DAF-2/IIR activates a kinase cascade, culminating in the phosphorylation of the DAF-16/FOXO transcription factor. In unfavorable environments, DAF-16 enters the nucleus, where it potentiates the expression of genes required for stress resistance, dauer formation, and longevity (reviewed by Coleen et al., 2003).²¹²

*daf-22* encodes the *C. elegans* ortholog of human sterol carrier protein SCP2, which catalyzes the final step in peroxisomal fatty acid beta-oxidation. *daf-22* mutants lack the production of the pheromone responsible for the dauer formation, so *daf-22* mutants do not form dauers even if crowded and starved.²¹³
eat-2 mutants are typically used as the C. elegans model of dietary restriction, and can live up to 50% longer than wild type.\textsuperscript{214}

PHA-4 is a FOXA homolog required for eat-2 induced longevity.\textsuperscript{215} PHA-4 acts independently of daf-16 and IIS to regulate lifespan extension in dietary restricted worms. Interestingly, it was disclosed that DAF-16 and PHA-4 had consensus DNA binding sites, making possible that these two transcription factors could regulate the same genes.\textsuperscript{215} However, analysis of the expression levels of the sod genes revealed differential regulation in response to IIS and DR. While the response to IIS involves the DAF-16 regulation of sod-1, sod-3 and sod-5, the response to DR involves the PHA-4 dependent expression of every sod gene except for sod-3. It is thus concluded that a different form of ROS production may be induced under conditions of reduced IIS than is induced under conditions of dietary restriction.

In this experiment, the mRNA levels of all the daf genes tested were higher in C. elegans fed with the pathogenic bacteria E. coli LF82, when compared to wild-type (Figure 18). However none of the results was statically significant.

Of the pharyngeal pumping related genes tested, eat-2 was found downregulated, whereas pha-4 had increased expression on C. elegans infected with LF82 (Figure 18). Since the results obtained are not statically significant, repetition of the analysis is required in order to validate the results obtained.

4.4. Gas Chromatography and GC-MS

The fatty acid composition of total lipids in C. elegans was examined by direct trans-methylation of lipids, followed by gas chromatography (GC). As free fatty acids may sometimes be hard to analyze, fatty acid methyl esters (FAMEs), were investigated in this study. Total fatty acids are isolated, methylated and later analyzed by GC. The fatty acids identified were assigned using standards of known reference.

In this experiment, wild-type worms fed on empty vector RNAi bacteria were tested, as well as worms treated with RNAi bacteria to knockdown the genes elo-1, elo-2, elo-5 and elo-6. Worms were fed either in control HT115 bacteria or pathogenic LF82 during 4 days. FuDR was added to the NGM plates in order to prevent the worms from having progeny.
4.4.1. Lipid profile of control *C. elegans* fed with L4440 RNAi

Image 19: Relative abundance of fatty acid species in wild-type *C. elegans* expressed as percentage of total fatty acid as determined by gas chromatography analysis. Wild-type *C. elegans* fed on *E. coli* LF82 have significantly higher levels of C15iso and 9,10-methylene hexadecanoic acid (C17Δ) than wild type worms fed on *E. coli* HT115. Error bars represent the standard error of the mean (SEM). Unpaired t-test was performed using GraphPad Prism version 5.00 (*, p < 0.05).

4.4.2. Lipid profile of *C. elegans* treated with elo-1 RNAi

Image 20: Relative abundance of fatty acid species in elo-1 (RNAi) *C. elegans* expressed as percentage of total fatty acid as determined by gas chromatography analysis. *C. elegans* fed with dsRNA bacteria for elo-1 until L4, afterwards fed on *E. coli* LF82 has significantly higher levels of Eicosatetraenoic acid (C20:4n3) than elo-1 knockdown worms fed on *E. coli* HT115. Error bars represent the standard error of the mean (SEM). Unpaired t-test was performed using GraphPad Prism version 5.00 (**, p < 0.01; *, p < 0.05).
4.4.3. Lipid profile of *C. elegans* treated with elo-2 RNAi

Image 21: Relative abundance of fatty acid species in elo-2 (RNAi) *C. elegans* expressed as percentage of total fatty acid as determined by gas chromatography analysis. *C. elegans* fed with dsRNA bacteria for elo-2 until L4, afterwards fed on *E. coli* LF82 has significantly lower levels of Myristic Acid (C14:0) and cis-Vaccenic acid (C18:1 n7) than elo-2 knockdown worms fed on *E. coli* HT115. Error bars represent the standard error of the mean (SEM). Unpaired t-test was performed using GraphPad Prism version 5.00 (**, p < 0.01; *, p < 0.05).

4.4.4. Lipid profile of *C. elegans* treated with elo-5 RNAi

Image 22: Relative abundance of fatty acid species in elo-5 (RNAi) *C. elegans* expressed as percentage of total fatty acid as determined by gas chromatography analysis. *C. elegans* fed with dsRNA bacteria for elo-5 until L4, afterwards fed on *E. coli* LF82 has significantly higher levels of Palmitoleic acid (C16:1 n7) than elo-5 knockdown worms fed on *E. coli* HT115. Error bars represent the standard error of the mean (SEM). Unpaired t-test was performed using GraphPad Prism version 5.00 (**, p < 0.01; *, p < 0.05).
4.4.5. **Lipid profile of C. elegans treated with elo-6 RNAi**

Image 23: Relative abundance of fatty acid species in elo-6 (RNAi) C. elegans expressed as percentage of total fatty acid as determined by gas chromatography analysis. C. elegans fed with dsRNA bacteria for elo-6 until L4, afterwards fed on E. coli LF82 has significantly higher levels of C15iso, 9,10-methylene hexadecanoic acid (C17Δ) and γ-Linolenic acid (C18:3 n-6) than elo-6 knockdown worms fed on E. coli HT115. Error bars represent the standard error of the mean (SEM). Unpaired t-test was performed using GraphPad Prism version 5.00 (**, p < 0.01; *, p < 0.05).

By analyzing the fatty acid profiles above (Figure 19 to 23), depicting the relative abundance of fatty acid species on C. elegans fed with control or LF82 bacteria, it is possible to conclude that infection with LF82 induces fatty acid changes on the nematode.

In wild-type C. elegans treated with empty vector RNAi bacteria (L4440), there are significantly higher levels of C15iso and C17Δ (9,10-methylene hexadecanoic acid) on worms fed with E. coli LF82, when compared to wild-type worms fed on E. coli HT115 (Figure 19).

In C. elegans treated with RNAi bacteria producing dsRNA for elo-1, Eicosatetraenoic acid (C20:4n3) was found to have increased its relative abundance in worms fed on E. coli LF82, when compared to worms fed on E. coli HT115 (Figure 20).

For C. elegans fed with dsRNA bacteria for knockdown of elo-2, significantly lower levels of Myristic Acid (C14:0) and cis-Vaccenic acid (C18:1 n7) were found on worms fed on E. coli LF82, when compared to worms fed on E. coli HT115 (Figure 21).

*Elo-5 (RNAi) animals fed on E. coli LF82 have significantly higher levels of Palmitoleic acid (C16:1 n7), when compared to animals fed on E. coli HT115 (Figure 22).

Lastly, *elo-6 (RNAi)* animals displayed significantly higher levels of C15iso, C17Δ (9,10-methylene hexadecanoic acid) and γ-Linolenic acid (C18:3 n-6) when fed on pathogenic E. coli LF82 (Figure 23).
The analysis of the fatty acid profile of the worms also allowed to insure that the RNAi treatment applied was being efficient, in the sense that elo-5 mutants displayed undetectable levels of C15iso and C17iso fatty acids, whereas elo-6 mutants had reduced levels of C17iso, as expected (Supplementary Figure 1).

The results allow us to conclude that infection with E. coli LF82 has an effect on the lipid profile of C. elegans. It is however hard to withdraw any further conclusion based only on this data, since there seems to be no pattern on which fatty acids have increased or decreased abundance in each of the strains tested. Between the experiments performed there was also great variation on the abundance of the fatty acids, hence the size of the error bars. Gas Chromatography is a powerful technique that allows determining the fatty acid composition, however a more sensitive approach using mass-spectrometry-based methods, should be attempted in order to do the lipid profiling of C. elegans infected with E. coli LF82.

4.5. RNAi against autophagy gene atg-12 and elongation gene elo-3 at L4

Some worms were subjected to RNAi treatment only from L4 stage, since RNAi at early stages altered their normal development. This was the case of elo-3 mutant worms for which the knockdown of this gene was lethal when feeding dsRNA elo-3 expressing bacteria at L1, and also for the atg-12 knockdown worms due to the important role that autophagy genes have during development.179

The worms were kept in the RNAi bacteria for 48h at 20ºC to incorporate the dsRNA. It has been shown previously that L4 worms need to be feed at least for 36h at 22ºC in order to produce a strong RNAi effect, since feeding times shorter than that were not sufficient.195 However, in order to keep the strength of the treatments, the worms should be continuously transferred to fresh plates with RNAi bacteria,172 which is not possible in this case, since we replace C. elegans food source for pathogenic bacteria in order to access longevity.

For this study we consider that the feeding time of 48h is sufficient. Therefore, the L4+2 worms were placed in the killing plates being this the Day 0 in the survival curves.

4.5.1. Survival of atg-12

As discussed above, autophagy has been linked with Crohn’s disease, as a polymorphism in the atg gene ATG16L1 has been related with genetic susceptibility to the disease. C. elegans possess an ortholog to this gene, namely atg-16.2. The hypothesis to test on this study was to knockdown this autophagy gene by RNAi, and to investigate if the nematode exhibited increased susceptibility to E.
coli LF82, which was originally isolated from a patient with Crohn’s disease. Unfortunately, the RNAi library on our lab did not possess the E. coli producing dsRNA for atg-16.2, so another autophagy gene was silenced: atg-12 (also called lgg-3).

Figure 24: RNAi against the autophagy gene atg-12 resulted in no significant alterations on lifespan of C. elegans. Survival curves of wild-type nematodes (L4440 control vector RNAi) and atg-12 RNAi nematodes fed on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk at 25ºC. L4 synchronized nematodes were placed onto the killing plates and scored for survival every second day. C. elegans lifespan had no significant alterations in worms fed with dsRNA to the autophagy gene atg-12, when compared to control worms (*, p=0.0005). Kaplan-Meir method was used for the statistical analysis.

RNAi against atg-12 at worms on the L4 stage had no effects on lifespan of C. elegans. Moreover, atg-12 mutants do not display increased susceptibility towards infection with E. coli LF82 (Figure 24).

4.5.2. Survival of elo-3

The last C. elegans elongase gene available on the RNAi library from the lab was elo-3. Similar to elo-4 and elo-7, little or no research has been published about this gene. Its in-vivo function is also not known. Surprisingly, in this study, when synchronized N2 worms at L1 stage were placed on dsRNA producing bacteria for elo-3 knockdown, the worms died. That is the reason why RNAi treatment was applied to adult worms. When applied to adults, the nematode appearance under the microscope was normal, with no developmental or movement defects noticed.
RNAi treatment for the elongase gene elo-3 at the L4 stage also did not affect C. elegans lifespan. Additionally, elo-3 (RNAi) worms fed on E. coli LF82 had the same median lifespan as elo-3 worms fed on HT115 bacteria, suggesting that elo-3 knockdown has no consequence on C. elegans susceptibility to infection (Figure 25).

Table 7: Median lifespan of N2 worms treated with different RNAi at the adult stage, respectively with L4440 (control), atg-12 and elo-3, on E. coli HT115 (DE3)/pEGFPk and E. coli LF82*/pEGFPk. Data shown is based in one experiment. P values were calculated with Log-rank (Mantel-Cox) test using GraphPad Prism version 5.00.

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<th>Bacteria</th>
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<th>Lifespan change (%)</th>
<th>p-value vs. control</th>
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4.6. Autophagy assay

The *C. elegans* DA2123 transgenic strain expressing GFP-tagged LGG-1 was used in order to determine if the pathogenic bacteria *E. coli* LF82 exerted an effect on the autophagy levels. This strain is commonly used to access autophagy in *C. elegans*. LGG-1 is an ortholog of yeast Apg8/Aut7p and mammalian MAP-LC3, which during autophagy localizes to the preautophagosomal and autophagosomal membranes. When autophagy is occurring, *lgg-1* have a punctate staining pattern, contrasting with its diffuse pattern in the absence of autophagy.179

L4 animals of the second generation fed either on control HT115 bacteria or pathogenic *E. coli* LF82 were collected, observed under a fluorescence microscope and the number of LGG-1::GFP puncta per seam cell was calculated. Three independent experiments were performed.

Wild-type animals (DA2123) exhibited a significant higher level of LGG-1::GFP puncta when fed on pathogenic *E. coli*, compared to DA2123 animals fed on control bacteria (Figure 26). This observation indicates that autophagy is induced on *C. elegans* upon infection with the pathogenic *E. coli* LF82.

![Figure 26: *E. coli* LF82 increases *C. elegans* autophagy levels. Bars represent the mean number of LGG-1::GFP-containing puncta per seam cell in non-starved wild type (DA2123) and Mah-34 mutants. Worms were grown at 25°C and the second generation at the larval stage L4 was used. Nematodes were fed on *E. coli* HT115 and *E. coli* LF82. *C. elegans* autophagy was significantly enhanced when feeding on LF82 compared to the HT115 control (***, p<0.0001). Statistical analyses were performed by unpaired two-tailed t-test (with Welch’s correction if variances were significantly different) using GraphPad Prism version 5.00 (GraphPad Software). Mean ± SEM is shown.](image-url)
Besides wild-type animals, Mah-34 worms were also used. These are eat-2 mutants who also express LGG-1 tagged with GFP. The reason to use these mutants was to determine if LF82 would have some effect disturbing autophagy on a strain that has normally high levels of autophagy. The eat-2 mutants have a defect in the pharyngeal function leading to an insufficient food intake, resulting on a DR model. This mutant pumps at a reduced rate comparing to wild-type. Eat-2 mutants can have their lifespan lengthened by 50%. An increased level of LGG-1::GFP-containing puncta in seam cells was described for eat-2 mutants, and also on dietary restricted animals.

As expected, Mah-34 mutants had significant higher levels of autophagy when compared to DA2123 wild-type mutants (Figure 26). However, when fed on E. coli LF82 the increase on autophagy was not as significant as the one described with the DA2123 strain. Intriguingly these Mah-34 mutants fed on LF82 were the hardest to analyze under the microscope, having their puncta slightly diffuser than the others. For that reason, also less worms and puncta were scored, which can have a reflection on the acquired results. Repetition of the experiment is necessary in order to validate the results obtained from the use of Mah-34 mutants.

Illustrative images of LGG-1::GFP puncta observed under the microscope are given below.

**Figure 27: Representative fluorescence images of DA2123 animals fed on HT115 bacteria (top) or LF82 bacteria (bottom).** There is a significant increase of LGG-1::GFP puncta (arrows) per seam cell on *C. elegans* fed with pathogenic bacteria *E. coli* LF82.
Figure 28: Representative fluorescence images of Mah-34 animals fed on HT115 bacteria (left) or LF82 bacteria (right). Mah-34 mutants have autophagy levels superior to those of wild-type, which reflects on an increased number of LGG-1::GFP puncta (arrows) per seam cell.

4.7. Effect of pathogenic *E. coli* LF82 on subcellular DAF-16 localization

DAF-16 is a FOXO transcription factor which plays a central role in the control of the stress response and longevity in *C. elegans*.

In wild-type well fed worms, DAF-16 is phosphorylated by AKT and therefore it is equally distributed throughout the cells.\(^{216}\) In response to stress, DAF-16 translocates from the cytoplasm to the nucleus, in order to enhance the expression of numerous target genes to protect the worm from stress.\(^{217}\) Heat stress also causes DAF-16 nuclear localization, a response that uses the JNK signaling pathway, since JNK-1 was found to interact directly with DAF-16 and modulate its nuclear translocation\(^{218}\).

Using the reporter strain TJ356, which expresses DAF-16::GFP, it was attempted to analyze the influence of infection with *E. coli* LF82 on the subcellular distribution of DAF-16. As a positive control in this study, TJ356 worms fed on *E. coli* OP50 were subjected to mild thermal stress, by placing the plates at 30°C during 4h. Under thermal stress, nematodes should display complete nuclear localization of DAF-16, however that was not the case in this study (Figure 29). A previous report stating that *B. thuringiensis* infection on *C. elegans* induced DAF-16 nuclear localization, after 4 hours of infection,\(^{219}\) was used as a reference for planning this experiment. On this experiment, the positive control used were nematodes exposed for the same time periods to heat shock at 30°C, however a careful, extensive analysis of the literature denotes that the most commonly used temperature to induce thermal stress in *C. elegans* is 35-37°C.\(^{217}\) Therefore, it is suggested the repetition of this assay, using as a positive control worms under thermal stress exposed to higher temperatures, namely temperatures of 37°C.
Image 29: Representative fluorescence image of TJ356 worms exposed to 3 hours of thermal stress of 30°C. The animals were fed with OP50 control bacteria. After 3 hours of exposure to 30°C, DAF-16 localization remained cytosolic. Representative animals under a 5 x objective.

Translocation of DAF-16::GFP from the cytosol into the nucleus was not apparent in any of the experiments performed (Image 30). It could be assumed that exposure to pathogenic *E. coli* LF82 does not cause DAF-16 nuclear localization, but since the positive control on this experiment also failed to show nuclear localization of DAF-16, unfortunately no conclusions can be drawn from this experiment.
Image 30: Effect of *E. coli* LF82 infection on the subcellular DAF-16 localization. Representative fluorescence images of TJ356 worms exposed for 3 hours to (A) HT115 control bacteria and (B) LF82 bacteria. Subcellular distribution of DAF-16 was performed in two independent trials with an average of 15 nematodes per experiment analyzed under a fluorescence microscope. A 10x objective was used.

### 4.8. Effect of pathogenic *E. coli* LF82 on markers of the Unfolded Protein Response

Expression of *hsp-4*, encoding an ER chaperone protein, is induced under conditions of ER stress. HSP-4 is a homologue of GRP78/BiP, a chaperone and sensor of ER misfolded protein stress. As a chaperone, it helps refold misfolded proteins in this ER, and also engages ER-specific stress sensors that carry out UPR functions.

The strain SJ4005, which harbors the HSP-4::GFP gene construct was used to determine if the unfolded protein response was stimulated upon infection of *C. elegans* with LF82. Two different methods were used to access that. In one of the experiments L4 worms of the second generation of worms feeding with control bacteria or *E. coli* LF82 were used to detect *hsp-4*::gfp fluorescence under the microscope, and on the second experiment, L4 worms of the first generation were used, after being fed during 24 hours with control or pathogenic LF82 bacteria.

On L4 *C. elegans* from the second generation, GFP levels appear to be higher in worms fed with pathogenic LF82, comparing to worms fed in control bacteria (Figure 31). On the other hand, worms fed only during 24 hours with pathogenic bacteria, have a similar expression pattern of *hsp-4*::gfp, and the intensity of the fluorescence does not seem to be increased, when compared to worms on control bacteria (Figure 32).

In addition, comparing the intensity between the two experiments, worms from the second generation had higher fluorescence than worms of the second generation, condition that may be explained by the fact that the worms of the second generation were exposed not only more time to the pathogenic bacteria, but also to temperatures of 25ºC, which may enhance ER stress.
Image 31: ER stress response on *C. elegans* upon infection with *E. coli* LF82. Representative DIC and fluorescence images of SJ4005 worms exposed to HT115 control bacteria (left panel) and LF82 bacteria (right panel). L4 worms of the second generation were used in this experiment. Hsp-4::GFP expression is slightly increased on *C. elegans* exposed to pathogenic bacteria. The images are representative of fluorescence profile of worms from one experiment. Approximately 10-15 worms were visualized under fluorescence microscopy.

Image 32: ER stress response on *C. elegans* upon infection with *E. coli* LF82. Representative DIC and fluorescence images of SJ4005 worms exposed during 24 hours to HT115 control bacteria (left panel) and LF82 bacteria (right panel). L4 worms of the first generation were used in this experiment. Hsp-4::GFP expression is similar on both bacteria. The images are representative of fluorescence profile of worms from one experiment. Approximately 10-15 worms were visualized under fluorescence microscopy.
5. Discussion and Conclusion

The link between lipid metabolism and immunity in *C. elegans* is still not elucidated. As discussed in this thesis, lipids are very important molecules, not only to provide energy but also for signaling and structural functions. The present work aimed to address the role of polyunsaturated fatty acids on the innate immune response of *C. elegans*, bringing more knowledge to the ongoing research that uses the nematode as a model host.

This study began by investigating the importance of the elongation genes (*elo*) on *C. elegans* lifespan. To achieve that goal, RNAi technique was used, in order to knockdown selectively these genes, and to be able to identify which ones were important upon *C. elegans* infection with *E. coli* LF82.

It was possible to identify the elongase genes *elo-1* and *elo-2* as being important for the lifespan of the worm infected with LF82, since knockdown of these genes resulted in reduced mean lifespan of *C. elegans*. Interestingly, a previous study by Nandakumar et al., shows that *elo-1* mutants are more resistant to infection with *P. aeruginosa*. The results found in this work contradict what is described before, so more investigation using *elo-1* mutants is necessary, to understand its role on the innate immunity of *C. elegans*. Furthermore, as *elo-1* and *elo-2* act together on the elongation of PUFAs, it would be interesting to use a double mutant for these genes in order to elucidate the role of C20 PUFAs on defense against pathogens.

Similar results were found when silencing the genes *elo-4* and *elo-7* which is interesting, since not much attention has been put on these genes. However, the knockdown of these genes only decreased the lifespan of the nematode, and did not increase the susceptibility towards infection. A previous study shows that mRNA levels of *elo-7* were up regulated upon infection with *P. aeruginosa* strain PA14. To our knowledge, there are no more published papers relating *elo-7* with innate immunity, but following the evidences from that paper and from this work, it would be interesting to further deepen investigation around this gene relating to determine its importance for *C. elegans* defense against pathogen attack.

*elo-5* mutants, which lack C15iso and C17iso, had a decrease in the mean lifespan of almost 20%, when challenged with LF82, also suggesting a role for these monounsaturated fatty acids on *C. elegans* defense against pathogenic attack. *elo-6* mutants fed with LF82 have a lifespan similar to the control worms fed with LF82, thus knockdown of this gene does not increases susceptibility towards infection. Interestingly, the data acquired through gas-chromatography reveals that *elo-6* mutants have significantly higher levels of γ-linolenic acid when infected with *E. coli* LF82.
Using fat-3 mutants which lack a Δ6 desaturase, it was possible to show that the enhanced susceptibility towards infection with LF82 which these mutants exhibited was successfully rescued by dietary supplementation of the worms with 4mM of γ-linolenic-acid.

These data and data from previous works, seem to suggest an implication of this fatty acid on C. elegans immune response. It would be interesting to study in detail the role of gamma-linolenic acid on pathogenicity on C. elegans and also how these findings can translate into mammals, particularly humans.

On the contrary, genes that were knockdown by RNAi at the L4 stage tested in this study, namely elo-3 and atg-12 did not seem to affect C. elegans longevity, neither its susceptibility towards infection.

A previous study using RNAi for the atg-12 gene has shown a slight decrease on the lifespan of the worm, when compared to control worms. However, this effect was only seen when the second generation of worms was used. An interesting finding is that atg-12 RNAi in daf-2 mutants had a stronger shortening effect on lifespan, suggesting that autophagy may contribute to lifespan regulation at least in part through the insulin/IGF-1 pathway.

On the opposite hand, a study shows that among many other autophagy genes tested, lgg-3 mutants have extended lifespan compared to control and therefore they show that suppression of some autophagy genes can extend lifespan.

Therefore, previous studies where autophagy genes were suppressed seem to have different results concerning longevity on C. elegans. More studies on this area are of great interest, since clearly autophagy has an implication on the nematode lifespan.

RNAi against elo-3 on C. elegans at the larval stage L1 revealed to be lethal for the nematode. This effect could not be confirmed on the literature, so repetition of this experiment under different conditions is advisable, and to be confirmed, this is an interesting fact, since elo-3 may be required for proper development of C. elegans. A problem with the stock of dsRNA bacteria for elo-3 may not be excluded, since the bacteria may have undergone a mutation leading to C. elegans dead in this case.

The mRNA level of several genes was examined. All detoxification genes tested, including sod and gst genes, were found up-regulated on worms fed with pathogenic E. coli LF82.

A previous study shows that deletion of sod-1 slightly decreases lifespan of C. elegans, and on the other hand, its overexpression extended the lifespan of the worm, pointing a role for cytosolic O$_2^-$ toxicity in limiting the lifespan of C. elegans. However, it was later found that this sod-1 overexpression was leading to activation of DAF-16, and this should be the cause for the lifespan
This study by Cabreiro et al. shows the same effect in lifespan extension for the sod-2 gene.

sod-3 is a well-known target gene of DAF-16. The sod-3 gene is up-regulated in daf-2 mutants, in dauer worms, and also as a response to oxidative stress. Although the results are not statistically significant, they suggest that sod-3 is up regulated on C. elegans infected with E. coli LF82, which may result from the activation of the transcription factor DAF-16.

In a previous paper, using C. elegans as an infection model for E. faecalis, it was shown that the worm produced more ROS as a response to this pathogen, when compared to nonpathogenic bacteria Bacillus subtilis and Escherichia coli. This overproduction of ROS was accompanied with an increased expression of oxidative stress enzymes, particularly of sod-3, leading the authors to suggest a model in which the intestinal cells of C. elegans would produce ROS as a defense mechanism against E. faecalis, while the intestinal cells would produce intracellular antioxidants to protect any cellular damage caused by those ROS.

It is thus interesting to verify that all enzymes responsible for detoxification tested in this experiment had increased expression in C. elegans fed with pathogenic bacteria, when compared to worms fed on control bacteria. The reason for such a result may rely on the fact that the worm increased the levels of ROS production as a protective stress response to pathogen attack, and later used the superoxide dismutases to scavenge these species, and protect itself from the internal damage they can cause.

Expression of genes encoding superoxide dismutase gst-4 and gst-5 was also measured. Both genes were up regulated on C. elegans fed on LF82; however the results are not statistically significant.

A study shows that overexpression of gst-4 results in increased resistance to paraquat, but not to increased lifespan. Conversely, RNAi against this gene resulted in reduced daf-2 mutant longevity.

Interestingly, RNAi knockdown of the gene gst-5, but not of other gst genes tested, decreased lifespan in C. elegans.

It is still not clear the role that these genes have on C. elegans lifespan and longevity; however the data shown in this work, points out to an implication of these genes on the worms’ response to pathogenic bacteria, knowledge that can be further broaden using strains carrying GST::GFP reporter strains.

Expression of the FOXA homolog, pha-4, was also found up regulated on worms fed with pathogenic bacteria LF82.

PHA-4 was previously found up regulated as a response to dietary restricted worms. Moreover, this transcription factor was found to be required for autophagy. It would be interesting
to discover what genes act downstream of *pha-4* to find out its potential role on the *C. elegans* immunity, as well as for autophagy since its implication on this process is not completely elucidated.

The autophagy related genes tested (*atg* and *lgg-1* genes) were also found up regulated on *C. elegans* fed with pathogenic bacteria. These evidences suggest that there is enlarged stress on *C. elegans* upon infection, and that certain mechanisms such as autophagy are triggered.

Little by little, connections between immunity and autophagy are being made. In this study, it was possible to show that autophagy was increased on wild-type worms fed with LF82, by assessment of the number of LGG-1::GFP positive puncta per seam cell. Despite knockdown of the autophagy gene *atg-12* resulted in worms feeding with LF82 bacteria having a lifespan similar to worms feeding with wild-type bacteria, the data acquired from fluorescence microscopy, and the fact that autophagy genes are up regulated on worms infected with LF82 as determined by qRT-PCR, strongly indicates the implication of autophagy in bacterial pathogenesis on *C. elegans*.

The dynamic changes in the fatty acid composition were also observed in *C. elegans* infected with pathogenic *E. coli* LF82. In this work, it was possible to verify that the lipid profile of the worms was altered upon infection with LF82. The changes on the lipid composition may contribute to the *C. elegans* defense against pathogens, however further studies are necessary to conclude that.

To address the role of DAF-16 in *C. elegans* infected with LF82, a transgenic strain was used in order to determine the subcellular localization of this transcription factor. The results obtained indicate that DAF-16 remained on the cytoplasm, however as the positive control used on this experiment did not work properly, the experiment needs to be repeated in order to decipher the implication of this transcription factor on *C. elegans* infected with pathogenic bacteria. In fact, as sod-3 expression was found up regulated following infection with LF82 in this study, and as sod-3 is a known target of DAF-16, it is most likely that DAF-16 translocates into the nucleus to increase the expression of its target genes.

Additionally, a previous study reports DAF-16 as being necessary for the response mounted by *C. elegans* towards infection with *E. coli* LF82, as worms lacking this transcription factor are more susceptible to the pathogen.37

This experiment should therefore be repeated, and different times for exposure to the pathogenic bacteria should also be tested.

Finally, looking at the fluorescence expression pattern of *hsp-4::gfp*, it was possible to observe that *C. elegans* infected with LF82 displayed higher fluorescence levels, than worms cultivated in control bacteria. Elevated expression of *hsp-4::gfp* is indicative of the unfolded protein response, meaning that protein damage may be occurring.
Collectively, the results from this study demonstrate that *C. elegans* is capable of mounting a response to pathogenic attack caused by the adherent-invasive *E. coli* LF82. *C. elegans* has proved to be an efficient host-model towards infection with this fairly novel type of bacteria. Results show that silencing some genes responsible for PUFAs formation can increase the susceptibility towards this pathogenic bacterial strain, suggesting a role for these fatty acids on *C. elegans* immunity.

The study of the mechanisms of host defense, pathogen detection and host-pathogen interaction in *C. elegans* can elucidate the fundamental principles of innate immunity, and expand our knowledge and understanding about these processes in higher order hosts. Therefore, studies towards the understanding these mechanisms should be continued.
6. Future Perspectives

As previously said, there is still much work to do in order to understand how *E. coli* LF82 infects the worm, and how the nematode recognizes this attack and protects itself against infection.

It would be of great interest to study the necessary mechanisms to pathogen recognition by the intestinal epithelial cells. Due to the natural environment where *C. elegans* is inserted, the ability to distinguish the pathogenicity of the food source is of major importance. Understanding how the *E. coli* strain LF82 elicits an immune response in the worm intestinal cells would bring more knowledge about infections that result from a failure in the recognition process of pathogens and that are related to many human disorders like irritable bowel syndrome and Crohn’s disease.

The qRT-PCR performed shed some light on some of the changes happening at the expression level of some genes. It would be interesting to study the post-infection period not only after 24h, but also after 48h and 72h as it would reveal the worms’ response to bacteria. As the work by Simonsen *et al.* demonstrates, there are more regulated proteins after 72h of infection rather than at 24h, suggesting that the majority of immune effectors identified are implied in bacterial clearance and not so much on the pathogen recognition strategy. Therefore, the experience performed in this study should be repeated, using the temperature-sensitive germ-line mutant *glp-4* or FuDR in an attempt to avoid degradation of RNA, as discussed before.

Since *C. elegans* cuticle has lipids in its composition, and due to the importance that this organ represents in the worms’ resistance to pathogens as a barrier, it would be interesting to study the exact role of fatty-acids in this context. Using a pathogen like *D. coniospora*, which produces spores that adhere to *C. elegans* cuticle, one could do screenings and survival assays using mutants for genes involved in lipid metabolism and possibly discover lipid species essential for the nematode immunity. For example, *fat-3* mutants show defects on the cuticle composition suggesting that LC-PUFAS are important fatty-acids for this structure.

Another interesting application for *C. elegans* is to use the nematode in drug screenings, in order to unravel new medicines with anti-microbial or immune enhancer effects. The possibility to perform high-throughput drug screening is being investigated and should further be explored, since the small round nematode holds great promise to the discovery of new compounds to tackle human pathogens.
The study about DAF-16 implication on infection with pathogenic bacteria should also be repeated. A previous work shows that knockdown of \textit{daf-16} increases \textit{C. elegans} susceptibility to infection with LF82, suggesting that this transcription factor is required for the immunity response mounted by the worm.\textsuperscript{37}

Besides DAF-16, another transcription factor that controls life span, HSF-1, has also recently been shown to affect pathogen resistance. HSF-1 regulates several heat shock proteins involved in the heat shock response.\textsuperscript{232} In this way, it would be interesting to study the possible involvement of other transcription factors and not only DAF-16.
7. Supplementary information

**Supplementary Table 1: Primers** - Primer sequences used in this study.

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<th>GENE</th>
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Supplementary Figure 1: Relative abundance of fatty acid species in *C. elegans* treated with RNAi for L4440, elo-1, elo-2, elo-5 and elo-6, and fed with *E. coli* HT115, expressed as percentage of total fatty acid as determined by gas chromatography analysis.

Supplementary Figure 2: Relative abundance of fatty acid species in *C. elegans* treated with RNAi for L4440, elo-1, elo-2, elo-5 and elo-6, and fed with *E. coli* LF82, expressed as percentage of total fatty acid as determined by gas chromatography analysis.
8. References


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