Caenorhabditis elegans as a model for *Candida albicans-Pseudomonas aeruginosa* co-infection and infection induced prostaglandin production

By

Nthabiseng Zelda Mokoena

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Study Leader: Prof. C.H Pohl-Albertyn

Co-study leader: Prof. J. Albertyn

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Declarations

"I, Nthabiseng Zelda Mokoena declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/published articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification of Microbiology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education."

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Signature Date

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"I can do all things through Christ who strengthens me" Phillipians 4:13

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Summary

The discovery of substantial commonality between microbial pathogenesis in mammals and invertebrate model hosts, such as the nematode *Caenorhabditis elegans*, has provided the foundation for genetic analysis of microbial virulence factors in live animal models. In most cases, *Candida albicans* yeast cells inhabit the human intestines, yet this opportunistic pathogen can led to host tissues invasion, causing life-threatening infections in immunocompromised hosts. Given the importance of this fungus to human health and its co-existence with other pathogenic microbes, particularly bacteria, such as emerging Gram-negative *Pseudomonas aeruginosa,* thus we used *C. elegans* as an infection model to study interactions between *C. albicans* and *P. aeruginosa*. Our goal was to firstly, successfully propagate and monitor the life cycle of *C. elegans* at 15 °C. Secondly, for this reason, we established a liquid medium assay using *C. elegans* model for *C. albicans* or *P. aeruginosa* monomicrobial infections. We demonstrate that the *C. albicans* yeast form establishes an intestinal infection in *C. elegans,* while the hyphal form is not required to efficiently kill the nematode. Furthermore, investigating mutants and genetically engineered *C. albicans* strains, we proved that hyphal formation is indeed not required for full virulence in this system. Thirdly we demonstrated that polymicrobial interactions are more virulent to *C. elegans* than monomicrobial species. We also aimed to understand the genetic mechanisms of virulence observed in *C. elegans in vitro*, since it was shown that not only does *C. albicans* and *P. aeruginosa* kill the nematode *C. elegans,* but also that *C. albicans* and *P. aeruginosa* virulence factors required for mammalian pathogenesis might also be required for efficient killing of *C. elegans*. Here our *in vitro* results suggested that there are multiple virulence factors of *P. aeruginosa* that may cause virulence, including pyoverdine, pyocyanin and swarming motility. Another factor that contributes to virulence is the hydrolytic enzyme production, known to facilitate pathogenicity of bacteria, protozoa, and pathogenic yeasts. Our results demonstrated that although *C. albicans* and *P. aeruginosa* possess a wide range of hydrolytic enzymes, proteinases are more predominantly associated with virulence. Furthermore, when comparing the effect of infection on the microbial burden of specific pathogens, from monomicrobial infections, it is clear that the virulence observed in killing of nematodes was not due to number of cells but rather specific virulence factors of the different strains. Surprisingly, from polymicrobial infections, we see that for both *P. aeruginosa* strains, co-infection resulted in an increased microbial burden. This is due to the fact that virulence of co-infection is strongly influenced by microbial burden and that this is dependent on the specific strains in the co-infection. For further understanding of the influence of virulence that underlie susceptibility to this pathogens, we used this pathogen model system to further evaluate the influence of

infection towards the nematodes fatty acid composition. Total lipids of *C. elegans* were extracted using chloroform and methanol [2:1 ratio (v/v)]. Fatty acids composition of the extracted total lipids was converted to their corresponding fatty acids methyl esters (FAMEs) and analysed by gas chromatography (GC). From the nematodes feeding on control *E. coli* OP50, we identified twenty-three different fatty acids ranging from 12 to 22 carbons in length, with 35 % being saturated, while 65 % being unsaturated. We then only focused on major unsubstituted long chain fatty acids (LCFAs), with margaric acid (17:0) being the predominant saturated fatty acid, comprising of an average of 24 % total major fatty acids. Through this process, after *C. albicans* and *P. aeruginosa* infection, we identified three fatty acids that have varying degrees of influence in *C. elegans*, namely linoleic acid (18:2n6), eicosapentaenoic acid (20:5n3) and docosenoic acid (22:1n9). Therefore, we observed changes in fatty acid profile being strain dependent, however there is no clear correlation between production of fatty acid and virulence. We further extended the usage of *C. elegans* infection models to investigate the influence of signalling molecules called prostaglandins, on infection. Here we show that only the co-infection synthesize prostaglandin E_2. Together, these results expand the use of *C. elegans* in the field of polymicrobial pathogenesis and provide further evidence of the likely importance of polymicrobial interactions. Since there is an urgent need for development of new antimicrobial agents, *C. elegans* which is known to evaluate different chemical compounds libraries could be used to solve some of the main obstacles in current antimicrobial discovery.

CHAPTER 1

Literature review

1.1 Motivation

Polymicrobial intra-abdominal infection involving fungi result in mortality rates between 50 % and 75 %, compared to 10 to 30 % for polymicrobial bacterial infections (Dupont *et al.*, 2002; Blot *et al.*, 2007). These infections have become resistant and this complicates diagnosis and treatment. Most of the polymicrobial fungal intra-abdominal infection are caused by the fungus *Candida albicans* (de Ruiter *et al.*, 2009; Hasibeder and Halabi, 2014). *Candida albicans* is most often discovered in a community with Gram negative bacteria, *Pseudomonas aeruginosa*. These microorganisms are both nosocomial pathogens that cause opportunistic infections in similar host niches. In addition to the ample evidence supporting polymicrobial interactions between *C. albicans* and *P. aeruginosa*, not only with each other, but also with their host, it is evident that the interaction is multifaceted. Various mechanisms contribute to the virulence of *C. albicans*, such as secreted hydrolytic enzymes, biofilm formation, adhesion/invasion molecules, and morphogenesis (yeast to hyphae switching), and can cause damage to hosts in order to establish aggressive and rapid colonization and infection (Mayer *et al.*, 2013; Ells *et al.*, 2014). For instance, the ability of *C. albicans* to transition between the yeast and hyphae state has been found to be significant for host infection. As such, during infection, the yeast form facilitate in dissemination, while the hyphal form is important for tissue invasion (Berman and Sudbery, 2002; Saville *et al.*, 2003; Mayer *et al.*, 2013). Moreover, it is also evident that several secreted virulence determinants of *C. albicans* (including farnesol) and *P. aeruginosa* (including *N*-acyl homoserine lactones, *Pseudomonas* quinolone signal, pyocyanin and various peptides) form radicals, triggering oxidative damage not only to one another, but also to the host (Fourie *et al.*, 2016). Despite many descriptions of *C. albicans* and *P. aeruginosa* co-inhabiting the same niches in the mammalian host and the frequency with which they are associated with polymicrobial infections, few studies have specifically evaluated how these two pathogens interact and affect each other in a particular host environment (Hofs *et al.*, 2016).

Non-mammalian infection models, such as, larvae of the greater wax moth (*Galleria mellonella*) and *Drosophila melanogaster* have been remarkably useful in the study of host-pathogen interactions (Brennan *et al.*, 2002; Mylonakis and Aballay, 2005; Chamilos *et al.*, 2006). However, the free living nematode, *Caenorhabditis elegans*, offers much better advantages over these models (Brenner, 1974; Corsi *et al.*, 2015). These include a fully sequenced genome, known cell lineages and a transparent body (Brenner, 1974; Kurz and Ewbank, 2000; Corsi *et al.*, 2015). Furthermore, its hermaphroditic nature (self-fertilization) allows reproduction of a large number of homozygous animals in a short period, and the

presence of males helps transfer mutations between strains (Brenner, 1974; Corsi *et al.*, 2015). It also has the advantage of a fast reproductive cycle, as the complete life cycle of *C. elegans* takes during two to three days at 20 °C (Brenner, 1974; Corsi *et al.*, 2015).

Moreover, the simple anatomy of the nematode and its wide range of forward and reverse genetic tools make these models ideal for discovering new biological functions and regulation for lipid metabolism (Vrablik and Watts, 2013). Lipids play an important role as building blocks of membranes, energy storage or second messengers and signal transducer. However, the lipid functional studies have been significantly underdeveloped, simply due to the difficulties in applying genetics and molecular methods to tackle the complexity related with lipid biosynthesis, metabolism, and also function. In the past decade, researchers have made attempts to analyse the roles of lipid metabolism and physiological functions using different animal models, and they also attempted to combine some of the genomics, genetics, and biochemical approaches. Remarkably these pioneering efforts together have not only provided understanding regarding lipid functions *in vivo* but have also established feasible methodology for future studies. Despite fatty acids being the major player in pathogen lipid signalling, they are not the only lipid molecules that affects signalling pathways in infections.

Although there has been studies pertaining the biological function of lipid mediators in monomicrobial pathogens and their hosts, there is still a gap that exists with regards to the knowledge concerning the production and role of lipid mediators of *C. albicans* and *P. aeruginosa* polymicrobial infections in host (Noverr *et al.*, 2001; Erb-Downward and Noverr, 2007; Fourie *et al.*, 2016). As a result of both pathogens possessing the ability to produce significant amounts of prostaglandins and other eicosanoids from exogenous arachidonic acid, this could affect the dynamics of this co-infection as well as host survival during infection (Erb-Downward and Noverr, 2007; Fourie *et al.*, 2016). This warrants investigation in order to completely gain insight of the polymicrobial interactions of *C. albicans* and *P. aeruginosa* with regards to eicosanoid production, specifically prostaglandins. This will enable us eventually to understand the role of these eicosanoids in the *C. elegans* model during co-infection.

1.2 Introduction

Non-human species are considered models to science because they are subjectable to laboratory experimentation. Studying these species is important to understand biological processes and the infection processes leading to human and animal diseases. Some important model organisms and the significant contribution they have made to biology are listed in Table 1. Among those models the *Caenorhabditis elegans* (nematode) (Brenner, 1974), *Drosophila melanogaster* (fruit fly), *Galleria mellonella* (wax moth) and *Mus musculus* (mouse) are now used as model systems in microbial pathogenesis, innate immunity, drug discovery and development (Mylonakis *et al.*, 2005; Marsh and May, 2012). Mammalian models (e.g. *M. musculus*), however, present several bottlenecks for broad pathogenic studies, such as laboratory expense, long reproduction and strong bioethical issues. Therefore animal models like *C. elegans* and *Drosophila* have been considered better alternatives (Brenner, 1974; Corsi *et al.*, 2015). Often these models are inexpensive and reproduce rapidly and can be easily cultivated and maintained under laboratory conditions. In addition, unlike mammalian models, the insect and nematode models are not subjected to ethical regulations for experimentation.

In the early 1970s *C. elegans* was selected as an experimental model organism, mostly due to its short life cycle and production of large number of progeny (Brenner, 1974; Corsi *et al.*, 2015). The life cycle of this nematode includes embryonic stage, larva stages (L1-L4) and adulthood (Corsi *et al.*, 2015). In the laboratory, nematode is typically grown monoxenically, feeding on *Escherichia coli* laboratory strains (Brenner, 1974; Corsi *et al.*, 2015). It can be preserved in a laboratory incubator at a temperature between 16 °C and 25 °C, most preferably at 20 °C (Maniatis *et al.*, 1982; Sulston and Hodgkin 1998). In addition, unfavourable environmental conditions such as absence of food and high temperature can trigger the *C. elegans* larva to undergo an unusual larval survival stage called dauer (Cassada and Russell, 1975; Golden and Riddle 1984; Hu, 2007). Furthermore, like humans, the nematode has specialized cells (neurons and muscles) and complex systems (digestive, reproductive and excretory) (Corsi *et al.*, 2015). It is also observed that this nematode and humans share genes and cellular processes (Culetto and Satelle 2000; Kaletta and Hengartner, 2006; Shaye and Greenwald 2011). Thus, all this advantages makes many *C. elegans* relevant to study human health and disease.

Studies showed that *C. elegans* can be infected by most bacterial and fungal pathogens that are of clinical relevance (Tan *et al.*, 1999; Pukkila-Worley *et al.*, 2009). For instance, *Pseudomonas aeruginosa*, which is a human opportunistic pathogen, was the first bacteria used to infect and cause death in *C. elegans* (Tan *et al.*, 1999; Mahajan-Miklos *et al.*, 1999;

Darby *et al.*, 1999). Furthermore, the nematode has been used in many studies to evaluate the virulence factors of the yeast, *Candida albicans*, which is also a human opportunistic pathogen (Pukkila-Worley *et al.*, 2009, 2012). Specifically Pukkila-Worley and co-workers (2012) showed that hyphal formation plays a crucial role in *C. albicans* virulence in *C. elegans* and that mutant strains of the yeast unable to form hyphae have decreased virulence. It is evident that most virulence factors contributing to the killing of *C. elegans* are also considered important in causing virulence in mammals (Rahme *et al.*, 1995; Tan *et al.*, 1999; Saville *et al.*, 2003; Pukkila-Worley *et al.*, 2009; Natalia *et al.*, 2013).

The nematode can activate protective mechanisms when confronted with pathogens (Irazoqui *et al.*, 2010). Unlike humans, the nematode lacks an adaptive immune system so it only depends on its innate immune defence (Pukkila-Worley *et al.*, 2011). Fascinatingly, this nematode shares similar innate immunity signalling cascades with mammals, in response to pathogen invasion, making it valuable to investigate infection. The immune response to these pathogens when introduced individually into *C. elegans* has been well studied (Irazoqui *et al.*, 2010; Pukkila-Worley *et al.*, 2011; Marsh and May, 2012) and interestingly, it was found that there are separate immune responses activated, depending on if the infection is due to a bacterium (such as *P. aeruginosa*) or a yeast (such as *C. albicans*). Most microorganisms of clinical relevance, elicit specific innate immunity mechanisms, which subsequently provoke expression of antibacterial or antifungal polypeptide. Lines of defence of the nematode is believed to be regulated by signalling pathways, such as, transforming growth factor β (TGFβ)/DBL-1, p38 mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), and insulin signalling/DAF-2 pathways. Despite the intensive research on using *C. elegans* model to study the monomicrobial infection of *C. albicans* and *P. aeruginosa* (Peleg *et al*, 2008), little is known about the polymicrobial interaction between *P. aeruginosa* and *C. albicans* specifically in this infection model. Studies on these *C. albicans* and *P. aeruginosa* interactions are very important, since microbes normally exists in nature as polymicrobial communities and often cause polymicrobial infections (Hogan and Kolser, 2002; Wang *et al.*, 2004).

Recent work in *C. elegans* has identified many regulatory proteins and downstream effector genes responsible for lipid homeostasis (Zheng and Greenway, 2012). Thus, capacity for polyunsaturated fatty acid synthesis makes *C. elegans* an excellent system to genetically dissect the biological function of various fatty acids with resolution not possible in mammals. Despite fatty acids being the major player in pathogen lipid signalling, they are not the only lipid molecules that affects signalling pathways in infections. Eicosanoids are one such family of lipids that plays roles in a number of pathogenic microbes (Fourie *et al.*, 2016). Despite the information that is currently available pertaining production of eicosanoids, with their

respective roles during monomicrobial infections, there is not enough knowledge available concerning the role of eicosanoids production during polymicrobial infections, as well as on the host (Fourie *et al.*, 2016).

Therefore this literature review will focus on the relevance of the *C. elegans* infection models. Important aspects of *C. elegans*, such as the life cycle and laboratory growth requirements will be highlighted. Also we will review *C. elegans* as model for the co-infection of prokaryotes-eukaryote pathogens. Moreover, although extensive studies have been done on *C. elegans*, the metabolomics and lipidomics studies on *C. elegans* are scarce, but number of publications related to this topic is increasing steadily. Within this review we also aim to give a general overview of lipids present *C. elegans*, and influence of infection on these lipids.

Table 1. Biological infection models (Gonzalez-Moragas *et al.*, 2015).

	Invertebrates		**Vertebrates**	
	Caenorhabditis elegans	***Drosophila melanogaster***	***Danio rerio***	***Mus musculus***
Common name	Worm	Fruit fly	Zebrafish	Mouse
Habitat	Terrestrial (soil)	Terrestrial	Aquatic (freshwater)	Terrestrial
Cultivation	Inexpensive and easy	Inexpensive and easy	Inexpensive and easy	Expensive
Space	Hundreds of animals in a 100 mm petri dish in laboratory	Bottles in a dedicated room	45-L aerated aquaria in a dedicated room	Cages in a dedicated room
Food	Bacteria	Fly food (water, agar, sugar, corn meat)	Adult shrimp	Pelleted mouse feed
Environmental conditions	16-25 °C	18-29 °C	25-31 °C	18-23 °C
Adult size	-1 mm x 70-90 µm	-3 mm x -2 mm	-4.5 cm x -1 cm	- 17 cm
Adult weight	-4 µg	200-250 µg	150-250 µg	17-25 µg
Gender	Hermaphrodite and ♂	♂ and ♀	♂ and ♀	♂ and ♀
Life cycle	Short (2-3 days)	Short (10 days)	Long (2-4 months)	Long (2-3 months)
Life span	2-3 weeks	-30 days	2-3 years	2-3 years
Number of offspring	-300	-400	100-200 eggs/clutch	40-100
Year genome sequenced	1998	2000	2013	2002
% Homology with humans	60-80 %	50-80 %	70 %	Up to 99 %
Automated high throughout assays	Possible at all stages	Only possible with larvae	Only possible with embryos	Not possible

1.3 Life cycle of *Caenorhabditis elegans*

Before the establishment of *C. elegans* model system, it is important to fully understand the life cycle of this small, free-living soil nematode. The nematode has an anatomically simple body structure that is an unsegmented, cylindrical body shaped with a tail that has tapered end (Figure 1) (Altun and Hall, 2005). The typical adult body plan consist of about 1000 somatic cells with different tissue types (Figure 1) (Altun and Hall, 2005). It has two sexes such as the hermaphrodites which are self-fertile and known to maintain homozygous mutations and males which are used mostly for genetic crosses (Altun and Hall, 2005; Corsi, 2006). The life cycle of *C. elegans* consist of different stages growing drastically in different sizes, this includes the embryonic stage, four larva stages (L1-L4) and adulthood respectively (Altun and Hall, 2005). Important to note is that at the end of each larval stage (L1-L4), the nematode undergo moulting process, whereby an old stage-specific cuticle is shed-off, while a new one is produced.

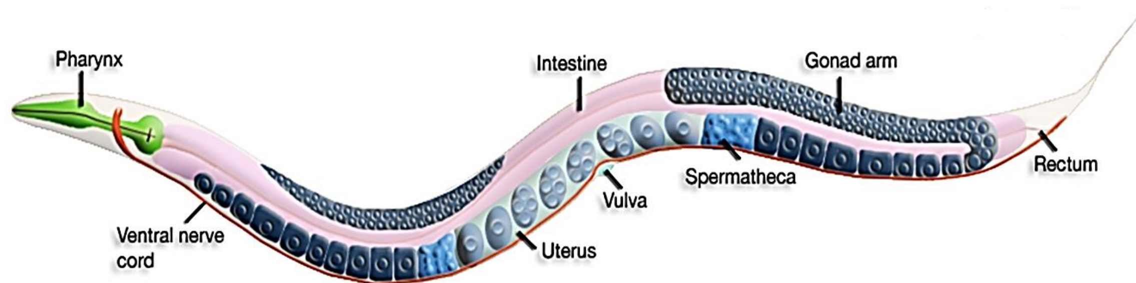


Figure 1. Schematic diagram of anatomical structures of *C. elegans* adult hermaphrodite (Altun and Hall, 2009).

1.3.1 Embryo

Embryogenesis begins inside the hermaphrodite, whereby the first cleavage takes place at about 40 min at 22 °C (Corsi *et al*., 2015). The embryo is then laid into the environment at about 150 min, through the vulval opening as it reaches the 30 cell stage. Once the embryo is outside the hermaphrodite, it takes about 13 hours for embryogenesis to be completed. The process of embryogenesis consist of the proliferation and organogenesis phase (Sulston *et al*., 1983). During proliferation, the single cells undergo cell division to a large number of undifferentiated cells (von Ehrenstein and Schierenberg, 1980; Wood, 1988; Bucher and Seydoux, 1994). In organogenesis, cells undergo terminal differentiation without additional cell

divisions. There is also a threefold elongation of the embryo, forming differentiated tissues and organs. In the threefold elongation stage, the nematode becomes motile inside the eggshell by rolling in pretzel configuration, indicating advanced motor system development. It is observed that after cell cleavage the embryo starts pharyngeal pumping at 760 min and immediately undergo hatching at 800 min (von Ehrenstein and Schierenberg, 1980; Sulston *et al.*, 1983; Bird and Bird, 1991). Therefore at the end of the embryogenesis, the main body plan of the nematode becomes evident, and does not change during postembryonic development.

Postembryonic development

Based on the availability of food, the larva is triggered to undergo the postembryonic development program which proceeds three hours after hatching (Ambros, 2000). However, in the absence of food, the development of embryos stops and can last 6-10 days without food. Once there is food, the embryos undergo normal moulting and development (Johnson *et al.*, 1984; Slack, and Ruvkun, 1997).

1.3.2 Larval stages

Following completion of embryogenesis, *C. elegans* first larvae (L1) emerge from the eggshell and begin feeding. Under certain conditions, such as, low population and plentiful food, the larvae undergoes different characteristic developmental events in each of the four larval stages (L1-L4) before moulting into reproductive adults (Figure 6). Also, at the end of each larva stage, the nematode undergoes moulting, where stage-specific cuticle is shed off and the new one develops (Raizen *et al.*, 2008). This process consists of three steps (Cassada and Russell, 1975; Corsi *et al.*, 2015). The first step is an apolysis, whereby the old cuticle separates from the nematode hypodermis. This is followed by the second step whereby new cuticle develops from the hypodermis, and finally, ecdysis or shedding of the old cuticle. However, before apolysis, there is a ceasing in pharyngeal pumping, leading to the nematode entering a lethargus state (Bird and Bird, 1991; Raizen *et al.*, 2008; Corsi *et al.*, 2015).

L1 Larva

The presence of food after hatching enables the larva to enter the larval L1 stage, which lasts for 12 hours. The length of the larva is about 250 μm as it enters the L1 stage. As depicted in figure 2, in the nervous system of this nematode, about five classes (ASn, VAn, VBn, VCn and VDn) of the eight classes (ASn, DAn, DBn, DDn, VAn, VBn, VCn and VDn) of motor neurons

are created from 13 precursors (W, P1-P12) at the end of L1 stage (Sulston *et al.*, 1976; Sulston and Horvitz, 1977; Chalfie and White, 1988).

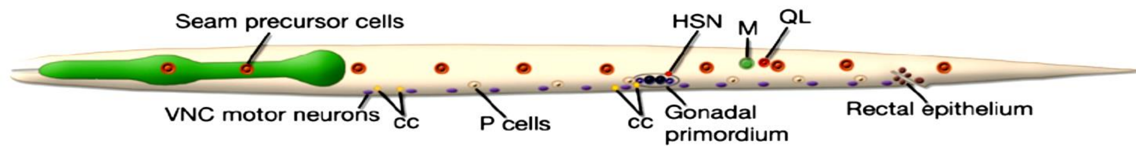


Figure 2. The L1 larva. Located on the right are the M cell and the anterior ventral pair of coelomocytes (cc). In the middle plane are the rectal epithelial cells, and at the ventral midline are the VNC motor neurons. Some of the remaining cells are located at the left lateral side. The seam precursor cells are H1 (anterior most), H2, V1-6 and T (posterior most) cells. The P cells are P1/2 at the anterior to P11/12 at the posterior. HSN are called hermaphrodite-specific neuron (Altun and Hall, 2005).

It is also observed that other types of neurons are created from H2, G1, Q and T blast cells. In the reproductive system, during the second half of L1, the Z1 and Z4 somatic gonad precursors normally give rise to 12 cells, while Z2 and Z3 germ line precursors divide accordingly (Figure 3) (Kimble and Hirsh, 1979). While in the coelomocyte system, two dorsal coelomocytes are produced by the M mesoblast in the hermaphrodite.

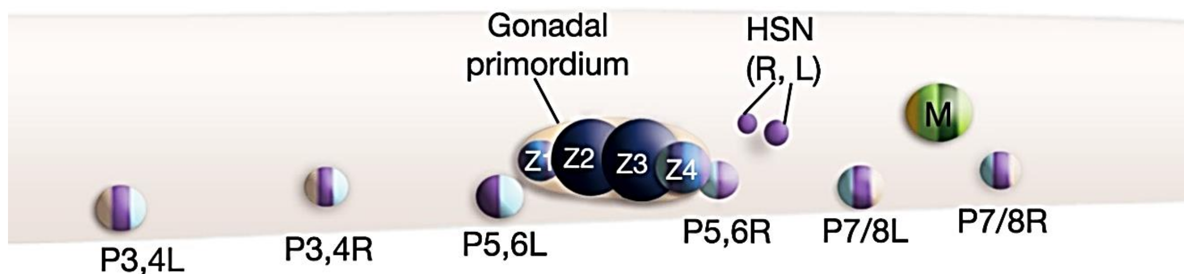


Figure 3. The L1 gonad primordium (Z1-Z4) (Lints and Hall, 2009).

L2 Larva

Following the L1/L2 moulting process, the nematode enters the larva L2 stage that lasts for eight hours. During the L2 stage, V5.pa produces postdeirid sensilla, while G2 specifically forms two ventral ganglion neurons in the nervous system. Meanwhile, in the reproductive system, Z2 and Z3 germ cells undergo cell divisions while Z1 and Z4 somatic gonad precursors does not. Then the somatic and germ cells rearrange and are organised like future gonads (Kimble and Hirsh, 1979). The gonad further elongates, and this is led by the distal tip cells (DTC).

L3 Larva

Following the L2 stage, the nematode enters the larval L3 stage. This stage lasts for eight hours and the nematode has now elongated to about 490-510 µm in length. Here, the somatic gonad precursors give rise to about 143 cells producing the anterior and posterior gonadal sheaths, uterus and spermathecae in the reproductive system (Kimble and Hirsh, 1979). The gonad arms continue elongating in opposite directions until the middle of L3. The distal tip cells stop and reorient themselves slowly in dorsal directions (Antebi *et al.*, 1997). One can also trace the vulva precursor fates and the committed cells divide to produce vulva terminal cells by early L4. Moreover, from the two sex myoblasts produced in L3, 16 sex muscle cells are generated.

Dauer Larva

As mentioned above, availability of food is of great importance during the life cycle of this nematode. The presence of food enables the larva to undergo four stages punctuated by moulting at the end of each stage, while its absence arrests the L1 larva (Slack, and Ruvkun 1997; Hu, 2007; Corsi *et al.*, 2015). This arrested stage is named dauer larval stage, and its signalling begins in the middle of the L1 (Golden and Riddle 1984; Corsi *et al.*, 2015). Research also showed that absence of food is not the only environmental factor that triggers this stage, but also high temperature and presence of pheromones (Riddle, 1988). The nematode can last for about 4 months in this state. During this stage, dauers are non-feeding and developmentally arrested with restricted movement (Cassada and Russell, 1975).

Dauers possess specialized adaptations that allows them to survive harsh conditions for a long period of time (Figure 4). They have a cuticle that is very thick protective that covers the whole body protecting the body from harsh environmental factors such as detergents and toxins (Cassada and Russell, 1975).The thickened cuticle also occludes the buccal opening

to prevent feeding and dauers cease pharyngeal pumping (Albert and Riddle, 1983). The dauer body within the cuticle becomes radically constricted with cells being compacted. This radical constriction are dependent on the autophagy pathway, known to remove and recycle waste and intracellular materials (Meléndez *et al.*, 2003). The autophagy pathway shrinks the muscles, intestines and hypodermal cells through catabolizing cellular components not required during diapause. Thereafter, the remaining cellular structures are enclosed by the dauer cuticle to a smaller volume (Cox *et al.*, 1981). The thin and stiff dauer body structure plays an important role in the specialized dauer behaviours including characteristic locomotion patterns and nictation. Once the conditions are favourable, the nematode is able to exit the dauer stage within one hour of presence of food. Thereafter it begins feeding after two to three hours and moults to the L4 stage after 10 hours (Cassada and Russell, 1975; Riddle, 1988; Sulston, 1998).

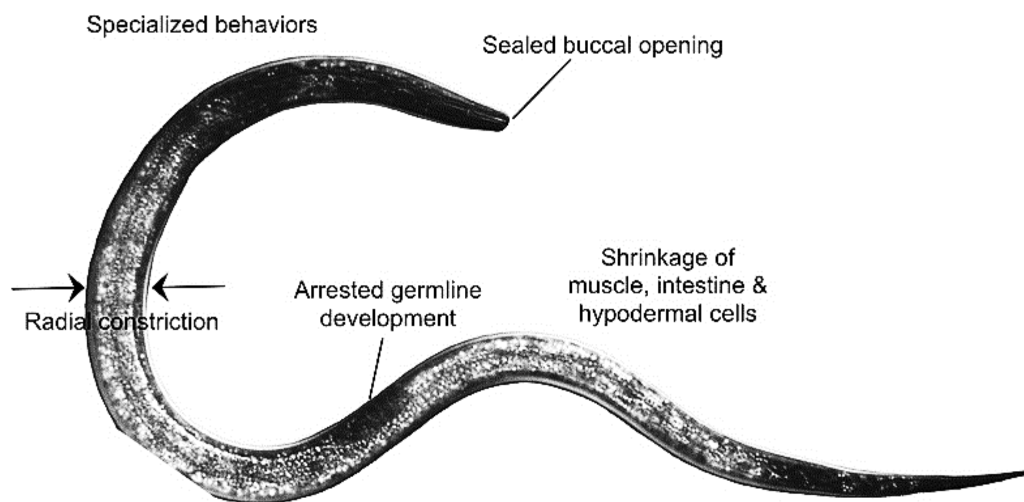


Figure 4. Adaptations of the dauer larvae favouring survival and dispersal. Dauers exhibit a thickened cuticle that overlies and seals the buccal opening, preventing feeding. Dauers are radially constricted with shrunken tissues and display specialised behaviours including nictation and characteristic locomotory patterns. Germline development is arrested (Wolkow and Hall, 2015).

L4 Larva

The last stage of larva is called the L4 stage which lasts for 10 hours. In the reproductive system, there is completion of the process of gonadogenesis, which was established seven hours after hatching. Also the distal gonad arms continue elongating in a circular manner along the dorsal body wall muscles and complete this at the L4/adult moult (Antebi *et al.*, 1997). Furthermore, at L3/L4 moult, meiosis in the germ line takes place in the arms of the gonads and germ cells turn into matured sperm. Then at L4/adult moult, production of sperm stops and the remainder of the germline cells undergo meiosis and differentiate to become oocytes. In the reproduction system there is also generation of vulva and uterine terminal cell followed by tissue morphogenesis. This structures further association with egg-laying neurons and sex muscles to form the egg-laying apparatus in the nematode (Figure 5) (Greenwald, 1997).

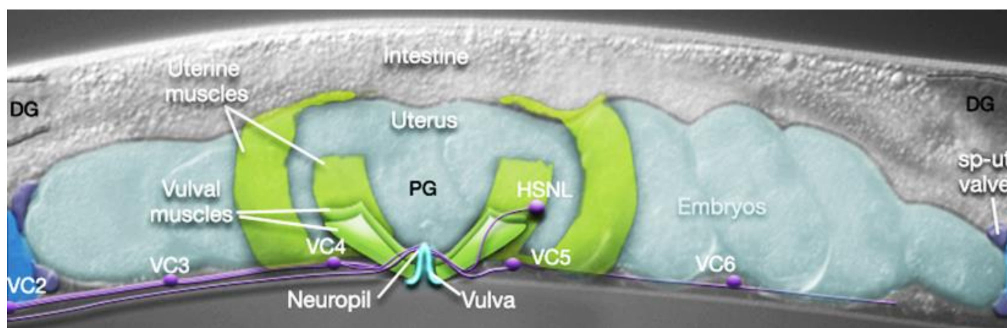


Figure 5. Reproductive system of *C. elegans*. Lateral left side. Dorsal gonad (DG), proximal gonad (PG) and motor neurons (VC1-6 and HSNL) (Lints and Hall, 2009).

1.3.3 Adulthood

The nematode enters adulthood when it is about 900-940 μm long as a young adult, where it takes about 8 hours to become a matured adult (1110-1150 μm) (Figure 6) (Corsi *et al.*, 2015). During hermaphrodite development, about 1090 somatic cells are generated and from this 131 are known to undergo programmed cell death (Driscoll, 1995). This leaves the adult hermaphrodite with 959 somatic nuclei with 302 being neurons and 95 being body wall muscle cells (Chalfie and White, 1988; Driscoll, 1995). Once the young adult is matured, it is able to lay eggs and this leads to the nematode completing its reproductive life cycle within three days (Byerly *et al.*, 1976; Lewis and Fleming, 1995). After reproduction the adult nematode can survive for about 10-15 days before it dies. A self-fertilising hermaphrodite produces

approximately 300 progeny compared to a hermaphrodite that mates with a male which produces 1200-1400 progeny.

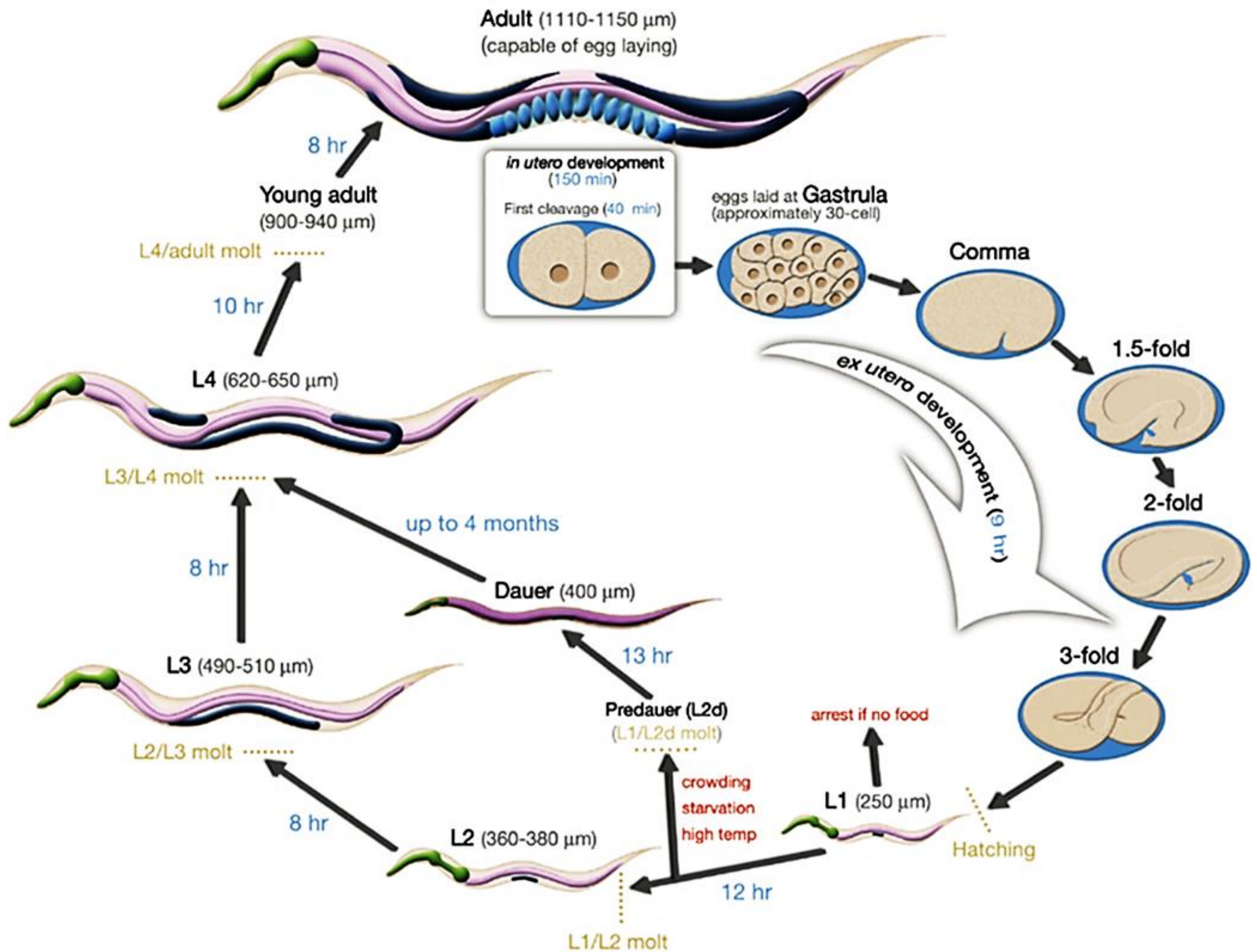


Figure 6. The typical life cycle of *C. elegans* at 22 °C (Altun and Hall, 2009).

1.4 Laboratory growth requirements

In natural environments, *C. elegans* is mostly isolated from temperate soil environments and feed on bacterial species that decay plant decompositions (Nigon, 1949; Nicholas *et al.*, 1959; Barrière and Félix 2014; Corsi *et al.*, 2015). Even though often characterised as soil nematode, its larval and adult forms have also been isolated from rotting fruit, organic-rich garden soils, plant stems, and compost (Félix and Duveau, 2012). This nematode mostly favours compost-like environments since they are more abundant in bacterial food sources. Moreover, the

dauer larvae is also found outside of rotting fruits and stems (Figure 7). However, in the laboratory, the nematode is maintained on Nematode Growth Medium (NGM) agar, where it is grown monoxenically, feeding on uracil auxotroph *Escherichia coli* OP50 strain (Brenner, 1974). This strain is advantageous because it shows limited bacterial lawn on the NGM plates, improving the probabilities of better observation and mating of the nematodes (Brenner, 1974; Corsi *et al.*, 2015). Importantly, the nematodes need to be subcultured, to avoid overgrowth or starvation. After plating *C. elegans*, one can observe growth of bacteria first, then after two to three days, trails of the nematode become visible on the lawn of bacteria.

It is of great importance to ensure that stocks of *C. elegans* are preserved between 16 °C and 25 °C, preferably 20 °C (Maniatis *et al.*, 1982; Sulston and Hodgkin, 1998). In some instances, this stocks can be contaminated with other bacteria, yeasts or moulds. Bacteria and yeasts can easily be removed using hypochloride solution and are not necessarily harmful to the nematode. This treatment destroys the contaminant and all nematodes that are without egg shells. In contrast, moulds can be removed by permitting the nematode to move away from the moulds, and through chunking and serial transfer. For long term preservation, *C. elegans* can be kept as frozen stocks and kept in liquid nitrogen (-196 °C) (Brenner, 1974). Interestingly, methods used to store stocks of this nematode in liquid nitrogen were similar to those used for mammalian cell lines, further facilitating their use as model organisms (Sulston and Hodgkin, 1998). There are specific factors that must be considered when freezing. For instance, freshly starved young larva (L1-L2) should be used and 5 % final volume of glycerol should be added to the freezing media as cryoprotectant. Freezing should also be gradual at -80 °C. After about 12 hours at -80 °C, the frozen stocks can be transferred in liquid nitrogen for long term storage (Brenner, 1974). About 35-45 % of the total number of frozen nematodes can be recovered from the stocks stored in liquid nitrogen.

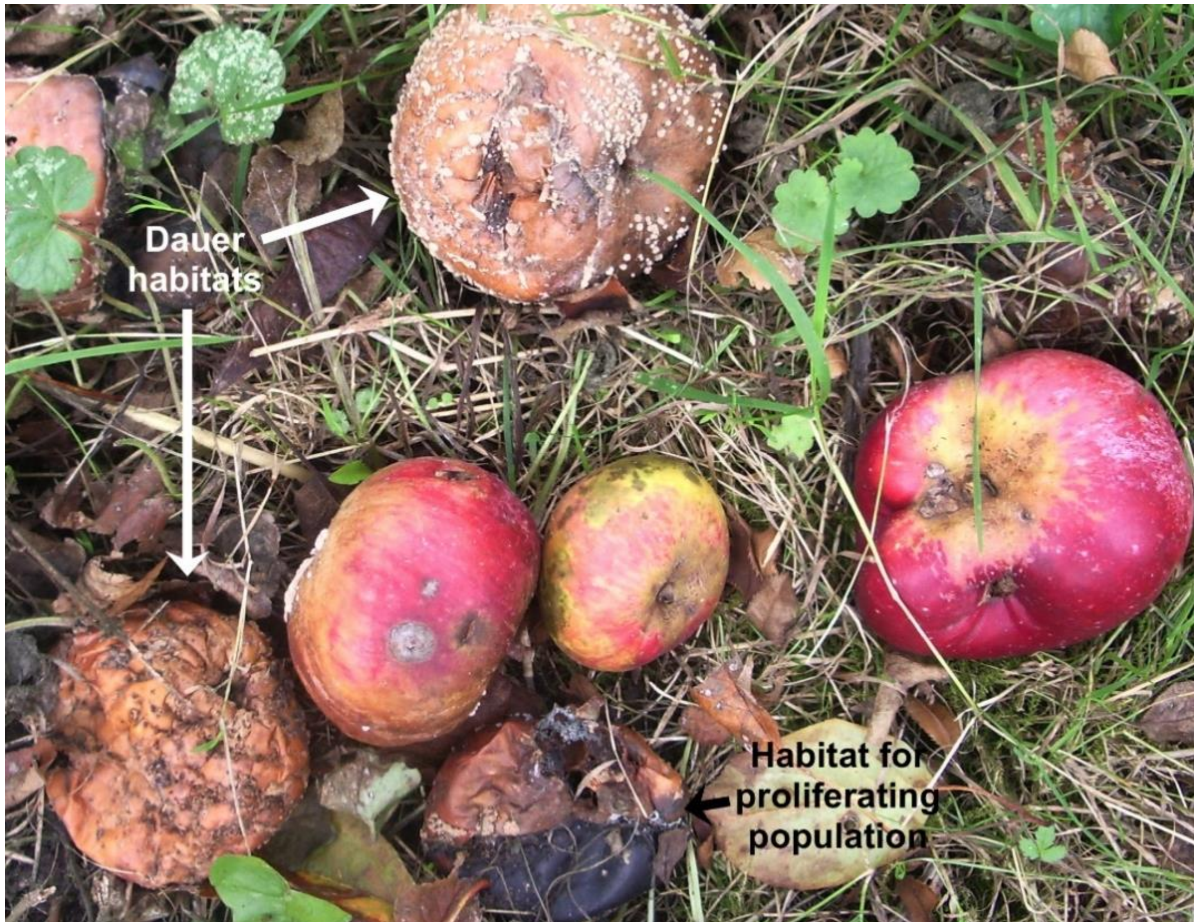


Figure 7. Fruit orchard as a natural habitat for *C. elegans***.** As indicated at the lower center, there are proliferating *C. elegans* populations growing in a decayed fruit. At the left lower and upper the fruits at a lesser stage of decay contained only dauers (Wolkow and Hall, 2015).

1.5 Relevance of *Caenorhabditis elegans* infection model

Given the advantages of *C. elegans* under laboratory conditions, such as simple cultivation and ease manipulation using variety of molecular and genetic methods (Brenner, 1974; Kurz and Ewbank, 2000). And also a short life cycle with a single adult able to produce a large number of progeny (Corsi *et al.*, 2015). The fact that it is transparent enables better observation of cellular events throughout its life cycle and allow usage of fluorescent markers for gene expression (Chalfie *et al.*, 1994; Corsi, 2006). Furthermore, it is one of the multicellular organisms that has a complete sequenced genome and through this a conservation of genes and cellular mechanisms has been observed similar to that of humans (Culetto and Satelle, 2000; Kaletta and Hengartner, 2006; Shaye and Greenwald, 2011). Importantly, there are no ethical regulations for experimentation with these nematodes. In addition to being a genetically tractable model, *C. elegans* has also been used in studies of

microbial pathogenesis, focusing on host-pathogen interactions, innate immunity and drug discovery and development (Alper *et al.*, 2008; Marsh and May, 2012). These organisms are very useful to investigate specific virulence traits of the pathogen and their role in infection. The fact that they are highly susceptible to human pathogens, makes it advantageous to be regarded as an infection model in order to perform intensive studies on host-pathogen interactions (Tan *et al.*, 1999; Pukkila-Worley *et al.*, 2009). At present, more than 40 human pathogens, including bacterium, *Pseudomonas aeruginosa*, and yeast, *Candida albicans*, are known to cause diseases in *C. elegans*. Thus, infection processes in *C. elegans* has been shown to closely resemble chronic infection in humans. In addition, the nematode shares similar signalling cascades of innate immune response with humans, such as extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinases (MAPKs), transforming growth factor β (TGFβ)/DBL-1),and β-catenin and insulin signalling/DAF-2 pathways (Kurz and Ewbank, 2003; Alper *et al.*, 2008; Zugasti and Ewbank, 2009). *Caenorhabditis elegans* is particularly useful in the studies of intestinal epithelial innate defences since like mammalian intestinal epithelia, it has 20 cells that are not shed and non-renewable (Irazoqui *et al.*, 2010). This is advantageous because it allows the *in vivo* studies of defence functions, without confounding tissue repair and cell proliferation. Therefore this biological model allows studies to be done entirely on epithelial innate defences since there is no adaptive immunity (Sifri *et al.*, 2005). Moreover, due to their small size and the possibility to perform assays in microdilution plates, *C. elegans* offers an excellent model to perform large screenings of antimicrobial compounds. Overall, screening of the whole animal *C. elegans* can facilitate in the identification of compounds that would be difficult to identify with *in vitro* screens or even be expensive and inefficient when using an *in vivo* mammal approach (Pukkila-Worley *et al.*, 2011). However, there are important limitations that need to be highlighted concerning the use of *C. elegans* model system in order to study human diseases. It is important to perform assays at lower temperature, since the physiological temperature (37 °C) is lethal to *C. elegans* (Maniatis *et al.*, 1982; Sulston and Hodgkin, 1998). Despite the fact that there is conservation of pathogenesis mechanisms between the nematode and mammals, there are also huge differences. In mammalian hosts, pathogens are often internalized then disseminate throughout the host, in contrast majority of infections in *C. elegans* are not intracellular colonized or disseminated throughout the host.

1.6 *C. elegans* as infection model for pathogenic yeasts

1.6.1 *C. elegans-C. albicans* infection model

Since the establishment of *C. elegans* model system, research towards usage of this nematode as an infection model has expanded rapidly, and now investigations are based on wide range of human pathogens. Recently, researchers are making use of *C. elegans* model to closely study many yeasts species (Aballay and Ausubel, 2002; Arendrup *et al.*, 2002; Pukkila-Worley *et al.*, 2009). For instance*, Candida albicans*, which is an opportunistic pathogen inhabiting the human microbiome causing infection in susceptible hosts, has been used to infect the *C. elegans*. The most studied virulence factors of *C. albicans* is the yeast-hyphal transition, where the yeast form switches to hyphal form. Importantly, this hyphal form facilitates in tissue destruction and host invasion (Berman and Sudbery, 2002). Researchers revealed that *C. albicans* is capable of establishing persistent infections in the intestines of *C. elegans* and produce hyphae that has the ability to penetrate host tissues and pierce the nematode cuticle (Breger *et al.*, 2007; Pukkila-Worley *et al.*, 2009). Therefore, like in mammals, this hyphal morphogenesis appears to be one of the important mechanism to kill the nematode (Breger *et al.*, 2007; Pukkila-Worley *et al.*, 2009). However mutants that cannot form hyphae seemed to be avirulent, implying that hyphal morphogenesis might be one of the mechanism of nematode killing (Breger *et al.*, 2007). Interestingly, the mode of entry for *Candida* infection in nematodes and human infection is both through gastrointestinal tract. Moreover, Pukkila-Worley *et al.* (2009) showed that the environmental factors within the nematode are similar to those in human gastrointestinal tract and they induce hyphal formation in *C. albicans*. Furthermore, *C. elegans-C. albicans* infection model used *in vivo* have more benefits compared to other models, in such that it is easier to evaluate the *Candida* dimorphism and visualize the formation of hyphae in nematodes (Pukkila-Worley *et al.*, 2009). The fact that the nematode is transparent, also enables proper examination of the stages of infection of fungal cells within the intestines (Pukkila-Worley *et al.*, 2009).

For instance, Pukkila-Worley and co-workers (2009) when examining the intestines they observed hyphae piercing cuticle of infected nematodes. They speculated that the hyphal formation was occurring post-mortem and thus was more of an indicator for aggressive infection, rather than a virulence determinant. In addition to *C. albicans*, studies were done on other fungal species (*Debaryomyces hansenii* and *Candida lusitaniae*) that are incapable of hyphal formation (Pukkila-Worley *et al.*, 2009). Results showed that the hyphal forming *C. albicans* strains (DAY185 and SC5314) were more virulent towards *C. elegans* than the other two yeast species incapable of forming hyphae (*D. hansenii* and *C. lusitaniae*) and also than *E. coli* OP50 bacterial food source (Figure 8A). Moreover, the two yeast species (*D. hansenii*

and *C. lusitaniae*) killed *C. elegans* at a slow rate, however only the yeast cells and no hyphae was observed in the intestines of the nematode, suggesting that they killed nematode using a yeast-dependent process (Figure 8C and D) (Pukkila-Worley *et al.*, 2009).

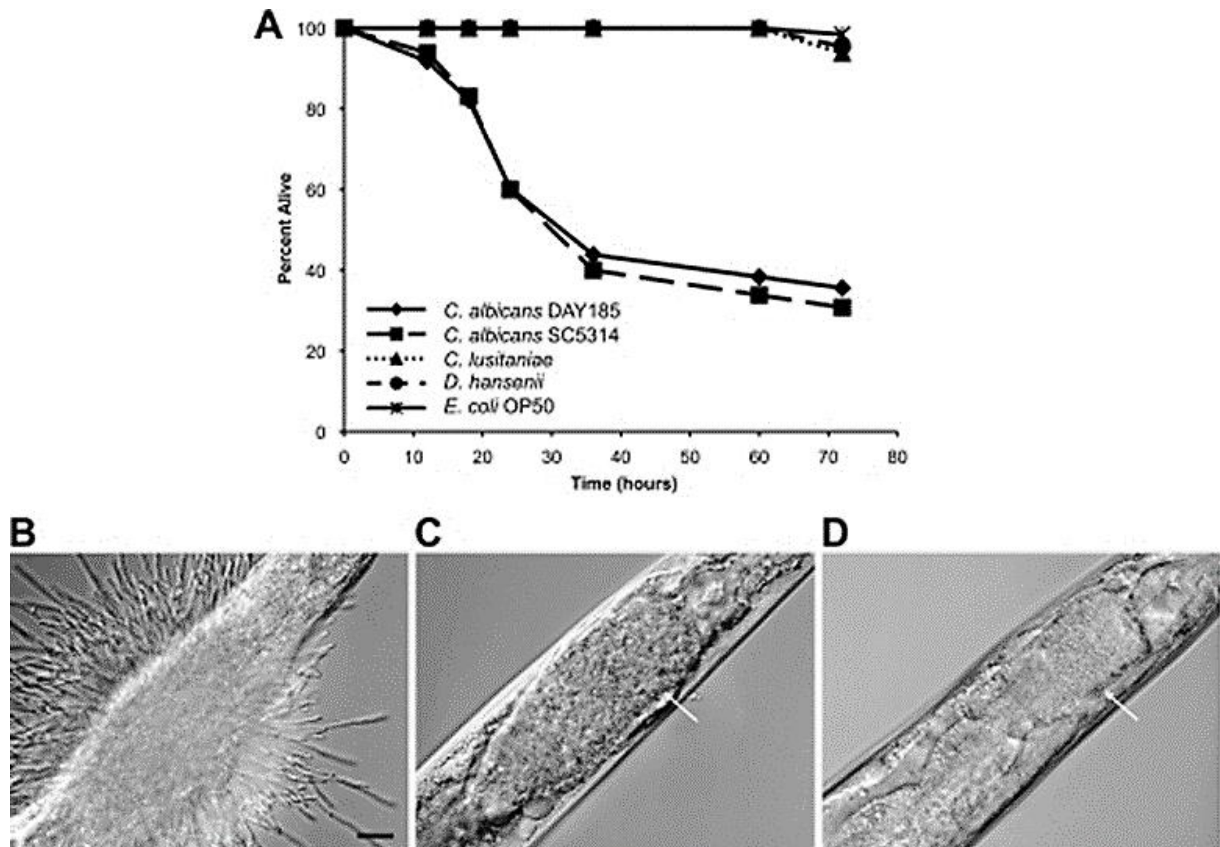


Figure 8. Yeast mediated killing of *C. elegans*. (A) The *C. albicans* SC5314 and the *C. albicans* DAY185 strains were more virulent towards *C. elegans* in a liquid medium killing assay than *D. hansenii*, *C. lusitaniae* and *E. coli* OP50 during the first 72 h of infection. Micrographs of nematodes infected with (B) *C. albicans* DAY185 with hyphae piercing the cuticle, (C) *D. hansenii* with only yeast cells, and (D) *C. lusitaniae* with only yeast cells. White arrows indicates to the intestinal lumen. The scale bar shown in figure 8B equates to 20 μm (Pukkila-Worley *et al.*, 2009).

1.6.2 *C. elegans* infection with other pathogenic yeasts

Other studies were done on virulence factors of *Cryptococcus neoformans* and *Histoplasma capsulatum* using *C. elegans* infection assays (Mylonakis *et al*., 2002, 2004; Johnson *et al*., 2009). Interestingly, *C. neoformans* uses a different approaches than *C. albicans* to facilitate killing in the nematode. Since cell of *C. neoformans* are larger than *Candida* cells, they are unable to persist in the intestines of *C. elegans*, instead they are mostly concentrated at the area directly distal to the pharyngeal grinder (the organ that functions to disrupt ingested organisms). Nematodes allowed to feed on cryptococcal lawn are capable of defecating the cryptococcal cells once they are transferred into the liquid media, helping to get rid of the cryptococcal infection (Mylonakis *et al*., 2002). However, it was suggested that capsule and/or melanisation is highly associated to the *C. neoformans*-mediated *C. elegans* killing (Mylonakis *et al*., 2002). Moreover, Johnson and co-workers (2009) reported that the yeast, *H. capsulatum* is able to transit the pharynx and pass through the intestine as intact yeast. They observed that only live, virulent strains produced rapid lethality in the nematode and that the rapid killing was due to production of toxic yeast metabolites (Johnson *et al*., 2009).

Despite the fact that this nematode is proven to be one of the most important model system to study fungal virulence and also for identification of novel antifungal compounds (Breger *et al*., 2007; Okoli *et al*., 2009; Arvanitis *et al*., 2013), challenges still arise that limits the nematode to fully replace mammalian models for studies on virulence factors as well as drug development and discovery. There is an evolutionary distance and also physiological differences between nematodes and humans. Additionally, most human pathogens survive at 37 °C and this restricts their studies in *C. elegans*, since high temperatures like 37 °C can be lethal to the nematode (Maniatis *et al*., 1982; Sulston and Hodgkin 1998).

1.6.3 Immune response

In nature, *C. elegans* nematodes encounter several threats from ingested pathogens (Irazoqui *et al*., 2010). Exposure of *C. elegans* to pathogens activates three major protective mechanisms. Firstly, it uses physical barriers such as cuticle and pharyngeal grinder. Secondly, an avoidance behaviour, which relies on chemosensory neurons that sense pathogens and induce escape. This is triggered by recognising the pathogen through Toll-like receptor, TOL-1, and detecting microbial molecules like cyclic pentadepsipeptide biosurfactant, serrawettin W2. Thirdly, if the pathogen cannot be avoided, the nematode triggers specific innate immunity mechanisms, then provokes expression of antimicrobial polypeptides (Irazoqui *et al*., 2010). Coordination of these defences involves several highly conserved elements that have mammalian orthologues (Kim *et al*., 2002; Pukkila-Worley *et*

al., 2011). However, unlike humans, the nematode relies solely on innate immune defence since it lacks an adaptive immune system (Pukkila-Worley *et al.*, 2011). Fascinatingly, there is a striking resemblance between the *C. elegans* intestinal epithelial cells and human intestinal cells and due to lack of both circulatory system and cells dedicated to the immune response in the nematode, their intestinal epithelium establishes the primary line of defence against ingested pathogens (Troemel *et al.*, 2008). Thus, it is possible to conduct appropriate analyses for innate immune mechanisms in this *C. elegans* model.

In the innate immunity of *C. elegans*, there are different proteins that are involved in response mediated processes to pathogen infection. When *C. elegans* is infected, firstly it uses receptors to recognize a pathogen or damages induced by a pathogen. *Caenorhabditis elegans* Toll-like receptors (TLRs) function by launching an appropriate immune response against a pathogen. The TLRs share a similar structure, and contain an intracellular Toll-Interleukin-1 receptor (TIR) domain and ectodomain of leucine-rich repeats. A TIR domain adaptor protein TIR-1, orthologous to mammalian sterile alpha and TIR motif-containing protein 1 (SARM), usually functions by activating the PMK-1 pathway in *C. elegans* innate immunity. Secondly, a complex innate immune response is triggered, involving signalling cascades that uses proteins and transcriptional regulators to direct changes in gene expression (Figure 9). The three well characterized core signal transduction pathways are DBL-1 pathway, mitogen-activated protein kinase (MAPK) and DAF-2/DAF-16 pathway (Murphy *et al.*, 2003). For instance, MAPK pathway has a central role in resistance to microbial pathogens. Furthermore, pivotal regulators of MAPK, such as p38, have been seen to have a critical role in MAPK pathway which regulates innate immunity in *C. elegans*. Interestingly subsequent studies have implicated this pathway in host resistance to majority of fungal and bacterial pathogens. Thirdly, some antimicrobial peptides (AMPs) and proteins, such as cytokines function as effector mechanisms of innate immune response to control infection (Figure 9).

Pathogens like *C. albicans*, with cell walls that consist of chitin polysaccharides, mannan and glucan can be easily recognised by the host (Gow and Hube, 2012; Gow *et al.*, 2012). The yeast form of *C. albicans* can cause an intestinal infection in *C. elegans*. So host response to this pathogen is mostly mediated by pattern recognition, pathogen-associated molecular patterns (PAMPs). Pukkila-Worley *et al.* (2011) discovered that in response to fungal pathogens, the transcription of antibacterial immune effectors of nematodes are selectively repressed. Thus, nematodes selectively mount specific antifungal defences at the expense of antibacterial responses (Pukkila-Worley *et al.*, 2011).

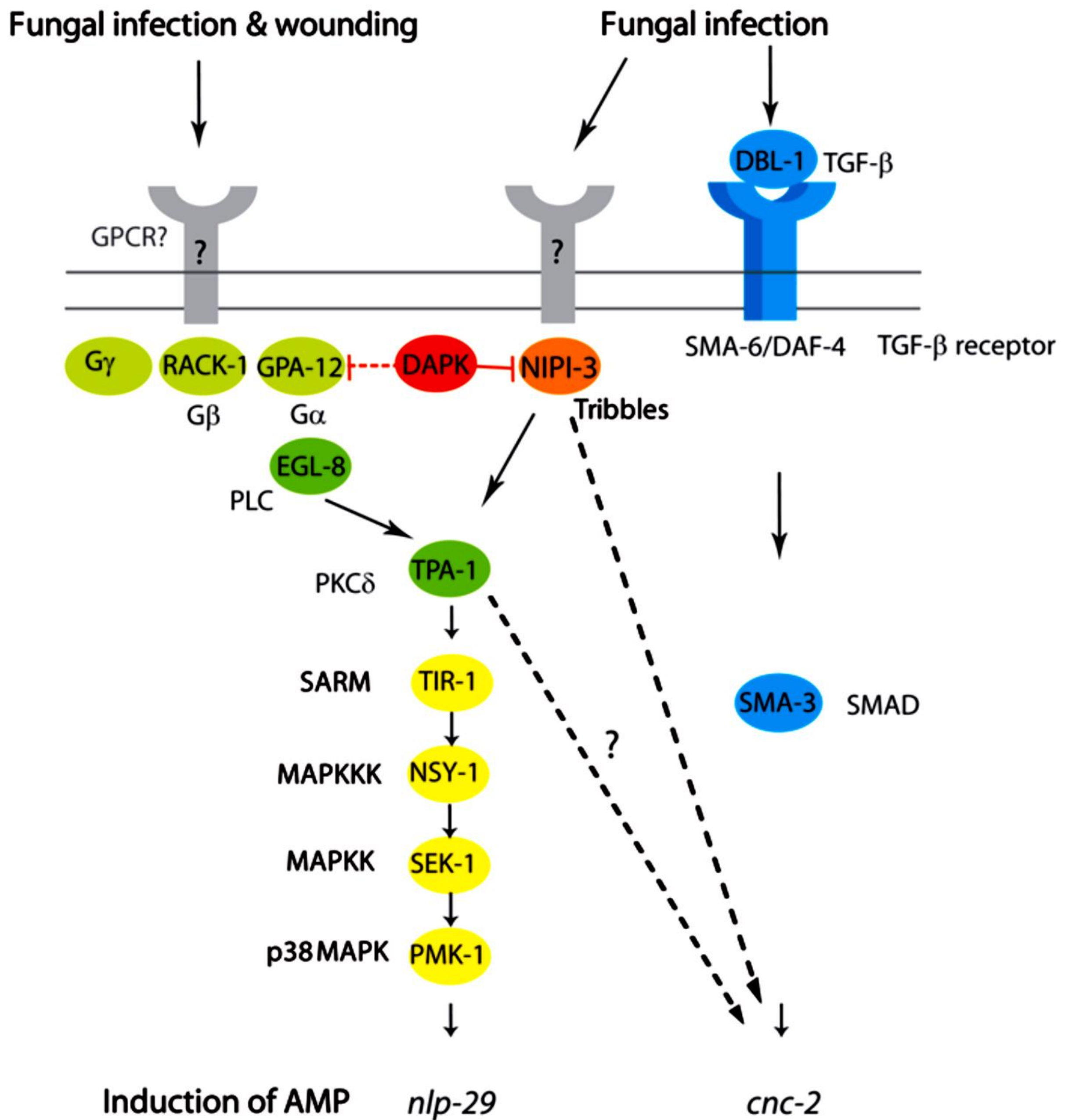


Figure 9. Schematic representation of the different signalling pathways and their components involved in the induction of antimicrobial peptides expression upon infection. Expression of the *nlp* genes is controlled by a PKC/SARM/p38 MAPK pathway and expression of *cnc* genes is controlled by a TGF-b pathway (Engelmann and Pujol, 2010).

1.7 *C. elegans* as infection model for *Pseudomonas aeruginosa*

1.7.1 *C. elegans-P. aeruginosa* infection model

Caenorhabditis elegans functions as a model organism to evaluate many bacterial infections caused by different bacterial species, especially *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhimurium. Pseudomonas aeruginosa* is an opportunistic pathogen causing life threatening infections in immunocompromised host (Wood, 1976; Doring, 1993; Govan and Deretic, 1996; Tan *et al.*, 1999). It is difficult to eradicate *P. aeruginosa* infection, because of high levels of innate antibiotic resistance and ever-increasing incidences caused by multidrug resistance strains of this bacterium (de Kievit *et al.*, 2001; Obritsch *et al.*, 2005; Fisher *et al.*, 2005). However, there are ongoing studies on determining the virulence mechanism of *P. aeruginosa* and development of treatments for the diseases caused by this bacterium. *Caenorhabditis elegans* has been used for the development of infection assay since it can mimic pathogenesis in humans (Powell and Ausubel, 2008).

Depending on the strain and culture conditions*, P. aeruginosa* is capable of causing infection-like processes in *C. elegans*. At this point five different *C. elegans* killing assays have been described *in vivo*, offering possibilities in the development of novel antibiotics through usage of whole-body infection system. In the first killing assay, named slow killing assay, *P. aeruginosa* PA14 strain can colonise the nematode intestines and kill it within a couple of days (Tan *et al.*, 1999; Papaioannou *et al.*, 2009). Slow killing is dependent upon active replication and accumulation of bacteria in the nematode intestines. However the nematode can recover from the infection if, after a brief exposure, it is removed from the pathogen source and also taking into consideration that the threshold is not exceeded, which might have an influence on the pathogen to induce intestinal pathology (Irazoqui *et al.*, 2010). So once this threshold is reached, the infection becomes persistent and nematode cannot recover (Tan *et al.*, 1999; Irazoqui *et al.*, 2010). However, in fast killing assay, the nematode is killed within a few hours. This is mediated by diffusible toxins such as phenazines, which are pigment compounds secreted by pseudomonads (Mahajan-Miklos *et al.*, 1999; Cezairliyan *et al.*, 2013). The third killing assay was discovered through infection with another *P. aeruginosa* clinical strain, PAO1, with a much lesser virulence potential than PA14. *Pseudomonas aeruginosa* PAO1 is capable of killing the nematode by rapid and lethal paralysis, by mediation of bacterial cyanide poison, which blocks respiratory electron transport (Darby *et al.*, 1999; Gallagher and Manoil, 2001). Therefore, this was observed when nematodes were treated with exogenous cyanide.

The fourth killing assay is named as red death and its lethality is mediated by consumption of phosphate in the media. It is observed that when a nematode is exposed to physiological stress such as starvation and heat shock, then infected with *P. aeruginosa* PAO1 , the nematode shows presence of red coloured *Pseudomonas* quinolone signal ferric ion (PQS-Fe_{3+}) complex in the pharynx and intestines (Zaborin *et al.*, 2009). Another recently describe assay is the liquid killing assay, mediated by pyoverdine, a bacterial siderophore, which induces a hypoxic response killing the nematode (Legendre *et al.*, 2011; Kirienko *et al.*, 2013). Individually these killing assays probe different virulence factors, such as phenazines, cyanide poison and pyoverdine, however they can complement one other in order to unfold and give better understanding of the precise virulence mechanisms involved in bacterial infections. Moreover, combination of this assays can be essential in the studies of pathogenesis inhibition by novel antibacterial therapies.

1.7.2 Immune response

As previously mentioned, there are shared traits within the innate immune system of humans and the nematode. Most of the nematode immunity characterisation has been through nosocomial bacterial pathogens, particularly *P. aeruginosa* (Kim *et al.*, 2002; Aballay *et al.*, 2003; Irazoqui *et al.*, 2008; Powell *et al.*, 2009). Pukkila-Worley and co-workers (2011) discovered that fungal and bacterial pathogens have remarkably distinct responses, for example, the immune response effectors that are upregulated by *P. aeruginosa* are downregulated by *C. albicans* during infection. The resistance of *C. elegans* to infection by *P. aeruginosa* triggers the DBL-1 pathway and specifically upregulates lysozyme gene (*lys-8*) of this pathway (Figure 10) (Mallo *et al.*, 2002). The gene *dbl-1* encodes one of 4 TGF-b-like ligands in the nematode. The binding of DBL-1 to heterodimeric DAF-4/SMA-6 receptor leads to phosphorylation and function via the SMA-2/SMA-3/SMA-4 SMAD complex to control gene expression. The SMA-2/SMA-3/SMA-4 complex translocate into the nucleus activating gene expression, acting in association with some of the multiple isoforms of SMA-9 (zinc finger transcription factor) (Liang *et al.*, 2003).

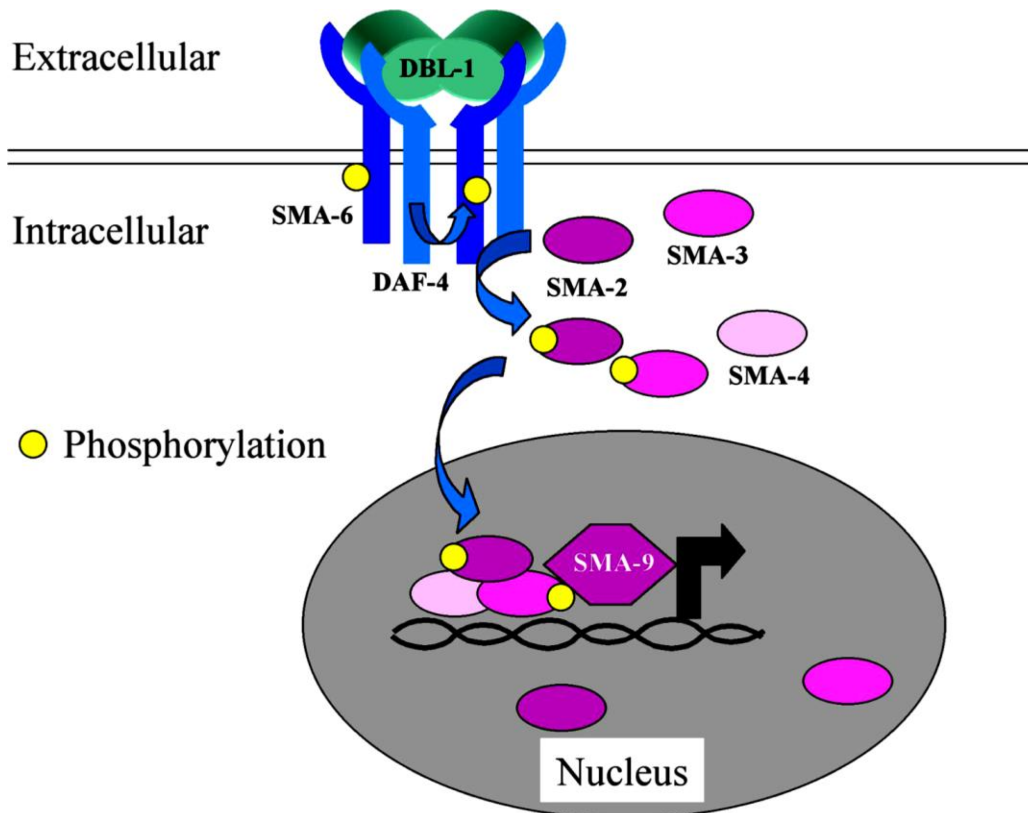


Figure 10. The DBL-1/TGF-b pathway. Binding of the TGF-b-like ligand to the heterodimeric DAF-4/SMA-6 receptor leads to the phosphorylation and activation of the SMAD proteins SMA-2, SMA-3 and SMA-4. Then they translocate into the nucleus to activate gene expression, in association with SMA-9 (Ewbank, 2006).

The DAF-2/DAF-16 pathway is another pathway involved in antibacterial defences and it highly regulates its expression (Figure 11) (Murphy *et al.*, 2003). It is well characterized for its function of controlling longevity in *C. elegans*. Briefly, as an agonist ligand is readily present, the insulin-like peptide DAF-28 activates the DAF-2 receptor, and this activation in return activates AGE-1 phosphatidylinositol-3 OH kinase, which act as a catalyst in the conversion of phosphatidylinositol bisphosphate (PIP_2) to phosphatidylinositol trisphosphate (PIP_3) (Figure 11A). The PIP_3 then binds to the AKT-1/AKT-2 complex, exposing the two phosphorylation sites. While also the PDK-1 kinase binds to PIP_3 recruiting it to the membrane in order to phosphorylate and activate AKT-1. Thereafter, AKT kinase phosphorylates DAF-16 transcription factor, ensuring its appropriate cytoplasmic retention. However, the presence of an antagonist ligand, INS-1, or in cases where *daf-2* lose its function mutant, then the DAF-2/DAF-16 pathway is deactivated (Figure 11B). This results in DAF-16 not being

phosphorylated, therefore it is translocated to the nucleus in order for it to regulate the expression of coupled antimicrobial genes and stress response.

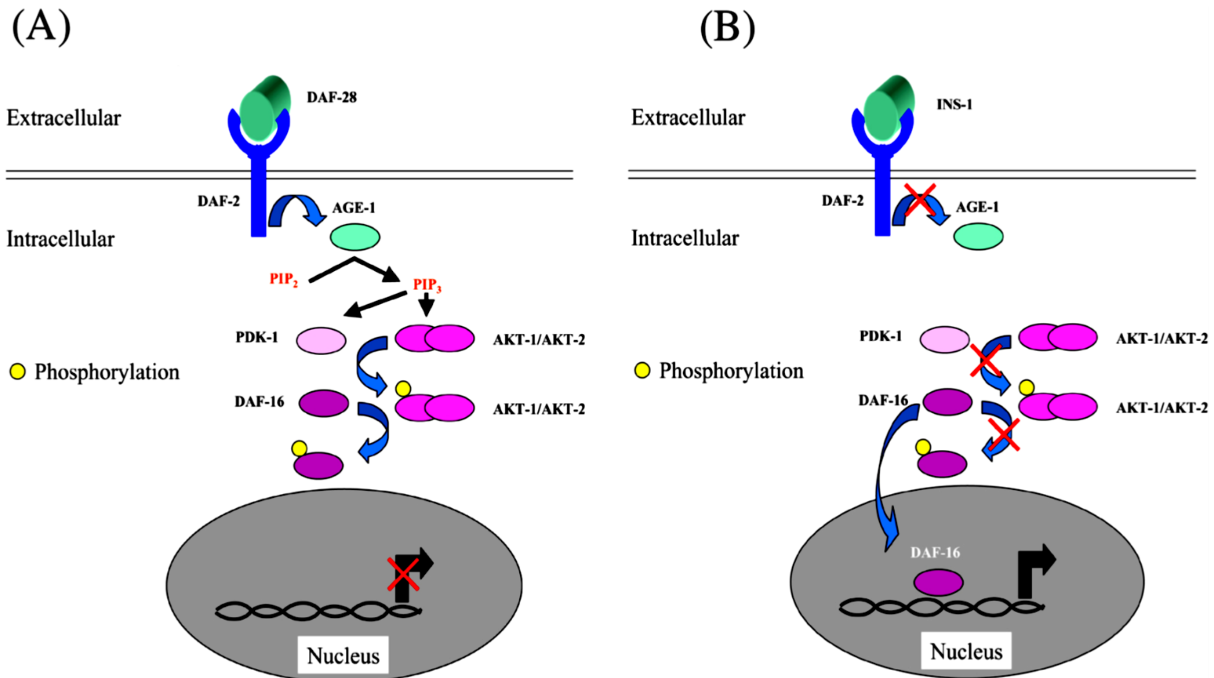


Figure 11. The DAF-2/DAF-16 pathway. (A) The presence of an insulin-like peptide DAF-28 agonist ligand activates the DAF-2 receptor and which in turn activates phosphatidylinositol bisphosphate (PIP_2), which is then converted into phosphatidylinositol trisphosphate (PIP_3). The AKT kinase in turn phosphorylates the DAF-16 transcription factor, ensuring its cytoplasmic retention. (B) The presence of INS-1 antagonist ligand, (or in a *daf-2* loss of function mutant), deactivates the pathway, thus DAF-16 is not phosphorylated. DAF-16 then translocate to the nucleus to regulate the expression of a set of antimicrobial genes and stress response (Ewbank, 2006).

Resistance to *P. aeruginosa* has been shown to involve a third pathway, a MAP kinase pathway, involving the MAP3K NSY-1 and the MAP2K SEK-1 that had originally been characterised as playing a role in determining asymmetric neuronal cell fate (Kim *et al.*, 2002). In wild-type nematodes, the chemoreceptor STR-2 is only expressed in one of the two AWC olfactory neurons, while in *nsy-1* and *sek-1* mutants, both sister neurons express the receptor (Sagasti *et al.*, 2001). Interestingly, during the genetic screening for nematodes hyper susceptible to *P. aeruginosa*, alleles of *nsy-1* and *sek-1* were identified and were shown to act together upstream of *pmk-1* (Figure 12). Moreover, an increased susceptibility to infection due to the RNAi against this gene, which encodes one of the nematode's three p38-family MAP

kinases can also be observed (Kim *et al.*, 2002). While in the case of cell determination, alleles of *nsy-1* and *sek-1* were shown to act downstream of *unc-43,* which encodes a calcium-calmodulin-dependent kinase (Figure 12) (Kim *et al.*, 2002). However, according to research this latter gene lacks a significant function in innate immune signalling (Kim *et al.*, 2002). Furthermore, when RNAi abrogate the function of *pmk-1* there is no cell determination phenotype provoked, and this suggest that there is differences in the downstream components of the pathway according to the two cases (Chuang and Bargmann, 2005). In addition, the TIR-1 adaptor protein was proven to function in both neuronal and defence pathways. Together all this findings were compared with human infection, and there was a striking high degree of resemblance between *P. aeruginosa* infection in *C. elegans* and humans. Thus there is a strong argument that *C. elegans* reveals features that makes it a relevant infection model to study host-pathogen interactions.

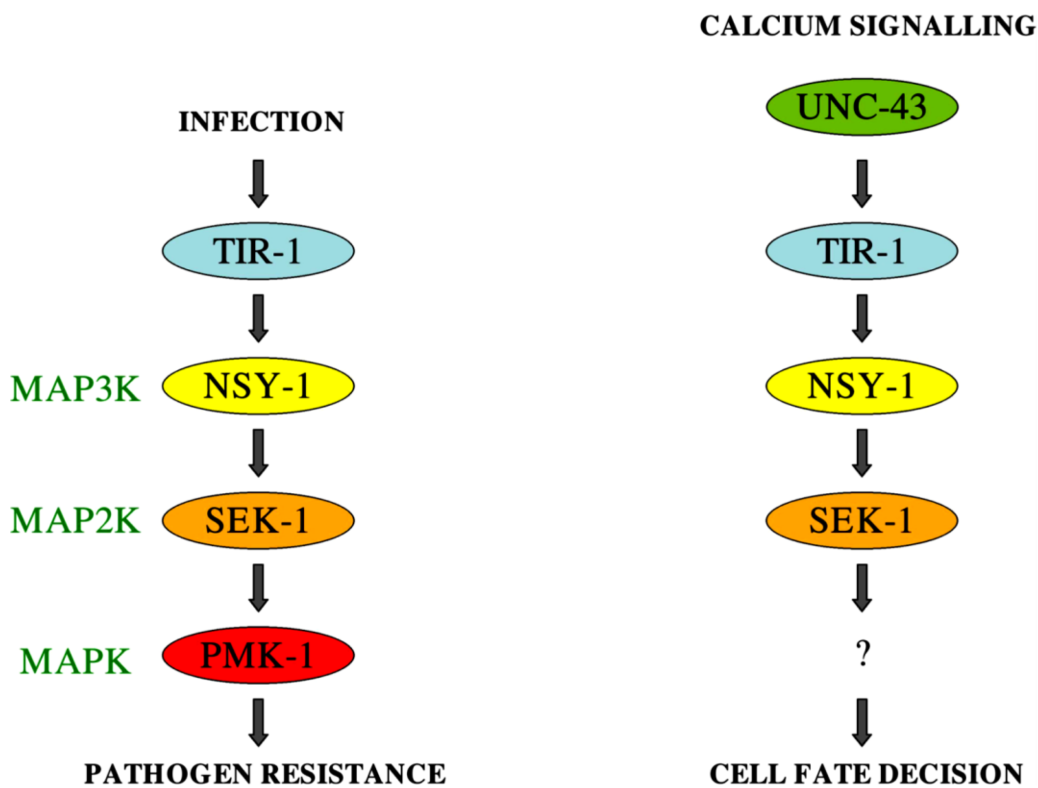


Figure 12. The *TIR-1/NSY-1/SEK-1* cassette functions in innate immunity and cell fate determination (Ewbank, 2006).

1.8 *Caenorhabditis elegans* as infection model for polymicrobial infections

The human host is often co-colonised or co-infected with different microorganisms (Figure 13) (Hofs *et al.*, 2016). The interactions can be detrimental to human health, with the combination of virulence factors promoting the survival of microbial populations during infection. At this present time, there is enormous research available describing specifically the *in vitro* antagonistic interaction between *C. albicans* and *P. aeruginosa* (Hogan and Kolter, 2002; Rinzan, 2009; Shirtliff *et al.*, 2009; Méar *et al.*, 2013; Lindsay and Hogan, 2014; Xu *et al.*, 2014; Fourie *et al.*, 2016). For instance it was reported that during the polymicrobial biofilm formation, *P. aeruginosa* can inhibit hyphae of *C. albicans* through the involvement of different virulence factors, such as the production of a phenazine pigment, pyocyanin and a quorum sensing molecule, 3-oxo-homoserine lactone (Kerr *et al.*, 1999; Hornby *et al.*, 2001; Brand *et al.*, 2008; McAlester *et al.*, 2008; Morales and Hogan, 2010).

While, *C. albicans* secretes a quorum sensing molecule, named farnesol, which reduces *Pseudomonas* quinolone signal with downstream reduction in phenazine production (Cugini *et al.*, 2007). Moreover, *C albicans* ethanol production can promote *C. albicans* biofilm formation and inhibit *P. aeruginosa* motility (Chen *et al.*, 2014). Other factors, such as iron sequestration, extracellular DNA as well as peptidoglycan and lipopolysaccharides can affect *C. albicans* and *P. aeruginosa* interaction (Xu *et al.*, 2008; Purschke *et al.*, 2012; Bandara *et al.*, 2013; Sapaar *et al.*, 2014; Trejo-Hernandez *et al.*, 2014).

Despite this, majority of research has been done on polymicrobial infections *in vitro*. There has been scarcity of *in vivo* models to investigate the biological and pathological mechanisms of interacting species. However, Peleg *et al.* (2008) showed that *C. elegans* can still be effectively used as a model to monitor the dynamics of a polymicrobial infection, precisely between eukaryotes and prokaryotes. This study was based on an antagonistic relationship between bacterial pathogen *Acinetobacter baumannii* and *C. albicans* where *A. baumannii* inhibited several virulence factors of *C. albicans* including biofilm formation and hyphal formation, resulting in reduced *C. albicans* pathogenicity (Peleg *et al.,* 2008). Interestingly, during their *in vitro* studies they were able to recapitulate *A. baumannii* inhibitory activity against *C. albicans* (Peleg *et al.*, 2008; Tampakakis *et al.*, 2009). Meanwhile, *C. albicans* secreted farnesol, which inhibited the growth of *A. baumannii* respectively (Peleg *et al.*, 2008). Interesting, *A. baumannii* and *C. albicans* independently killed the nematode at a rapid rate, however nematodes infected with both this pathogens survived significantly longer compared with those infected with *C. albicans* alone (Peleg *et al.,* 2008).

Moreover, Cruz *et al.* (2013) also supported the antagonistic observations in *C. elegans*, by investigating the interaction between *Enterococcus faecalis* and *C. albicans*. Here, a monomicrobial infection with either *E. faecalis* or *C. albicans* significantly reduced survival, however, *E. faecalis* and *C. albicans* polymicrobial infection caused less tissue damage to nematode, by preventing hyphal morphogenesis by *C. albicans* (Cruz *et al.*, 2013). Fascinatedly, the presence of *C. albicans* appeared to be able to protect *E. faecalis* from cell death. Apart from the fact that *C. albicans*-*P. aeruginosa* interactions has been well-studied, little is known about this interaction in the *C. elegans* infection model.

Recently, Bergeron and co-workers (2017), discovered that the co-infection of *C. albicans* and *P. aeruginosa* in the mucosa results in synergistic virulence and enhanced mortality when using Zebrafish swim bladder model. They discovered that monomicrobial infection causes little mortality, but polymicrobial infection are synergistically virulent. According to their observations, the polymicrobial infection mortality seemed to be more correlated to *C. albicans* invasive pathogenesis and higher microbial burden, which act as strong virulence determinants for mortality. Moreover, during the polymicrobial infection there are several important aspects of the proinflammatory immune response that are also enhanced. The enhanced *C. albicans* hyphal invasion and synergistic virulence in co-infections were unexpected based on known antagonistic interactions of the pathogens *in vitro* (Hogan and Kolter, 2002; Rinzan, 2009; Shirtliff *et al.*, 2009; Méar *et al.*, 2013; Lindsay and Hogan, 2014; Xu *et al.*, 2014; Fourie *et al.*, 2016). However, several *in vivo* studies reported that the enhanced virulence is consistent with most *C. albicans*-bacterial co-infection mouse models, such as organotypic models, which result in cytokine production, and/or fungal invasion (Villar *et al.*, 2005; Xu *et al.*, 2014; Nash *et al.*, 2014; Nash *et al.*, 2015).

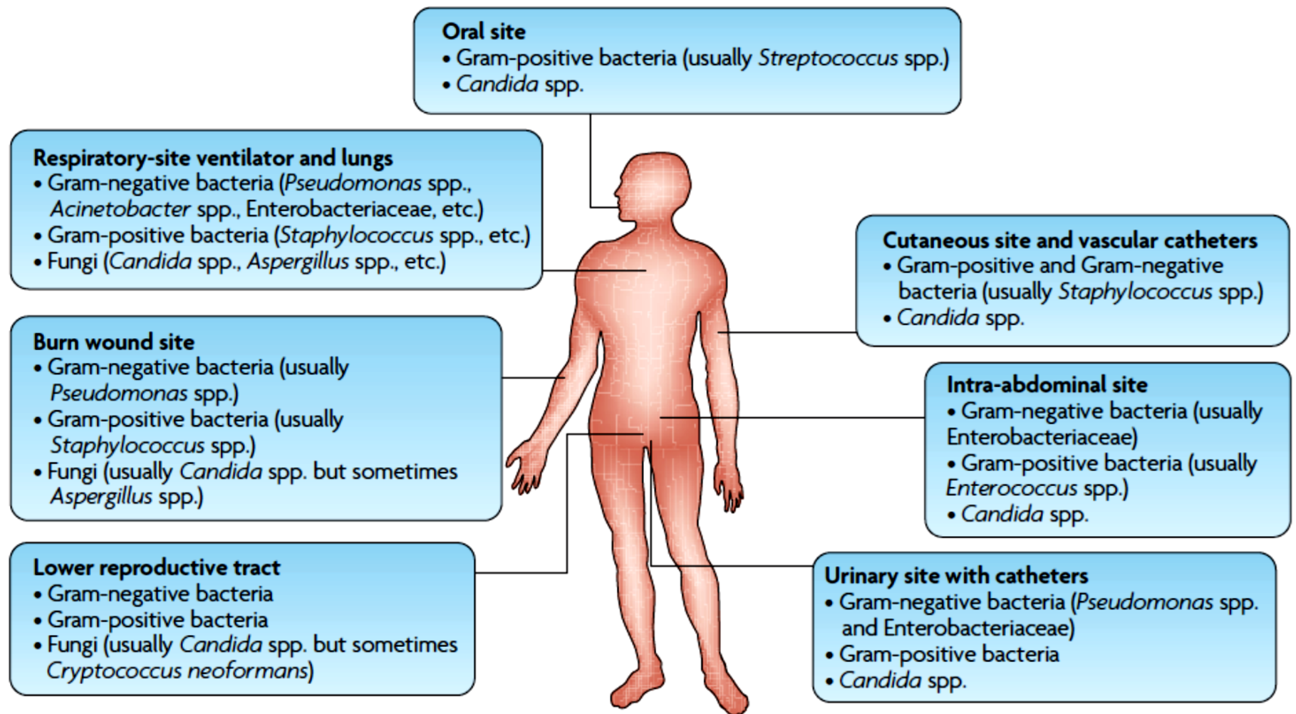


Figure 13. Scheme representing bacterial–fungal interactions found in clinically important sites of a critically ill patient in the intensive-care unit. The boxes describe, the microorganisms with each site they are found (Peleg *et al*., 2008).

1.9 Lipid metabolism in mammals

Lipids are small organic molecules that are insoluble in water, but are soluble in organic solvents. Biochemically, they originate entirely or in part from carbanion-based condensations of thioesters, forming fatty acids, which are components of triacylglycerols (TAGs), phospholipids, and sphingolipids; or by isoprene units, resulting into isoprenol derivatives (sterols) (Fahy *et al*., 2009). Lipids are fundamental to all biological systems and fulfil several cellular functions, including storage of energy, building blocks of membranes, and signalling. Their highly reduced state renders them efficient energy storage molecules. They are the hydrophobic units of bilayers that form cellular and organelle membranes, and they are potent signalling molecules. For instance phospholipids serve as integral parts of the membrane for separation and compartmentalisation of cells; moreover these membranes serve as scaffolds for interactions along membrane-associated moieties. Also for host-pathogen interactions membranes play crucial roles, because they are the first line of defence against pathogens. Several lipids act as second messengers of signal transduction for example lysolipids,

phosphoinositides and steroids. Lipidomic analyses, which seek to define all lipid species present in a system, indicate that the lipid composition of eukaryotic systems is enormously complex (Wenk, 2010).

1.9.1 Fatty acids

Fatty acids are carboxylic acids with long aliphatic chains of about 14-20 carbons (Tanaka *et al.*, 1996; Watts and Browse, 2002). They can be classified either as saturated, without any double bonds, as monounsaturated, containing one carbon-carbon double bond, or as polyunsaturated, containing two or more carbon-carbon double bonds. For instance, monounsaturated fatty acids (MUFAs) are synthesized from saturated fatty acids (SFAs) to unsaturated fatty acids (UFAs) by lipogenic enzymes, namely Δ9 fatty acid desaturases (also referred to as stearoyl-CoA desaturases or SCDs). The Δ9 fatty acid desaturases functions by introducing a double bond between the 9th and 10th carbons in a SFA chain. In eukaryotes, the Δ9 desaturases are ubiquitous enzymes found in organisms from yeast to humans. Some of these organisms, including yeast, sheep and pigs, contain one Δ9 desaturase in contrast to humans, and also mice and nematode *Caenorhabditis elegans*, which contains a multiple Δ9 desaturases (Stukey *et al.*, 1989; Watts and Browse, 2000; Miyazaki *et al.*, 2003; Ren *et al.*, 2004; Wang and Dey, 2005). It is important to maintain a proper ratio between MUFAs and SFAs by regulation of Δ9 desaturase genes, since alteration in this ratio can cause various diseases in humans such as diabetes, heart disease, atherosclerosis, cancer and obesity (Warensjo *et al.*, 2005; Lee *et al.*, 2018). Another group of fatty acids are polyunsaturated, which are about 18 carbon atoms or more long chain fatty acids in length, consisting of two or more double bonds. They can be characterized into three groups, based on the double bond position (n) closer to the fatty acid chain methyl end, such as, omega-3 (n3), omega-6 (n6) and omega-9 (n9) fatty acids. Very important to note that both the number of double bonds and the chain length highly influence the lipids physical properties that readily consist of them, as well as the function of the plasma membrane (Cribier *et al.*, 1993).

1.9.1.1 Fatty acids biosynthesis

In brief, mammalian fatty acids can be synthesized *de novo* by enzymatic activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), to yield 16-carbon SFA, known as palmitic acid (16:0) (Figure 14) (Leibundgut *et al.*, 2008). Thereafter, elongase enzymes elongates the palmitic acid with two-carbon units and fatty acid desaturase enzymes inserts double bonds. Each desaturase enzyme respectively functions by introducing a double bond

at a specific carbon in the fatty acid chain. For example, a Δ9 desaturase inserts a double bond specifically at the 9th carbon of palmitic or stearic acid, creating a MUFA. Surprisingly, in mammals, the 18-carbon and longer, omega-3 and omega-6 polyunsaturated fatty acid (PUFA) families are not synthesised *de novo*. This is due to lack of Δ12 and omega-3 fatty acid desaturase activities that enable them to convert oleic acid (18:1n9) to linoleic acid (18:2n6), or to convert omega-6 (n6) fatty acids to omega-3 (n3) fatty acids (Burr and Burr, 1930; Wallis *et al.*, 2002). As a result mammals have a dietary requirement for the fatty acids, such as, 18:2n6 and alpha-linolenic acid (18:3n3), which are produced through a series of elongase and desaturation reactions catalysed by elongase and desaturase enzymes, respectively (Burr and Burr, 1930). Furthermore, enzymes, such as, Δ5 and Δ6 desaturases, metabolise these dietary fatty acids in order to form a complex of PUFAs including arachidonic acid (20:4n6) and docosahexaenoic acid (22:6n3), required for mammalian growth and development (Figure 14) (Burr and Burr, 1930).

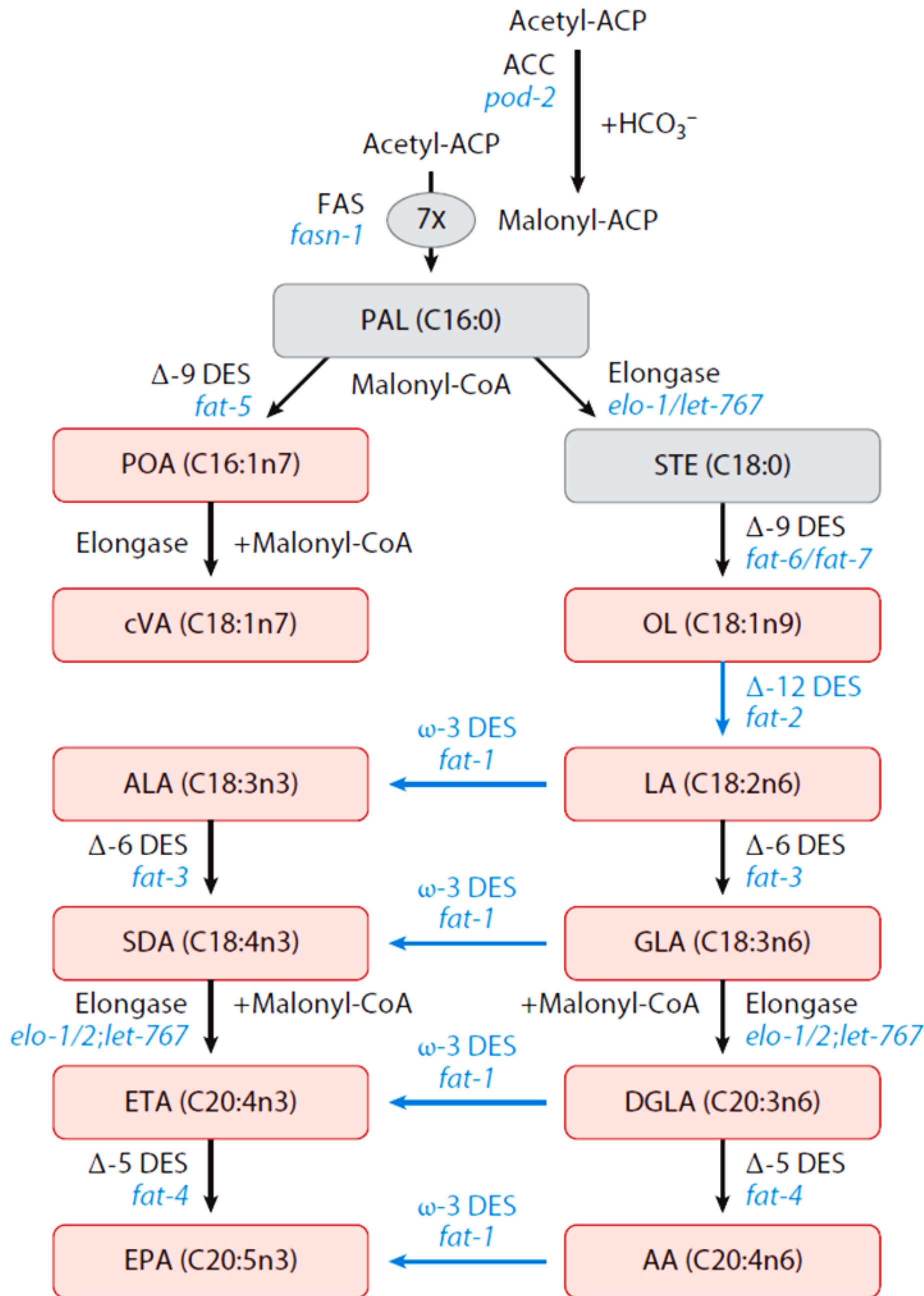


Figure 14. Fatty acid biosynthesis pathway. Abbreviations: AA, arachidonic acid; ACC, acetyl-CoA carboxylase; ACP, acyl carrier proteins; ACS, acyl-CoA synthase; ALA, α-linolenic acid; BCKAD, branched-chain α-keto acid dehydrogenase complex; cVA, cis-vaccenic acid; C5ISO, isovaleric acid; C13ISO, 11-methyldodecanoic acid; C15ISO, 13-methyltetradecanoic acid; C17ISO, 15-methylhexadecanoic acid; DES, desaturase; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; FAS, fatty acid synthase; GLA, γ-linolenic acid; KAR, 3-ketoacyl-CoA reductase; LA, linolenic acid; OL, oleic acid; PAL, palmitic acid; POA, palmitoleic acid; PUFA, polyunsaturated fatty acid; SDA, stearidonic acid; STE, stearic acid (Zhu and Han, 2014).

1.9.1.2 Biological function of fatty acids

Fatty acids are essential building blocks and precursors of membrane and storage lipids, such as cholesterol esters, phospholipids, triglycerides and sphingolipids which function in diverse cellular processes such as energy storage, membrane function, and also serve as mediators of signal transduction (Enoch *et al.*, 1976; Ntambi, 1999). The role of lipids in these cellular processes highly depend on their fatty acid compositions, thus it is crucial to regulate synthesis of MUFAs. For instance, the phospholipids fatty acid composition can have tremendous effect on membrane properties, including fluidity, curvature, tension, thickness, and the production of membrane microdomains. Importantly, these membrane properties can affect cellular processes that are responsible to alter membrane structure, including cytokinesis, as well as activity of membrane-associated proteins (Kremmyda *et al.*, 2011; Vrablik and Watts, 2012). Another biological role of fatty acids is seen where lipase enzymes are capable of cleaving phospholipids and triacylglycerols, to release several fatty acids that can be processed by enzymatic activity into signalling molecules or function independently.

Similarly, like MUFAs, PUFAs are also important components of membrane phospholipids functioning to maintain membrane fluidity and membrane function, and also regulators of gene expression (Kahn-Kirby *et al.*, 2004; Kubagawa *et al.*, 2006). Importantly, PUFAs function as precursors for signalling molecules called endocannabinoids and eicosanoids, which are involved in many physiological processes and biological processes, that includes neuroreception, development, reproduction, inflammation and immune response (Funk, 2001; Glass and Olefsky, 2012; Battista *et al.*, 2012; Hirata *et al.*, 2012; Katona and Freund, 2012). In mammals, composition of PUFA in phospholipids significantly influences many cellular processes such as endocytosis, exocytosis, modulation of ion channels, and membrane-associated enzymes activities that are sensitive to most of the lipid membranes biophysical properties. Thus disruption of proper PUFA intake and metabolism is highly related to certain human disease states, including inflammatory disorders, hypertension, coronary artery disease diabetes, and cancer. Moreover, UFAs can also functions in movement of mitochondria and inheritance (Stewart and Yaffe, 1991). Since the PUFAs are the central fatty acids that makes up the backbone of sphingolipid, they are also important for cell viability (Dickson, 1998; Schneiter, 1999). In order to identity the biological roles of lipids, one of the approaches used is the forward or reverse genetic screens, which offer unbiased determinations of functions of specific lipid classes (Han, 2015; Zhang *et al.*, 2015). For instance mass spectrometry (MS) is a commonly used analytical platform for lipidomics due to its specificity, sensitivity, and accuracy.

1.9.2 Eicosanoids

1.9.2.1 Eicosanoids biosynthesis

Polyunsaturated fatty acid functions as precursors that are used to synthesise diverse lipid mediators such as eicosanoids and docosanoids, which are synthesised by cyclooxygenases or lipoxygenases (Funk, 2001; Spector and Kim, 2015). For instance, eicosanoid biosynthesis is triggered by a stimulus, which in return stimulates cytoplasmic phospholipase A_2 (PLA_2) to be transferred to the endoplasmic reticulum, Golgi apparatus, or nuclear membrane where it catalyses the release of a PUFA from a phospholipid membrane (Figure 15) (Soberman and Christmas, 2003). Now, at this point the pathway diverges, where some of the free PUFAs can be metabolised either by "linear pathway" to synthesise lipoxin/leukotriene or the "cyclic pathway" to synthesise prostaglandins/thromboxanes, family of eicosanoids (Funk, 2001). The cyclic pathway that synthesise prostaglandin and thromboxane is derived from a pentane ring with 5-carbon or a 6-member ether-containing ring of this eicosanoids. Importantly, different PUFA precursors are known to synthesise three classes of prostaglandin, such as, the F_1 class derived from dihomo-γ-linolenic acid (DGLA, 20:3n6), the F_2 class is derived from arachidonic acid (AA, 20:4n6) and the F_3 class derived from eicosapentaenoic acid (EPA, 20:5n3).

Briefly, during cyclic pathway (Figure 15), cyclooxygenase enzymes (COX-1/2) and prostaglandin endoperoxide, which are enzymes found in endoplasmic reticulum or nuclear envelope catalysing the insertion of two oxygen atoms in AA, function to process AA to form prostaglandins (Marnett *et al.*, 1999). Important to note about the cyclooxygenase enzymes in mammalian cells, is that they exists in two isoforms, for example, COX-1 enzyme that is expressed constitutively, and a highly induced COX-2 enzyme. Therefore the preliminary oxidation reaction of AA, which is facilitated by these enzymes, is able to produce prostaglandin G_2 (PGG_2). Thereafter, PGG_2 is reduced to prostaglandin H_2 (PGH_2) through peroxidase activity, this PGH_2 then becomes a substrate for other immunomodulatory compounds, such as thromboxanes and prostaglandins. For instance, PGH_2 can convert to prostaglandin E_2 (PGE_2) through further action by prostaglandin E synthase enzyme (Figure 15). This acts as the first rate-limiting step for synthesis of prostaglandin, and also a targeted step for non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen (Vane, 1971; Funk, 2001).

In the linear pathways (Figure 15), free PUFAs are metabolized by lipoxygenase enzymes (LOX) to produce leukotrienes or by cytochromes (CYPs) P450 to produce molecules such as hydro-peroxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and epoxyeicosatrienoic acids (EETs). For example, LOX are dioxygenases that catalyse insertion

of oxygen into PUFAs in most animals, plants and microbes (Kuhn and O'Donnell, 2006; Brodhun and Feussner, 2011). Briefly, this reaction of oxygenation starts with abstraction of hydrogen, followed by rearrangement of radical and the insertion of oxygen. In addition to hydroperoxidation and oxygenation, LOX enzyme, through the combination of various enzymatic activities can also catalyse the synthesis of hepoxilins, leukotrienes and lipoxins (Figure 15) (Kuhn and O'Donnell, 2006; Kremmyda *et al.*, 2011; Dennis and Norris, 2015). The LOX enzyme exist in various isoforms comprising of different stereospecificity and activities, such as 12/15-LOX, which specifically catalyse hydroperoxidation and oxygenation of PUFAs at carbon 12 or 15 position. Thus like the CYPs, the LOX enzymes (5-, 12-, 15-LOX) are position-specific and stereospecific.

In the third pathway, PUFAs is converted into oxygenated signalling molecules that are known to mediate inflammation, tumour growth and cardiac function through CYP450 enzymes (Figure 15) (Fleming, 2007; Panigrahy *et al.*, 2010). In addition, cytochrome P450s catalyse AA to synthesise epoxyeicosatetraenoic acids (EETs), with simultaneous modification to diHETEs, displaying different functions to the host. Thereafter this newly synthesized group of eicosanoids translocate out of the cell by either passive diffusion or through specific transporters, like multi-drug resistance protein 4 (Schuster, 2002; Russel *et al.*, 2008). Therefore, these eicosanoids act as transient signals, that lasts for about seconds to minutes activating the neighbouring cells G-protein-grouped receptors (GPCRs) and also by facilitating their own physiological effects (Funk, 2001). Then the cells take up the eicosanoids via carrier-mediated transport, to the cytosol where they are rapidly oxidized (Samuelsson *et al.*, 1975; Schuster, 2002; Nomura *et al.*, 2004; Shiraya *et al.*, 2010).

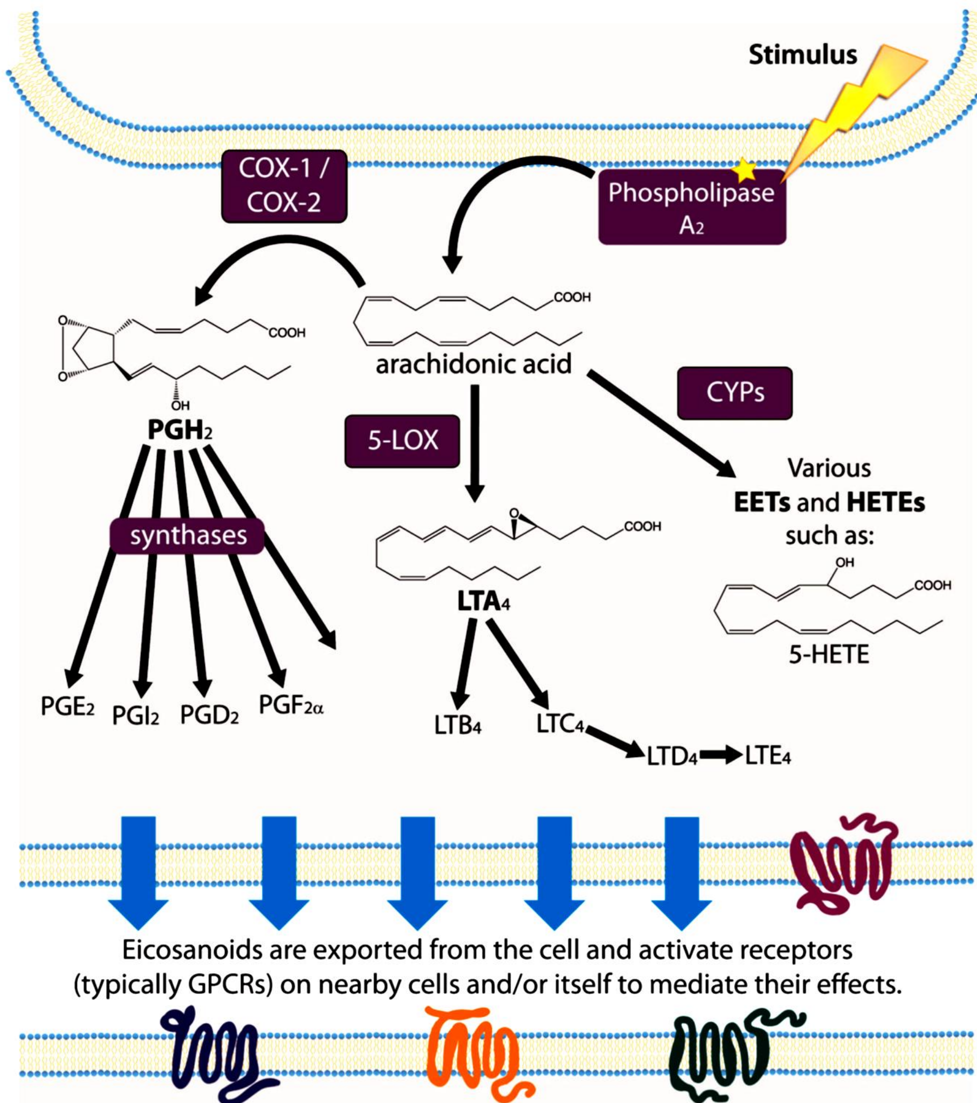


Figure 15. Eicosanoids biosynthesis pathway. Stimulus triggers phospholipase A_2 (PLA_2) to translocate to intracellular membranes, where PLA_2 cleaves and releases arachidonic acid (AA). For prostaglandin synthesis, cyclooxygenase enzymes (COX) create the cyclic intermediate prostaglandin H_2 (PGH_2), which can further be synthesised into a series of prostaglandins and thromboxanes through the activity of their respective synthase enzymes. For instance, prostaglandin/thromboxane synthases is able to catalyse PGH_2 into prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Alternately, AA can either be processed by lipoxygenase 5 (5-LOX) for synthesis of leukotriene or processed by cytochromes P450 (CYP450) enzymes to form other linear oxygenated fatty acids. Eicosanoids are then exported from the cell out and activates G-protein coupled receptors (GPCRs) on nearby cells or mediate their own effects (Vrablik and Watts, 2013).

1.9.2.2 Biological function of eicosanoids

In mammals, eicosanoids such as prostaglandins, prostacyclins, and leukotrienes function variously by applying stimulatory and inhibitory influences and also have profound effects on multiple aspects of organism's physiology, involving immunity (Funk, 2001; Kremmyda *et al.*, 2011). For example, prostaglandins and leukotrienes does not only act as pro-inflammatory mediators that are important during infection where they act as initial containment of infection, but also vital for the recruitment of immune cells including phagocytes, to a site of infection. A group of prostaglandins called PGE_2 serve as potent regulators of host immune responses, where they trigger both pro- and anti-inflammatory responses, and can act through one of four G-protein-coupled receptors (EP1 to EP4), depending on target cell. Although this PGE_2 can inhibit phagocytosis, Th1-type immune responses and lymphocyte proliferation, and Th2-type responses and tissue eosinophilia can also stimulate immunoglobulin E production. Amongst other prostaglandins that also function to counter inflammation, prostaglandin J_2 (PGJ_2), derived from prostaglandin D_2 (PGD_2), plays a vital role as a brake on the inflammatory response (Zurier *et al.*, 2009). Additionally, prostaglandin and prostacyclin functions in the bone response to mechanical loading. For example, an increase in the level of loaded bone tissue and also direct administration can result in bone formation (Chen *et al.*, 2014). However, prostaglandins biochemical blockage, such as indomethacin can lead to bones being unable to sense and respond to mechanical stimulation (Chen *et al.*, 2014). Moreover, prostaglandin signalling is important for numerous aspects of reproduction in mammals, such as fertilization, implantation, oocyte maturation, parturition and ovulation (Dey *et al.*, 2004; Wang and Dey, 2005; Takahashi *et al.*, 2006; Dorniak *et al.*, 2012). Another class of eicosanoids are lipoxins, derived from sequential activity of 5- and 15-lipoxygenases. This class function as stop signals, preventing leukocyte-mediated tissue injury, thus assisting with resolution of inflammation (Zurier *et al.*, 2009).

1.10 Lipid metabolism in *Caenorhabditis elegans*

1.10.1 Fatty acid biosynthesis in *Caenorhabditis elegans*

Metabolomics and lipidomic investigations on *C. elegans* are scarce, but the number of publications related to this topic is increasing steadily. The *C. elegans* model has become ideal for discovering new functions and regulation of lipid metabolism due to its simple anatomy and also wide range of forward and reverse genetic tools (Han, 2015; Zhang *et al.*, 2015). In addition, studies done using this nematode model can analysis lipid metabolism in the context of the whole organism, allowing for investigations on the functions of specific lipids

in development, lifespan, and also other important aspects of physiology. Unlike humans that store lipids in dedicated adipose tissue, *C. elegans* use intestinal and hypodermal cells to store lipids, however most aspects of lipid metabolism regulation are closely related to humans. Ashrafi and co-workers (2003) identified many genes in an RNAi screen that altered fat storage, which includes homologues that play a role in mammalian fat metabolism. In addition, many pathways like insulin-signalling pathways are able to regulate fat storage not only in mammals but also nematodes (Murphy *et al.*, 2003). *Caenorhabditis elegans* can directly absorb and incorporate dietary fats, and this enables researchers to modify the composition of fatty acids of live nematodes. These fatty acids may also be modified by elongation and/or desaturation. It can inherit fatty acids by maternal inheritance as well as synthesise a wide range of fatty acids *de novo* (Houthoofd *et al.*, 2002). For instance, *C. elegans* can synthesise 18- and 20-carbon PUFAs from *de novo*-synthesized 16:0 or dietary, thus there is no essential fatty acids (Wallis *et al.*, 2002; Perez and Van Gilst, 2008). *Caenorhabditis elegans* produces close to 10 major fatty acids, which includes SFAs like 16:0 and stearic acid (18:0), MUFAs like 18:1n9 and cis-vaccenic acid (18:1n11), and also PUFAs like 18:2n6 and 20:5n3 (Hutzell and Krusberg, 1982). At present several enzymes are shown to have a significant function during PUFAs biosynthesis in *C. elegans*, such as seven fatty acid desaturase enzymes (plant-like Δ12 and omega-3 desaturases (FAT-1 to FAT-7), one 3-ketoacyl-CoA reductase (LET-767) and fatty acid elongases (ELO-1 and ELO-2) (Spychalla *et al.*, 1997; Watts and Browse, 2002; Kniazeva *et al.*, 2003; Brock *et al.*, 2007; Entchev *et al.*, 2008). In *C. elegans,* FAT-5 desaturase acts specifically on 16:0, whereas 18:0 is desaturated by FAT-6 and FAT-7 desaturases (Watts and Browse, 2002).

Unsaturated fatty acid synthesis pathway in *C. elegans* starts with a precursor, 16:0, which is either synthesized *de novo* or obtained from the *E. coli* diet (Perez and Van Gilst, 2008). The 16:0 is then catalysed by FAT-5 to form palmitoleic acid (16:1) (Figure 16) (Watts and Browse, 2002). Then 16:1 is elongated to 18:1n11, which is the most abundant fatty acid in phospholipids and triglycerides (Tanaka *et al.*, 1996). The 16:0 can also be elongated to 18:0, the substrate for FAT-6 and FAT-7 desaturation to 18:1n9 (Watts and Browse, 2002). Both 18:1n11 and 18:1n9 are 18-carbon MUFAs however they function differently in the nematode (Spychalla *et al.*, 1997; Peyou-Ndi *et al.*, 2000; Watts and Browse 2002). For instance, 18:1n9 act as a precursor and is catalysed by FAT-2 Δ12 desaturase to form 18:2n6, however, FAT-2 does not act on 18:1n11. The 18:2n6 formed by FAT-2 is converted to an 18:3 by FAT-1 or FAT-3, this is a step not found in mammals (Spychalla *et al.*, 1997; Peyou-Ndi *et al.*, 2000; Watts and Browse 2002). The Δ6 desaturation provided by FAT-3 is required for elongation of 18-carbon PUFAs to 20-carbon PUFAs AA (20:4n6) by ELO-1 and ELO-2 (Watts and Browse,

2002). Both FAT-1 and FAT-4 modify the 20-carbon PUFAs to produce EPA (20:5n3) (Watts and Browse, 2002).

For experimentation purposes, usage of highly sensitive chromatographic and spectroscopic techniques have permitted wide-ranging lipid analysis in these models (Watts and Browse, 2002). For instance, fatty acids from nematode populations are commonly analysed using acidic methylation to produce fatty acid methyl esters (FAMEs), which are separated from each other by gas chromatography (GC), and detected with either mass spectrometry (MS) or flame ionization detection (FID) (Watts and Browse 2002). The laboratory *E. coli* OP50 strain, which act as food source and maintain *C. elegans* in the laboratory, contains 16:0, 16:1n7, vaccenic (18:1n7) and two cyclopropane fatty acids (C17D and C19D), but not 18:0, 18:1n9, mono methyl branched-chain (mmBCFA) nor PUFA (Tanaka *et al.*, 1996; Watts and Browse, 2002; Kniazeva *et al.*, 2004; Perez and Van Gilst, 2008).

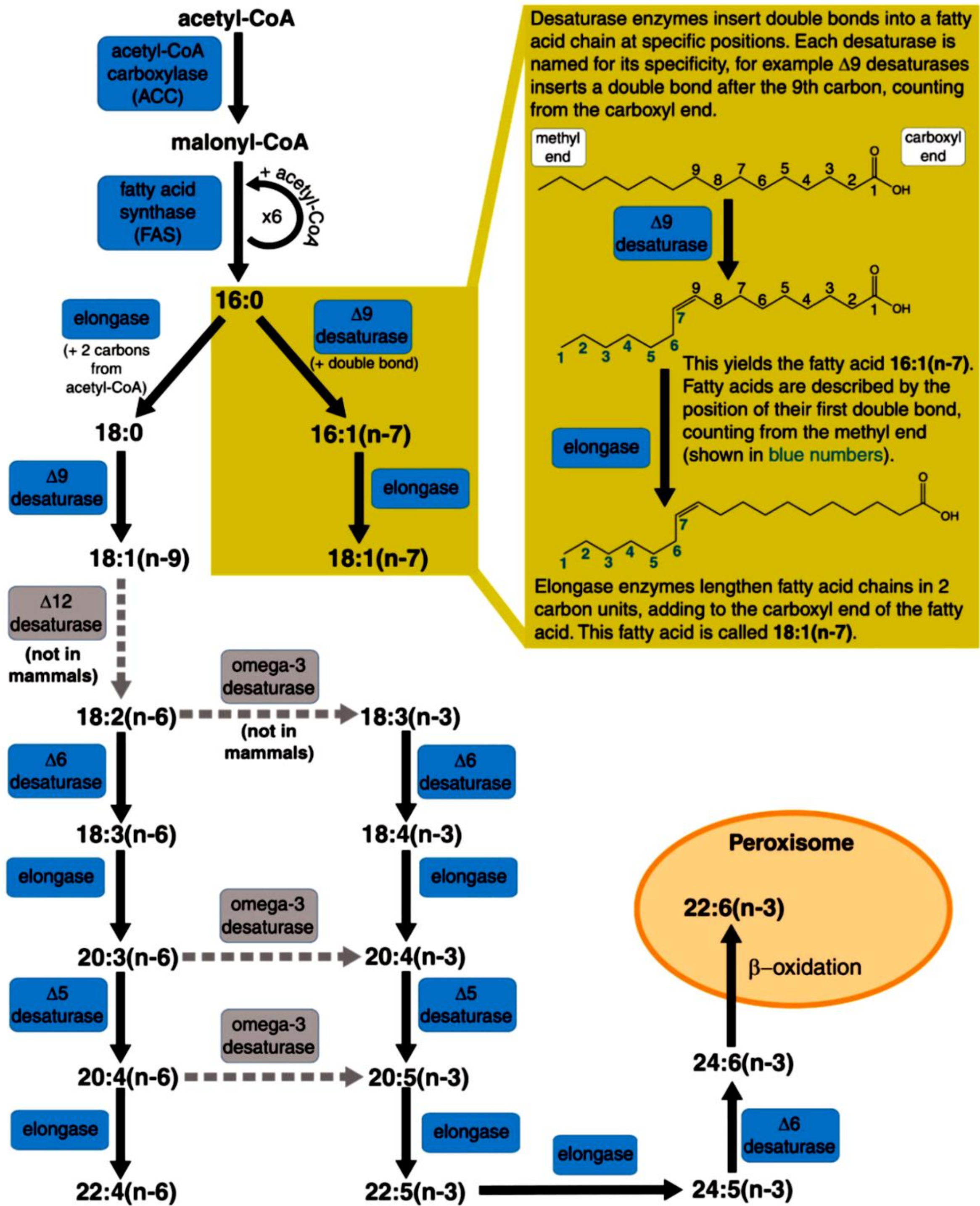


Figure 16. A general polyunsaturated fatty acids biosynthesis pathway. Mammalian enzymes are drawn in blue accompanied by solid black reaction arrows. The Δ12 and omega-3 desaturase enzymes (gray with dashed gray arrows) are included, as *C. elegans* possess these desaturase activities; however, *C. elegans* do not synthesize the 22 carbon PUFAs produced in mammals (Vrablik and Watts, 2013).

1.10.2 Eicosanoids biosynthesis in *C. elegans*

In *C. elegans*, eicosanoids are produced from PUFAs by CYP enzymes using two different pathways. One pathway uses v-hydroxylation to convert PUFAs such as AA to HETEs, while the other uses epoxygenase enzyme activity to produce EETs (Kulas *et al.*, 2008). Through the difficulty of identifying direct mammalian homologues of COX and LOX enzymes in *C. elegans*, the CYP family is represented by over 60 genes (Gotoh, 1998). For instance, PG G/H synthase or COX act as a bifunctional enzyme consisting of two types of different active sites that catalyse the cyclization of AA into PGG_2 and the peroxidase-mediated reduction of PGG_2 into PGH_2 (Chandrasekharan and Simmons, 2004) (Figure 17). The PG G/H synthase homologs are mostly found in tunicate, cnidarian and vertebrate genomes, but not in arthropod and nematode genomes. The most parsimonious explanation is that the prostaglandin synthesis is conserved evolutionarily in multicellular animals, however PG G/H synthases were lost in nematodes and arthropods (Stanley-Samuelson and Pedibhotla, 1996; Sommer *et al.*, 2003; Rowley *et al.*, 2005; Tootle and Spradling, 2008).

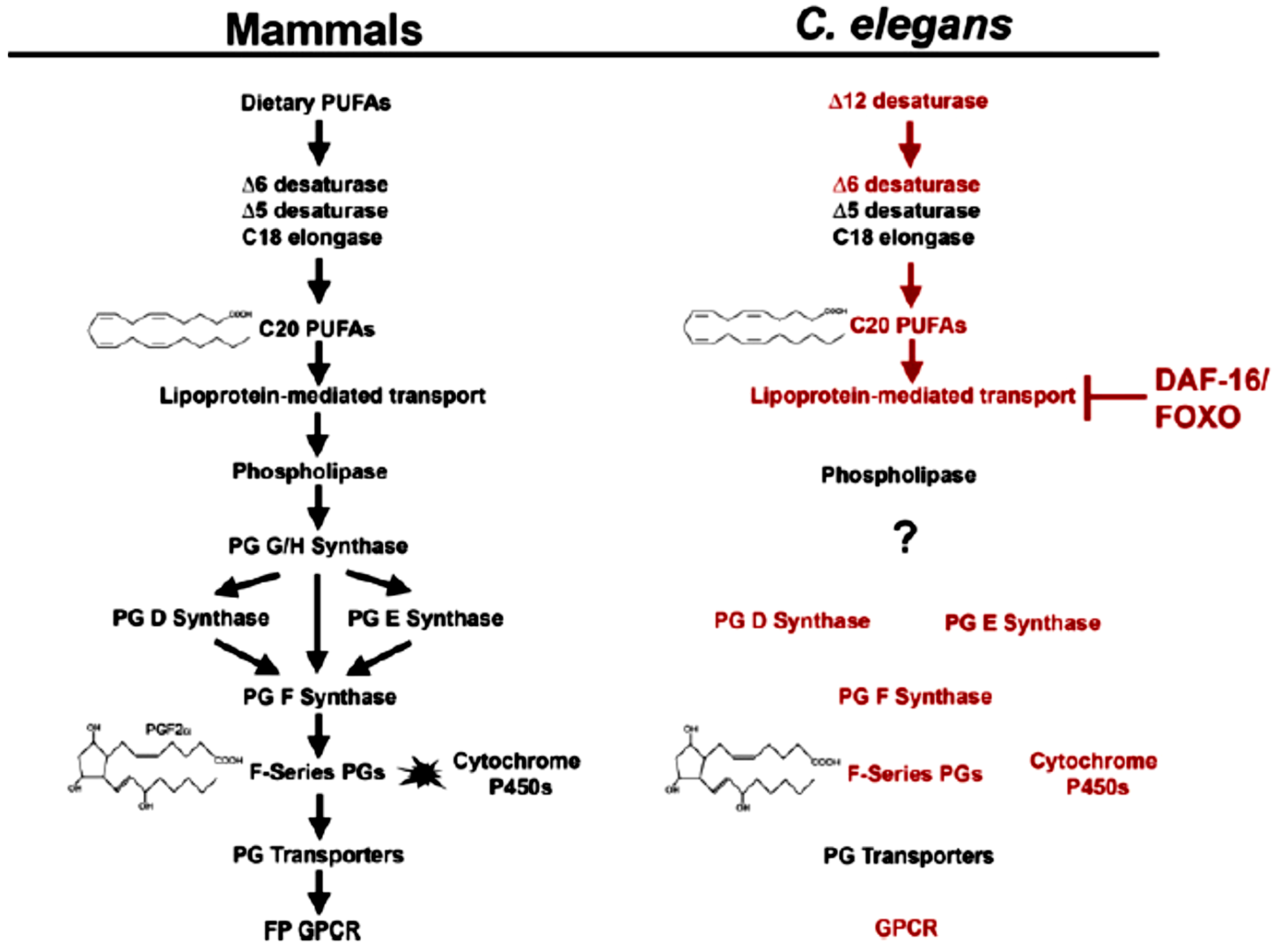


Figure 17. Prostaglandin synthesis pathway in mammals and predicted *C. elegans* pathway. The *C. elegans* genome share similar enzyme homologs with mammals, which synthesize and modify prostaglandins (PGs), however no PG G/H synthase is found in *C. elegans*. Red arrows indicate conserved steps in both mammals and *C. elegans.* Red colour indicates a process required for sperm guidance (Edmonds *et al.*, 2010).

Eicosanoid products of PUFAs have potent biological effects in *C. elegans* (Kulas *et al.*, 2008). Studies showed that the nematode can facilitate in describing the mechanisms that function in guiding the sperm to maturing oocytes (Kubagawa *et al.*, 2006; Edmonds *et al.*, 2010, 2011). For example, in order for fertilization to occur in *C. elegans*, the sperm senses the environment within the reproductive tract of the female, then locates a matured oocyte which has just fully completed meiotic maturation and ovulation. Sperm guidance during fertilisation was first found to depend on synthesis of 18- or 20-carbon PUFAs by the -6 and -12 desaturases FAT-2 and FAT-3, respectively (Hoang *et al.*, 2013). Follow up studies showed that these PUFAs, including AA and EPA, are synthesized in the intestine and transported to oocytes to be converted into F-series prostaglandins (a class of eicosanoid) to guide the sperm under the regulation of the insulin and TGF-β pathways (Figure 18) (Kubagawa *et al.*, 2006; Edmonds *et al.*, 2010; Hoang *et al.*, 2013). Briefly, in the intestine, there is a conversion of dietary fats to PUFAs, which are then incorporated into complexes of yolk lipoprotein (Kubagawa *et al.*, 2006). Thereafter the yolk is secreted into the nematode pseudocoelom, were it flows straight to the gonad. As it reaches the gonad it becomes endocytosed into oocytes by low-density lipoprotein receptor called receptor-mediated endocytosis 2 (RME-2) (Grant and Hirsh, 1999). In the oocytes, the yolk provides 20-carbon PUFAs that are in turn converted into about ten F-series prostaglandins that are structurally related, however they are independent of cyclooxygenase enzymes. The F-series prostaglandins are derived from the major precursors, DGLA, AA, and EPA, and consist of F1, F2, and F3 classes, that include prostaglandins $F_{1\alpha}$ ($PGF_{1\alpha}$) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stereoisomers (Kubagawa *et al.*, 2006; Hoang *et al.*, 2013).

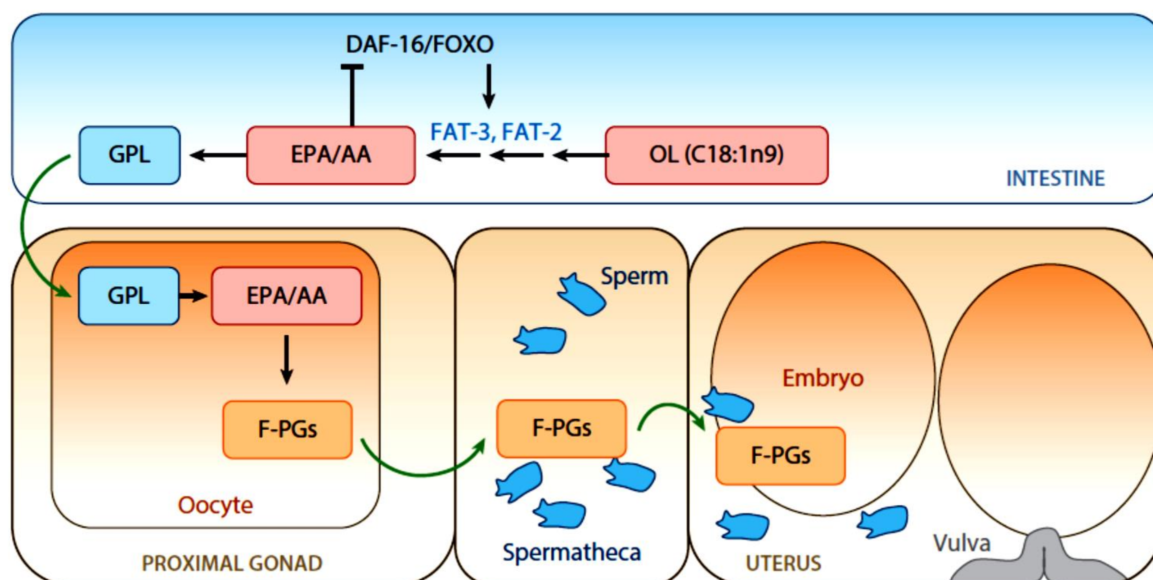


Figure 18. Guidance of sperm to the in spermatheca for egg fertilization in *Caenorhabditis elegans* by eicosanoids, F-series prostaglandins (F-PGs). In the intestines, DAF-16/FOXO promotes biosynthesis of polyunsaturated fatty acid (PUFA) (arachidonic acid, AA and eicosapentaenoic acid, EPA) through increased fat-family genes expression. The PUFAs regulates DAF-16 negatively to promote oocyte yolk endocytosis and PG synthesis. Then glycerophospholipid (GPL)-containing yolk translocates to oocytes in order to synthesize F-PGs that facilitates in sperm guidance. Intercellular transportation of lipid molecules such as GPLs and F-PGs are indicated by green arrows. Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; OL, oleic acid (Zhu and Han, 2014).

The importance of CYP-derived eicosanoids in the early growth phase of *C. elegans* was revealed by Benenati and co-workers (2009) where they demonstrated that depletion of two CYPs, CYP-31A2 or CYP-31A3, can result in polarity defects and arrest the embryo at the early stages. Moreover, the mutant embryos are unable to properly complete meiosis and also unable to establish their anterior-posterior axis correctly, thus suggesting the importance of specific lipid to facilitate in accurate execution of events in the early embryogenesis (Rappleye *et al*., 2003; Benenati *et al*., 2009). Recently, Olson *et al*. (2012) discovered that newly fertilized embryos form an envelope that underlies the eggshell acting as a permeability barrier. This permeable barrier is known to function in mediating important events in early embryogenesis, particularly the fully complete meiosis and also in the formation of anterior-posterior polarity. Interestingly, they also discovered that the biosynthesis of fatty acid and

activity of CYP-31A2/CYP-31A3, are required for establishment of the permeability barrier (Olson *et al.*, 2012).

Kosel and co-workers (2011) demonstrated that eicosanoid signalling can also function in pharyngeal cells, where it helps in the regulation of muscle contractions that are important for digestion. They also observed that one CYP isoform, CYP33E2, known to induce production of EET and HETE molecules in mammals, also functions in *C. elegans*. This CYP33E2 is one of the genes normally expressed in nematode pharynx (the organ responsible for rhythmically contraction to grind *E. coli* for digestion), however there can be a significant reduction in the pharyngeal contractions when the gene encoding CYP33E2 is depleted. Furthermore, another function of CYP-33E2 is to acts on DGLA and mediate the sterility that occurs after administration of dietary DGLA (Deline *et al.*, 2015). Other interesting studies demonstrated the function of eicosanoid biosynthesis, where the epoxygenase activity of CYP-13A12 is required for behavioural response to reoxygenation immediately after anoxia (Ma *et al.*, 2013; Keller *et al.*, 2014).

1.11 Lipid metabolism in *Candida albicans*

Candida albicans is an important fungal pathogen in humans, especially affecting individuals with implanted medical devices, those undergoing chemotherapy, or immunocompromised individuals. A number of virulence factors have been identified in *C. albicans* including secretion of hydrolytic enzymes, yeast to hyphal transitions, production of adhesions and biofilm formation (Calderone and Fonzi, 2001). For instance, the hyphae form penetrates tissues more easily and is, therefore, considered to be more pathogenic (Sobel *et al.*, 1984). Thus conversion to the hyphal form is required for virulence and invasiveness *in vivo*. Lipids govern many important physiological processes in *C. albicans,* including cellular permeability, enzymatic activity, morphogenesis, adhesion and virulence. For instance, fatty acids play a role in adherence and virulence since alterations in fatty acid synthesis decrease the adhesion of *C. albicans* to host epithelial cells (Hoberg *et al.*, 1986). The composition of fatty acids varies with the morphological form of the fungi, with the hyphal form containing more polyunsaturated fatty acids (Yano *et al.*, 1982; Ghannoum *et al.*, 1986; Sadamori, 1987).

1.11.1 Biosynthesis of lipids in *C. albicans*

1.11.1.1 Fatty acids biosynthesis

Cellular fatty acids are derived from three different sources: external supply, endogenous lipid turnover, and *de novo* synthesis and elongation (Figure 19A) (Tehlivets *et al*., 2007). Fatty acid biosynthesis and elongation in yeast begins with acetyl transferase enzyme, which binds acetyl CoA to a binding site on the fatty acid synthase complex to generate malonyl CoA (Figure 19B). Malonyl transacylation from CoA to the enzyme results in enzyme-bound malonate, followed by its condensation to 3-keto-acyl-enzyme by ketoacyl synthase. This is followed by the reduction of 3-ketoacyl to an intermediate, 3-hydroxyacyl, by ketoacyl reductase enzyme. Dehydratase catalyses the dehydration of this intermediate to 2, 3-trans-enoate and finally reduction of enoate to the saturated acyl-enzyme. The prosthetic group 4'-phosphopantetheine plays a central role in processing of intermediates (Schweizer and Hofmann, 2004). At the end of the process, palmitoyl transferase and thioesterase terminate chain elongation. Furthermore, yeast elongation elongate carbon 12 to carbon 18 acyl-CoA primers to carbon 18 to carbon 26 long chain fatty acids (Welch and Burlingame, 1973). About 70 % of the fatty acids found in *C. albicans* membranes are unsaturated fatty acids (Xu *et al*., 2009). For instance, PUFAs such as linoleic or linolenic acids are found in higher levels compared to other *C. albicans* fatty acids (Krishnamurthy *et al*., 2004). However, the balance between saturated fatty acids and unsaturated fatty acids is important in governing the fluidity of the membrane and morphogenesis in *C. albicans* (Krishnamurthy *et al*., 2004).

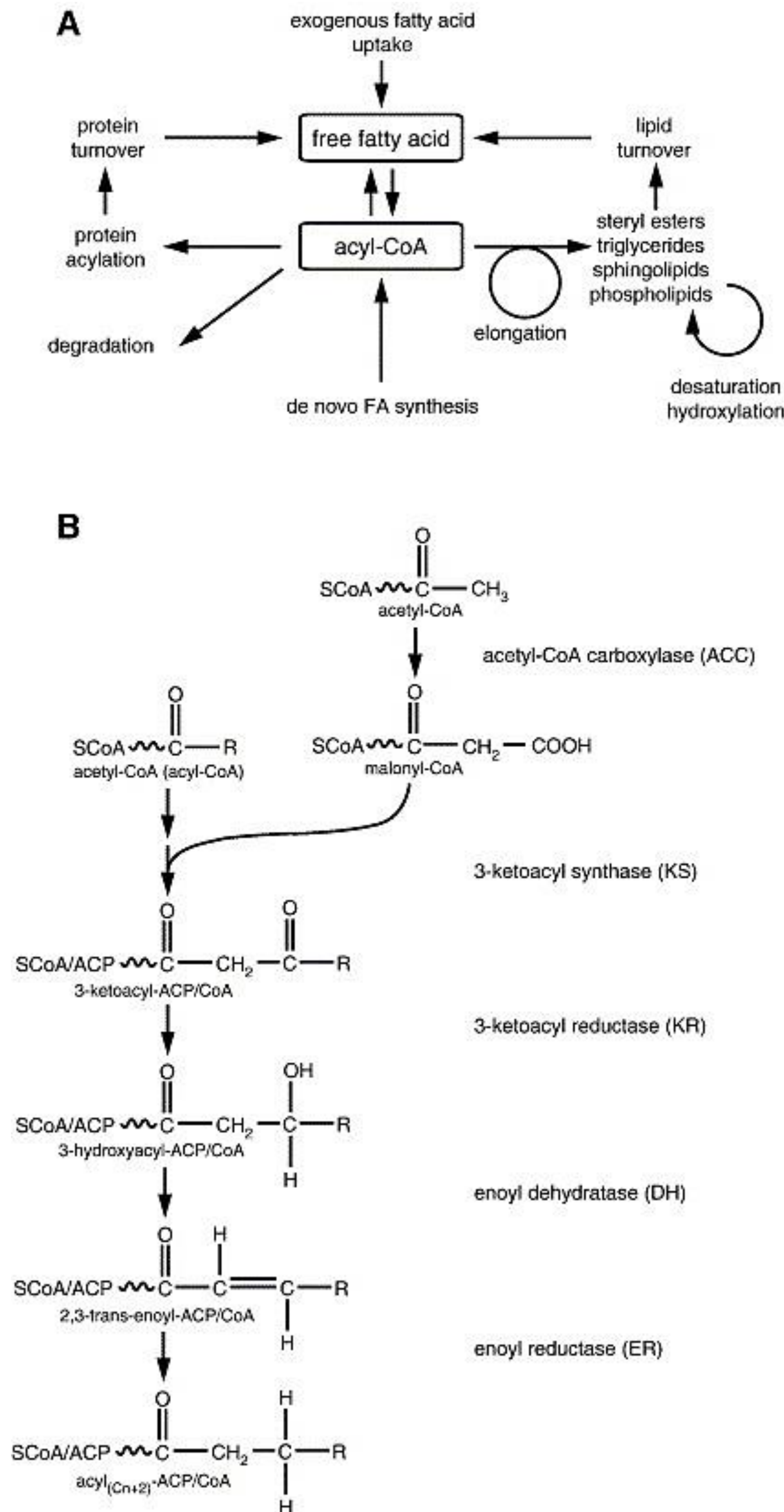


Figure 19. (A) A general schematic representation of fatty acid metabolism in yeast. (B) Reaction schemes of fatty acid synthesis and elongation (Tehlivets *et al.*, 2007).

1.11.1.2 Eicosanoids biosynthesis

Although a major player in fungal lipid signalling, fatty acids are not the only lipid molecules that affects signalling pathways in fungi. Eicosanoids such as PGE_2 and thromboxane B_2 are one such family of lipids that plays roles in a number of pathogenic fungi (Noverr *et al.*, 2001; Erb-Downward and Noverr, 2007). For instance *C. albicans* does not contain AA as part of its fatty acid repertoire; however, *C. albicans* is known to cause the release of AA from host tissues (Castro *et al.*, 1994). Unlike In mammalian cells, *C. albicans* lacks COX homolog, which catalyses conversion of AA into PGE_2. Instead, it was speculated that the fatty acid desaturase *OLE2*, the multicopper oxidase *FET3,* and also CYP450s facilitated in PGE_2 production (Erb-Downward and Noverr, 2007; Ells *et al.*, 2011; Krause *et al.*, 2015). Additionally, the fungus can use AA as a substrate for generation of 3,18-diHETE, which is related to hyphal formation (Deva *et al.*, 2000; Ells *et al.*, 2012). Moreover, studies revealed that in yeast, non-mitochondrial pathways are responsible for metabolizing majority of the AA, with the assumption that peroxisomal β-oxidation pathway metabolizes several fatty acids. *Candida albicans* is also able to produce the anti-inflammatory eicosanoid, resolvin E1, from EPA (Haas-Stapleton *et al.*, 2007).

1.11.2 Role of lipids on *C. albicans* morphology and pathogenicity

Lipids govern many important physiological processes in *C. albicans* including, but not limited to, cellular permeability, enzymatic activity, morphogenesis, adhesion and virulence. Fatty acids also fulfil diverse functions in the cell as they are important constituents of lipids, they can form side chains in co-enzymes and metabolites, and they also form covalent attachments to proteins and play a central role in energy storage and regulating the integrity and dynamics of cell membranes (Schweizer and Hofmann, 2004). Lipids are also involved in fungal development and pathogenicity (fa). For instance, fatty acids play a role in adherence and virulence (Hoberg *et al.*, 1986) since alterations in fatty acid synthesis decrease the adhesion of *C. albicans* to host epithelial cells. It has also been suggested that fatty acyl composition modulates chitin synthase enzyme activity (Hoberg *et al.*, 1983), and thus may play a role in cell wall biogenesis. Moreover, fatty acid profiles differ significantly between white and opaque cells with the ratio of unsaturated to saturated fatty acid being higher in opaque cells (Ghannoum *et al.*, 1990). Thus in addition to being important components of the cell membrane and governing the entry and exit of various molecules and metabolites, lipids also influence the activity of enzymes involved in cell wall morphogenesis and antifungal action.

In *C. albicans*, lipid molecules such as fatty acids and eicosanoids have been reported to modulate the yeast to hyphal transition. For instance, fatty acids, including butyric, capric, lauric, palmitoleic, oleic, linoleic, conjugated linoleic, and arachidonic acids, inhibited the yeast to hyphal transition induced under various conditions (McLain *et al.*, 2000; Noverr *et al.*, 2001; Murzyn *et al.*, 2010; Shareck and Belhumeur, 2011). The hypha-inhibiting activities of fatty acids were dependent on the medium. Moreover, although the effects of fatty acids on the yeast-hyphal transition have been described, their modes of action have not been studied extensively. In contrast to fatty acids, eicosanoids such as PGE_2 and thromboxane B_2 enhanced the yeast to hyphal transition (Odds, 1988; Noverr *et al.*, 2001; Erb-Downward and Noverr, 2007).

The interaction between *C. albicans* with the host, has the ability to trigger lipid mediator production from both *Candida* and host, and this can significantly influence immune responses. *Candida* produces both endogenous oxylipins (oxygenated polyunsaturated fatty acid) and novel eicosanoid products from exogenous AA (Deva *et al.*, 2000; Noverr *et al.*, 2001). Despite the fact that parts of the *C. albicans* fatty acid repertoire does not contain AA, *C. albicans* can trigger host tissue to release of AA (Noverr *et al.*, 2003). An important aspect of the determination of *C. albicans* virulence was the identification of a PGE cross reactive compound (PGEx) [(later identified as PGE_2 (Erb-Downward and Noverr, 2007; Fourie *et al.*, 2016)] produced by *C. albicans* from exogenous AA (Noverr *et al.*, 2001). Strong evidence revealed that this compound, was discovered to be bioactive on mammalian cells *in vitro* similar to PGE_2, thus it was concluded that it is PGE_2 (Noverr *et al.*, 2001). Moreover, all this indicated that the fungal oxylipins are capable of modulating host immune responses (Noverr *et al.*, 2001). As mentioned earlier, PGE_2 functions to elicit both pro- and anti-inflammatory responses, and can inhibit phagocytosis, lymphocyte proliferation and Th1-type immune responses (Sergeeva *et al.*, 1997; Strassmann *et al.*, 1994; McIlroy *et al.*, 2006). Moreover, it can also trigger, tissue eosinophilia, immunoglobulin E production and Th2-type responses (Erb-Downward and Noverr, 2007). Thus, during *Candida* infection, the formation of Th1-type immune response leads to protection and clearance, however Th2-type immune response is non-protective, leading to disseminating or chronic disease (Louie *et al.*, 1994). Therefore, PGE_2 may function as a vital factor in shifting the immune responses of host to a direction of those that trigger fungal colonization and chronic infection.

Researchers also reported that host and fungal PGE_2 enhance the morphogenesis of *C. albicans* (Noverr *et al.*, 2001). Additionally, supplementation of *C. albicans* cultures with exogenous AA significantly increased PGE_2 production. This was remarkable since *C. albicans* lack COX homologs found in mammalian cells which are responsible for PGE_2 production. Moreover there is an upregulation of candidal eicosanoid production during biofilm

formation and germ tube formation (Alem and Douglas, 2005). More studies showed that various COX-inhibitors including aspirin (ASA) have a drastic effect on the formation of biofilms and germ tubes (Alem and Douglas, 2004). However, the simultaneous addition of PGE_2 on *C. albicans* biofilm formation significantly abolished the inhibitory effect of ASA. Similarly, other mammalian eicosanoid inhibitors were also observed to inhibit candidal, morphogenesis, biofilm formation and eicosanoid production (Noverr *et al.*, 2001; Alem and Douglas, 2004). Together all these observations reveals the importance of the presence of an eicosanoid/oxylipin pathway in *C. albicans* in order to control morphogenesis and biofilm formation. Little is known about the direct effect of PGE_2 on *C. albicans*, though Levitin and Whiteway (2007) reported several alterations in the transcription profile of *C. albicans* upon exogenous exposure.

Studies also identified a HETE produced by *C. albicans* from AA namely 3, 18-diHETE, which is associated with hyphal forms and may play a role in adhesion during infection (Deva *et al.*, 2000; Ells *et al.*, 2012). Acetylsalicylic acid-sensitive 3-HETE produced by *C. albicans* was also speculated to be linked to *C. albicans* morphogenesis (Deva *et al.*, 2001). Additionally, 3-HETE can act as a precursor for COX-2 in mammalian cells forming 3-hydroxyeicosanoids with regards to their similarity with AA (Ciccoli *et al.*, 2005). More studies indicated that one of these metabolites, 3-OH-PGE_2, have a similar or even more robust effect on mammalian cells to elicit pro-inflammatory response.

Another class of lipids derived from omega-3-polyunsaturated fatty acids like AA is the resolvins (Rhome and Poeta, 2009). In mammals, these compounds are anti-inflammatory and affect migration of neutrophils in later stages of inflammation. Recently, Haas-Stapleton *et al.* (2007) described the synthesis of fungal resolvins in *C. albicans.* The synthesis of these resolvins was inhibited by lipoxygenase inhibitors, and production of these lipids by *C. albicans* affected neutrophil chemotaxis, phagocytosis, and intracellular killing at different concentrations. This finding is more evidence for the role of eicosanoid-derived lipids in fungal modulation of the host immune system. The anti-inflammatory lipid resolvin E1, which *Candida albicans* is able to produce from EPA, also plays a role in the interspecies signalling (Haas-Stapleton *et al.*, 2007). This compound was shown to protect *C. albicans* against host immunity at low doses, however, at higher concentration, this protective effect is lost. This is thought to facilitate the commensal carriage of *C. albicans* as part of the human microbiome. Although information is available regarding the eicosanoids produced, and their effect during single species infection, very little is known regarding the effect of eicosanoids produced by microbes on co-infecting pathogens, as well as on the host.

1.12 Lipid metabolism in *Pseudomonas aeruginosa*

1.12.1 Biosynthesis of lipids in *Pseudomonas aeruginosa*

1.12.1.1 Fatty acids biosynthesis

Fatty acid synthesis (FAS) is a primary metabolic pathway not only central to eukaryotes, but also to bacteria. A wide range of essential cellular processes are highly dependent on fatty acids, ranging from the biosynthesis of cellular structural components (lipoproteins, membrane phospholipids and lipoglycans), cofactors (biotin and lipoate), and also from energy storage reserves to diffusible secondary metabolites (siderophores, quorum-sensing signal molecules and biosurfactants). Fatty acids are usually assembled using two different types of biosynthetic pathways called FAS I and FAS II. In the mammalian FAS I pathway, a single multisubunit polypeptide acting as the acyl carrier protein (ACP) tether and also an important source of enzymatic activities for acyl chain elongation aims at synthesising the fatty acids. Most plants and bacteria, except some actinomycetes, use the disassociated FAS II pathway for production of *de novo* fatty acids (Schweizer and Hofmann, 2004; Lu *et al.*, 2004; White *et al.*, 2005; Gago, 2011). In this system, multiple discrete enzymes assemble the fatty acid chain on a freely diffusible phosphopantetheinylated ACP. The fact that bacterial FAS II system is disassociated and has a modular organisation, enables the synthesis of various fatty acid products that can be utilized by other multiple pathways, and this is in contrast to FAS I systems, that is more restricted to long-chain saturated fatty acids.

In the bacterial FAS II prototype pathway of *Escherichia coli* (Figure 20), the first committed step in fatty acid synthesis is the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). This reaction is carried out by the interacting AccBC and AccAD complexes (Cronan and Waldrop, 2002). The FabD (malonyl-CoA-ACP transacylase) catalyses the rapid equilibrium of malonyl groups between CoA and ACP. The condensation between malonyl-ACP and acetyl coenzyme A (acetyl-CoA) to form β-acetoacetyl-ACP is catalysed by the initiating enzyme β-ketoacyl ACP synthase III (FabH) (Tsay *et al.*, 1992). The β-keto carbonyl is next reduced to a hydroxyl group by the NADPH-dependent β-ketoacyl-ACP reductase (FabG) (Alberts *et al.*, 1964), dehydrated by β-hydroxyacyl-ACP dehydratase (FabZ/FabA) to form trans-2-enoyl-ACP (Kass *et al.*, 1967; Mohan *et al.*, 1994), and then reduced to a saturated acyl-ACP by NADH-dependent enoyl-ACP reductase (FabI) (Bergler *et al.*, 1994). The acyl-ACP intermediate is condensed with malonyl-ACP by the elongation β-ketoacyl ACP synthases I/II (FabB/FabF) (Garwin *et al.*, 1980), the product of which re-enters the reduction cycle for another round of elongation.

In the case of *E. coli*, synthesis of UFA usually occurs exclusively through anaerobic type II pathway and involves the activities of the *fabA* and *fabB* genes (Cronan *et al.*, 1969; Rosenfeld *et al.*, 1973; Clark *et al.*, 1983; Zhang *et al.*, 2002). The FabA is a β-hydroxydecanoyl-acyl carrier protein (ACP) dehydratase isomerase that forms *cis*-3-decenoyl-ACP, which is then elongated by the FabB condensing enzyme (Cronan *et al.*, 1969; Rosenfeld *et al.*, 1973; Clark *et al.*, 1983; Zhang *et al.*, 2002). Thus the proportion of UFA produced is governed by the levels of FabA and FabB expression. Like *E. coli*, *P. aeruginosa* also uses *fabA* and *fabB* for the anaerobic formation of UFA via the type II pathway that are co-ordinately expressed as a *fabAB* operon (Hoang and Schweizer, 1997). In *P. aeruginosa*, two oxygen-dependent desaturases, DesA and DesB, supplement the FabA pathway for UFA synthesis (Zhu *et al.*, 2006). The DesA introduces a double bond at the Δ9 position of fatty acyl chains attached to position 2 of existing membrane phospholipids, thus cells can quickly modify their membrane biophysical properties using this mechanism to adapt to changes in growth conditions (Zhu *et al.*, 2006). The DesB is also a Δ9-desaturase that introduces double bonds into acyl-CoAs that are produced from exogenous saturated fatty acids, thus allowing *P. aeruginosa* to modify the composition of exogenous fatty acids being transported into the cell (Zhu *et al.*, 2006).

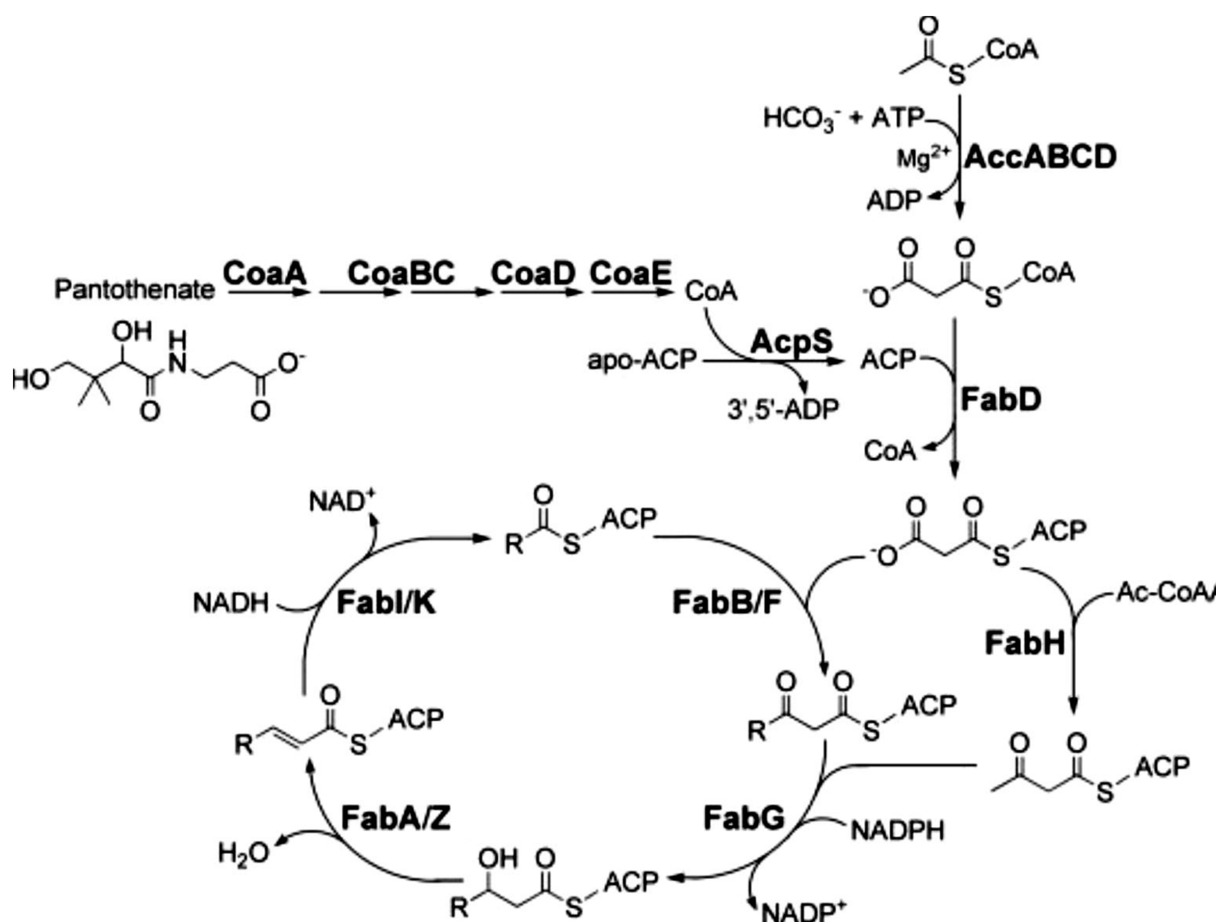


Figure 20. The bacterial type II fatty acid biosynthetic pathway. Here, bacterial membrane fatty acids are produced through catalysis of proteins which are encoded by separate genes capable of carrying out the individual enzymatic steps shown in the figure (Zhang *et al.*, 2006).

1.12.1.2 Eicosanoids biosynthesis

Pseudomonas aeruginosa is amongst the few bacterial species consisting of typical LOX genes. This bacterium possess a secretable 15-LOX, which is homologous to mammalian LOX, thus generating 15-HETE very similar to host 15-HETE and subsequently triggering anti-inflammatory effects on the host, therefore serving as a precursor for the formation of lipoxin (Serhan, 2002; Vance *et al.*, 2004). During *P. aeruginosa* infection, lipolytic enzyme PLA_2 cleaves membrane phospholipids releasing AA, which serves as the precursor to a diverse array of eicosanoids including hepoxilin A_3 (HXA_3) (Murakami and Kudo, 2002; Hurley and McCormick, 2008). The PLA_2-mediated liberation of AA is considered to be the rate-limiting step in the synthesis of all eicosanoids (Murakami and Kudo, 2002; Schaloske and Dennis, 2006). In addition during infection, large amount of AA are released mediated by ExoU, an

intracellular *P. aeruginosa* phospholipase, and this increase PGE_2 production (König *et al.*, 1996; Saliba *et al.*, 2005; Sadikot *et al.*, 2007; Agard *et al.*, 2013). Therefore, this ExoU function in the preliminary infection as well as infiltration by *P. aeruginosa*. In a case whereby ExoU was absent in *P. aeruginosa*, it led to diminished PGE_2 production and, also the infection became more severe (Agard *et al.*, 2013). For instance, the function of PGE_2 during *P. aeruginosa* infection was observed when a COX-2 inhibitor was employed, resulting in a decrease in severity of infection by this pathogen (Sadikot *et al.*, 2007). Moreover, a number of other virulence factors of *P. aeruginosa* also have tremendous effects on the levels of PGE_2. For example, production of QSM 3-oxo-HSL can induce COX-2, thus resulting in production of PGE_2 in human lung fibroblasts (Smith *et al.*, 2002; Fourie *et al.*, 2016).

1.12.2 Biological function of *P. aeruginosa* lipids

Bacteria regulate their membrane fatty acid composition to maintain an optimal fluidity to support growth under different environmental conditions (Zhang *et al.*, 2007). This is important since bacteria have evolved multiple mechanisms that controls fatty acid composition that is incorporated into membrane phospholipids, with fatty acid structure being consistent (de Mendoza and Cronan, 1983; Mansilla *et al.*, 2004). For instance, in adaptation to low temperature, the proportion of unsaturated fatty acids in membrane lipids increases (Russell and Fukunaga, 1990). The membranes of *P. aeruginosa* consist of straight-chain SFAs and UFAs. Interestingly, this bacterium has more UFAs than SFAs, with 1.8 UFA/SFA ratio, with cis-vaccinate (18:1n11) being the most predominant UFA identified in the wild type strain of *P. aeruginosa*, PAO1 (Zhu *et al.*, 2006). Moreover, 15-HETE synthesized by *P. aeruginosa* triggers anti-inflammatory effects on the host, as it acts as a precursor for generation of lipoxin (Serhan, 2002; Vance *et al.*, 2004). These lipoxin can somehow cause change in the severity of infection by causing inhibition on neutrophil recruitment and production of leukotrienes (Serhan, 2002).

1.13 Conclusions

There are several hosts used to model infections ranging from single cell protozoa to insects. For the best interrogation into host-pathogen interactions, researchers can select from a variety of invertebrate model hosts. *Caenorhabditis elegans* is one of the genetically tractable, whole animal model system used in most host-pathogen interactions. Most infection studies in *C. elegans* thus far have focused on clinically relevant pathogens, which have provided a

strong framework for studying infections in this host (Irazoqui *et al.*, 2010; Pukkila-Worley and Ausubel, 2012). Although these pathogens infect internal tissues, the transparency of *C. elegans* makes it possible to visualize and study pathogenic mechanisms of entry, replication and exit, as well as host defence mechanisms within whole animals. Understanding the earliest stages of the fungal-bacterial pathogen-host interactions in the nematode may thus enable us to gain a more complete picture of the innate immune response in isolation from the adaptive response. Further development of a virulence assay from this survival model, may entail the identification of components that are shared between the mammalian and invertebrate immune responses during *C. albicans* and *P. aeruginosa* pathogenesis.

While our knowledge of lipid signalling comes from studies in mammals, genetic model systems are being used to further our understanding of lipid signalling in development and reproduction. One emerging theme in this review is that fatty acid and eicosanoids appears to be fundamental for multicellular organisms, and often there are functionally relevant pathways in simple invertebrates. The early developmental events in *C. elegans* are amenable to experimental manipulation, allowing for genetic and biochemical approaches to determine roles for specific lipids in these processes. The nematode *C. elegans* has a highly simplified anatomy, rapid development, and an impressive conservation of fatty acid synthesis pathways. Moreover, studies with *C. elegans* are beginning to reveal roles for PUFAs, eicosanoids, and endocannabinoids in development and reproduction.

For future research, the evolution of synergistic, symbiotic, or antagonistic interactions between diverse organisms in nature or the clinical environment, especially those between prokaryotes and eukaryotes, is likely important for their pathogenesis toward a range of hosts, including humans. Thus the exploitation of the likely evolutionary defence mechanisms used by competing microbes may provide critical insights into novel therapeutic targets, which are desperately needed for pathogens such as *C. albicans* and *P. aeruginosa*. Further studies needed to extend the use of the *C. elegans* infection models to identify chemical compounds with antifungal activity against *Candida* species, the most common human pathogenic fungus. The search for novel antibacterial therapeutics greatly depends on representative infection models that mimic pathogenesis in humans and that can be used in a high-throughput fashion.

1.14 References

Aballay, A. & Ausubel. F. M. (2002). *Caenorhabditis elegans* as a host for the study of host-pathogen interactions. *Curr Opin Microbiol* **5**, 97–101.

Aballay, A., Drenkard, E., Hilbun, L. R. & Ausubel, F. M. (2003). *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signalling pathway. *Curr Biol* **13**, 47–52.

Agard, M., Asakrah, S. & Morici, L. A. (2013). PGE_2 suppression of innate immunity during mucosal bacterial infection. *Front Cell Infect Microbiol* **3**, doi: 10.3389/fcimb.2013.00045.

Alberts, A. W., Majerus, P. W., Talamo, B. & Vagelos, P. R. (1964). Acyl-carrier protein. II. Intermediary reactions of fatty acid synthesis. *Biochemistry* **3**, 1563–1571.

Albert, P. S. & Riddle, D. L. (1983). Developmental alterations in sensory neuroanatomy of the *Caenorhabditis elegans* dauer larva. *J Comp Neurol* **219**, 461-481.

Alem, M. A. S. & Douglas, L. J. (2004). Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. *Antimicrob Agents Chemother* **48**, 41–47.

Alper, S., Laws, R., Lackford, B., Boyd, W. A., Dunlap, P., Freedman, J. H. *et al*. (2008). Identification of innate immunity genes and pathways using a comparative genomics approach. *Proc Natl Acad Sci U S A* **105**, 7016-7021.

Altun, Z. F. & Hall, D. H. (2005). Handbook of *C. elegans* Anatomy. In *WormAtlas*. http://www.wormatlas.org/ver1/handbook/contents.htm.

Altun, Z. F. & Hall, D. H. (2009). Excretory system. In *WormAtlas*. doi:10.3908/wormatlas.1.17, http://www.wormatlas.org.

Ambros, V. (2000). Control of developmental timing in *Caenorhabditis elegans. Curr Opin Genet Dev* **10**, 428-433.

Antebi, A., Norris, C. R., Hedgecock, E. M. & Garriga, G. (1997). Cell and growth cone migrations. In the nematode *Caenorhabditis elegans* II, (eds. D.L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 583-609. New York: Cold Spring Harbor Press.

Arendrup, M., Horn, T. & Frimodt-Moller. N. (2002). *In vivo* pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection* **30**, 286–291.

Arvanitis, M., Glavis-Bloom, J. & Mylonakis, E. (2013). Invertebrate models of fungal infection. *Biochim Biophys Acta* **1832**, 1378–1383.

Ashrafi, K., Chang, F. Y., Watts, J. L., Fraser, A. G., Kamath, R. S. *et al.* (2003). Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421**, 268–72.

Bandara, H. M. H. N., K Cheung, B. P., Watt, R. M., Jin, L. J. & Samaranayake, L. P. (2013). *Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development. *Mol Oral Microbiol* **28**, 54–69.

Battista, N., Meccariello, R., Cobellis, G., Fasano, S., Di Tommaso, M., Pirazzi, V., Konje, J. C., Pierantoni, R. & Maccarrone, M. (2012). The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. *Mol Cell Endocrinol* **355**, 1–14.

Barrière, A. & Félix, M. A. (2014). Isolation of *C. elegans* and related nematodes, *WormBook* ed. The *C. elegans* Research Community, WormBook, doi: 10.1895/wormbook.1.115.2, http://www.wormbook.org.

Benenati, G., Penkov, S., Muller-Reichert, T., Entchev, E. V. & Kurzchalia, T. V. (2009). Two cytochrome P450s in *Caenorhabditis elegans* are essential for the organization of eggshell, correct execution of meiosis and the polarization of embryo. *Mech Dev* **126**, 382–393.

Bergeron, A. C., Seman, B. G., Hammond, J. H., Archambault, L. S., Hogan, D. A. & Wheeler, R. T. (2017). *Candida albicans* and *Pseudomonas aeruginosa* interact to enhance virulence of mucosal infection in transparent zebrafish. *Infect Immun* **85**, e00475-17.

Bergler, H., Wallner, P., Ebeling, A., Leitinger, B., Fuchsbichler, S., Aschauer, H., Kollenz, G., Högenauer, G. & Turnowsky, F. (1994). Protein EnvM is the NADHdependent enoyl-ACP reductase (FabI) of *Escherichia coli*. *J Biol Chem* **269**, 5493–5496.

Berman, J. & Sudbery, P. E. (2002). *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genet* **3**, 918-30.

Bird, A. F. & Bird, J. (1991). The structure of nematodes. Academic Press, California.

Blot, S. I., Vandewoude, K. H. & De Waele, J. J. (2007). *Candida* peritonitis. *Curr Opin Crit Care* **13**, 195–199.

Brand, A., Barnes, J. D., Mackenzie, K. S., Odds, F. C. & Gow, N. A. R. (2008). Cell wall glycans and soluble factors determine the interactions between the hyphae of *Candida albicans* and *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **287**, 48–55.

Breger, J., Fuchs, B. B., Aperis, G., Moy, T. I., Ausubel, F. M. & Mylonakis. E. (2007). Antifungal chemical compounds identified using a *Caenorhabditis elegans* pathogenicity assay. *PLoS Pathog* **3,** e18.

Brennan, M., Thomas, D. Y., Whiteway, M. & Kavanagh, K. (2002). Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol Med Microbiol* **34**,153–157.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Brock, T. J., Browse, J. & Watts, J. L. (2007). Fatty acid desaturation and the regulation of adiposity in *Caenorhabditis elegans*. *Genetics* **176**, 865–75.

Brodhun, F. & Feussner, I. (2011). Oxylipins in fungi. *FEBS* **278**, 1047–1063.

Bucher, E. A. & Seydoux, G. (1994). Gastrulation in the nematode *Caenorhabditis elegans*. *Sem Dev Biol* **5**, 121-130.

Burr, G. O. & Burr, M. M. (1930). On the nature and role of the fatty acids essential for nutrition. *J Biol Chem* **86**, 587–621.

Byerly, L., Cassada, R. C. & Russell, R. L. (1976). The life cycle of the nematode *Caenorhabditis elegans*. *Dev Biol* **51**, 23-33.

Calderone, R. A. & Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. *Trends Microbiol* **9**, 327–335.

Cassada, R. C. & Russell, R. L. (1975). The dauer larva, a postembryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev Biol* **46**, 326-342.

Castro, M., Ralston, N. V. C., Morgenthaler, T. I., Rohrbach, M. S. & Limper, A. H. (1994). *Candida albicans* stimulates arachidonic acid liberation from alveolar macrophages through alpha-mannan and beta-glucan cell wall components. *Infect Immun* **62**, 3138–3145.

Cezairliyan, B., Vinayavekhin, N., Grenfell-Lee, D., Yuen, G. J., Saghatelian, A. & Ausubel, F. M. (2013), Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*. *PLoS pathog* **9** (1), e1003101.

Chalfie, M. & White, J. (1988). The nervous system. In The nematode *Caenorhabditis elegans* (ed. Wood, W.B.). Chapter 11. pp 337-391. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.

Chamilos, G., Lionakis, M. S., Lewis, R. E., Lopez-Ribot, J. L., Saville, S. P., Albert, N. D. *et al.* (2006). *Drosophila melanogaster* as a facile model for large-scale studies of virulence mechanisms and antifungal drug efficacy in *Candida* species. *J Infect Dis* **193**, 1014–1022.

Chandrasekharan, N. V. & Simmons, D, L. (2004). The cyclooxygenases. *Genome Biol* **5**, 241.

Chen, A. I., Dolben, E. F., Okegbe, C., Harty, C. E., Golub, Y., Thao, S., Ha, D. G., Willger, S. D., Toole, G. A. O. *et al.* (2014). *Candida albicans* ethanol stimulates *Pseudomonas aeruginosa* WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog* **10**, doi: 10.1371/journal.ppat.1004480.

Chuang C. F. & Bargmann C. I. (2005). A Toll-interleukin 1 repeat protein at the synapse specifies asymmetric odorant receptor expression via ASK1 MAPKKK signaling. *Genes Dev* **19**, 270-281.

Ciccoli, R., Sahi, S., Singh, S., Prakash, H., Zafiriou, M. P., Ishdorj, G., Kock, J. L. F. & Nigam, S. (2005). Oxygenation by COX-2 (cyclo-oxygenase-2) of 3-HETE (3-hydroxyeicosatetraenoic acid), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3-hydroxyeicosanoids. *Biochem J* **390**, 737–747.

Clark, D. P., de Mendoza, D., Polacco, M. L. & Cronan, J. E. Jr. (1983). β-Hydroxydecanoyl thioester dehydrase does not catalyze a rate-limiting step in *Escherichia coli* unsaturated fatty acid synthesis. *Biochemistry* **22**, 5897–5902.

Corsi, A. K. (2006). A biochemist's guide to *Caenorhabditis elegans*. *Anal Biochem* **359**, 1–17.

Corsi, A. K., Wightman, B. & Chalfie, M. (2015). A Transparent window into biology: A primer on *Caenorhabditis elegans*. *Genetics* **200**, 387-407.

Cox, G. N., Staprans, S. & Edgar, R. S. (1981). The cuticle of *Caenorhabditis elegans*. II. Stage-specific changes in ultrastructure and protein composition during postembryonic development. *Dev Biol* **86**, 456-470.

Cribier, S., Sainte-Marie, J. & Devaux. P. F. (1993). Quantitative comparison between aminiphospholipid translocase activity in human erythrocytes and in K562 cells. *Biochim Biophys Acta* **1148**, 85-90.

Cronan, J. E., Jr, Birge, C. H. & Vagelos, P. R. (1969). Evidence for two genes specifically involved in unsaturated fatty acid biosynthesis in *Escherichia coli*. *J Bacteriol* **100**, 601–604.

Cronan, J. E, Jr. & Waldrop, G. L. (2002). Multi-subunit acetyl-CoA carboxylases. *Prog Lipid Res* **41**, 407–435.

Cruz, M. R., Graham, C. E., Gagliano, B. C., Lorenz, M. C. & Garsin, D. A. (2013). *Enterococcus faecalis* inhibits hyphal morphogenesis and virulence of *Candida albicans*. *Infect Immun* **81**, 189–200.

Cugini, C., Calfee, M. W., Farrow III, J. M., Morales, D. K., Pesci, E. C. & Hogan, D. A. (2007). Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. *Mol Microbiol* **65**, 896–906.

Culetto, E. & Sattelle, D. B. (2000). A role for *Caenorhabditis elegans* in understanding the function and interactions of human disease genes. *Hum Mol Genet* **9**, 869-877.

Darby, C., Cosma, C. L., Thomas, J. H. & Manoil, C. (1999). Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 15202–15207.

de Kievit, T. R., Parkins, M. D., Gillis, R. J., Srikumar, R., Ceri, H., Poole, K., Iglewski, B. H. & Storey, D. G. (2001). Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* **45**, 1761–1770.

de Mendoza, D. & Cronan, J. E. Jr. (1983). Thermal regulation of membrane lipid fluidity in bacteria. *Trends Biochem Sci* **8**, 49–52.

de Ruiter, J., Weel, J., Manusama, E., Kingma, W. P. & van der Voort, P. H. (2009). The epidemiology of intra-abdominal flora in critically ill patients with secondary and tertiary abdominal sepsis. *Infection* **37**, 522–527.

Deline, M., Keller, J., Rothe, M., Schunck, W. H., Menzel, R. & Watts, J. L. (2015). Epoxides derived from dietary dihomo-gamma-linolenic acid induce germ cell death in *C. elegans*. *Sci Rep* **5**, 15417.

Dennis, E. A. & Norris, P. C. (2015). Eicosanoid storm in infection and inflammation. *Nat Rev Immunol* **15**, 511-523.

Deva, R., Ciccoli, R., Kock, L. & Nigam, S. (2001). Involvement of aspirin-sensitive oxylipins in vulvovaginal candidiasis. *FEMS Microbiol Lett* **198**, 37–43.

Deva, R., Ciccoli, R., Schewe, T., Kock, J. L. F. & Nigam, S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochim Biophys Acta* **1486**, 299–311.

Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T. & Wang, H. (2004). Molecular cues to implantation. *Endocr Rev* **25**, 341–373.

Dickson, R. C. (1998). Sphingolipid functions in *Saccharomyces cerevisiae*: Comparison to Mammals. *Annu Rev Biochem* **67**, 27–48.

Doring, G. (1993). *Pseudomonas aeruginosa* infection in cystic fibrosis patients. In *Pseudomonas aeruginosa* as an opportunistic pathogen (eds. Campa, M., Bendinelli, M. & Friedman, H). Chapter 13. pp 245–273. Plenum Press, New York.

Dorniak, P., Bazer, F. W. & Spencer, T. E. (2012). Biological role of interferon tau in endometrial function and conceptus elongation. *J Anim Sci* doi: 10.2527/jas.2012-5845.

Driscoll, M. (1995). Methods for the study of cell death in the nematode *C. elegans*. *Methods Cell Biol* **46**, 323-353.

Dupont, H., Paugam-Burtz, C., Muller-Serieys, C., Fierobe, L., Chosidow, D., Marmuse, J. P., Mantz, J. & Desmonts, J. M. (2002). Predictive factors of mortality due to polymicrobial peritonitis with *Candida* isolation in peritoneal fluid in critically ill patients. *Arch Surg* **137**, 1341–1347.

Edmonds, J. W., Prasain, J. K., Dorand, D., Yang, Y., Hoang, H. D., Vibbert, J., Kubagawa, H. M. & Miller, M. A. (2010). Insulin/FOXO signaling regulates ovarian prostaglandins critical for reproduction. *Dev Cell* **19**, 858–871.

Edmonds, J. W., McKinney, S. L., Prasain, J. K. & Miller, M. A. (2011). The gap junctional protein INX-14 functions in oocyte precursors to promote *C. elegans* sperm guidance. *Dev Biol* **359**, 47–58.

Enoch, H. G., Catala, A. & Strittmatter, P. (1976). Mechanism of rat liver microsomal stearyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid. *J Biol Chem* **251**, 5095–5103.

Ewbank, J. J. (2006). Signaling in the immune response, *Wormbook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.83.1, http://www.wormbook.org.

Ells, E., Kilian, W., Hugo, A., Albertyn, J., Kock, J. L. F. & Pohl C. H. (2014). Virulence of South African *Candida albicans* strains isolated from different clinical samples. *Med Mycol* **52**, 246-253.

Ells, R., Kock, J. L. F., Albertyn, J. & Pohl, C. H. (2012). Arachidonic acid metabolites in pathogenic yeasts. *Lipids Health Dis* **11**, doi: 10.1186/1476-511X-11-100.

Ells, R., Kock, J. L. F., Albertyn, J., Kemp, G. & Pohl, C. H. (2011). Effect of inhibitors of arachidonic acid metabolism on prostaglandin E_2 production by *Candida albicans* and *Candida dubliniensis* biofilms. *Med Microbiol Immunol* **200**, 23–28.

Engelmann, I. & Pujol, N. (2010). Innate immunity in *C. elegans*. *Adv Exp Med Biol* **708**, 105-121.

Entchev, E. V., Schwudke, D., Zagoriy, V., Matyash, V., Bogdanova, A. *et al.* (2008). LET-767 is required for the production of branched chain and long chain fatty acids in *Caenorhabditis elegans*. *J Biol Chem* **283**, 17550–60.

Erb-Downward, J. R. & Noverr, M. C. (2007). Characterization of prostaglandin E_2 production by *Candida albicans*. *Infect Immun* **75**, 3498–505.

Fahy, E., Subramaniam, S., Murphy, R. C., Nishijima, M., Raetz, C. R., Shimizu, T., Spener, F., van Meer, G., Wakelam, M. J. & Dennis. E. A. (2009). Update of the LIPID MAPS comprehensive classification system for lipids. *J Lipid Res* **50**, S9–S14.

Fourie, R., Ells, R., Swart, C. W., Sebolai, O. M., Albertyn, J. & Pohl, C. H. (2016). *Candida albicans* and *Pseudomonas aeruginosa* interaction, with focus on the role of eicosanoids. *Front Physiol* **7**, 64.

Félix, M. A. & Duveau, F. (2012). Population dynamics and habitat sharing of natural populations of *Caenorhabditis elegans* and *C. briggsae*. *BMC Biol* **10**, 59.

Fisher, J. F., Meroueh, S. O. & Mobashery, S. (2005). Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. *Chem Rev* **105**, 395–424.

Fleming, I. (2007). Epoxyeicosatrienoic acids, cell signaling and angiogenesis. *Prostaglandins Other Lipid Mediat* **82**, 60–67.

Funk, C. D. (2001). Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* **294**, 1871-1875.

Gago, G., Diacovich, L., Arabolaza, A., Tsai, S. C. & Gramajo, H. (2011). Fatty acid biosynthesis in actinomycetes. *FEMS Microbiol Rev* **35** (3), 475-497.

Gallagher, L. A. & Manoil, C. (2001). *Pseudomonas aeruginosa* kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* **183**, 6207– 6214.

Garwin, J. L., Klages, A. L. & Cronan, J. E. Jr. (1980). Structural, enzymatic, and genetic studies of beta-ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli. J Biol Chem* **255**, 11949–11956

Ghannoum, M. A., Khamis, J. L. & Radwan, S. S. (1986). Dimorphism-associated variations in the lipid composition of *Candida albicans*. *J Gen Microbiol* **132**, 2367–2375.

Ghannoum M. A., Swairjo I. & Soll D. R. (1990). Variation in lipid and sterol contents in *Candida albicans* white and opaque phenotypes. *J Med Vet Mycol* **28**,103–115.

Glass, C. K. & Olefsky J, M. (2012). Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab* **15**, 635–645.

Golden, J. W. & Riddle, D. L. (1984). The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. *Dev Biol* **102**, 368-378.

Gonzalez-Moragas, L., Roig, A. & Laromaine, A. C. (2015). *C. elegans* as a tool for in vivo nanoparticle assessment. *Adv Colloid Interface Sci* **219**, 10–26.

Gotoh, O. (1998). Divergent structures of *Caenorhabditis elegans* cytochrome P450 genes suggest the frequent loss and gain of introns during the evolution of nematodes. *Mol Biol Evol* **15**, 1447–1459.

Govan, J. R. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539–574.

Gow, N. A. R. & Hube, B. (2012). Importance of the *Candida albicans* cell wall during commensalism and infection. *Curr Opin Microbiol* **15**, 406–412.

Gow, N. A., Van De Veerdonk, F. L., Brown, A. J. & Netea, M. G. (2012). *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat Rev Microbiol* **10**, 112–122.

Grant, B. & Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol Biol Cell* **10**, 4311–4326.

Greenwald, I. (1997). Development of the Vulva. In *C. elegans II* (ed. Riddle, D. L. *et al*.). Chapter 19. pp. 519-541. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Haas-Stapleton, E. J., Lu, Y., Hong, S., Arita, M., Favoreto, S., Nigam, S., Serhan, C. N. & Agabian, N. (2007). *Candida albicans* modulates host defence by biosynthesizing the pro-resolving mediator resolvin E1. *PLoS One* **2**, doi: 10.1371/journal.pone.0001316.

Han, M. (2015). Twists and turns-how we stepped into and had fun in the “boring” lipid field. *Sci China Life Sci* **58**, 1073–1083.

Hasibeder, W. & Halabi, M. (2014). *Candida* peritonitis. *Minerva Anestesiol* **80**, 470–481.

Hirata, T., Narumiya, S. & Frederick, W. A. (2012). Prostanoids as regulators of innate and adaptive immunity. *Adv Immunol* **116** (2012) 143-74.

Hoang, H. D., Prasain, J. K., Dorand, D. & Miller, M. A. (2013). A heterogeneous mixture of F-series prostaglandins promotes sperm guidance in the *Caenorhabditis elegans* reproductive tract. *PLoS Genet* **9**, e1003271.

Hoang, T. T. & Schweizer, H. P. (1997). Fatty acid biosynthesis in *Pseudomonas aeruginosa*: cloning and characterization of the *fabAB* operon encoding β-hydroxyacyl-acyl carrier protein dehydratase (FabA) and β-ketoacyl-acyl carrier protein synthase I (FabB). *J Bacteriol* **179**, 5326–5332.

Hoberg, K. A., Cihlar, R. L. & Calderone. R. A. (1983). Inhibitory effect of cerulenin and sodium butyrate on germination of *Candida albicans*. *Antimicrob Agents Chemother* **24**, 401–408.

Hoberg, K. A., Cihlar, R. L. & Calderone, R. A. (1986). Characterization of cerulenin-resistant mutants of *Candida albicans*. *Infect Immun* **51**, 102-109.

Hofs, S., Mogavero, S. & Hube, B. (2016). Interaction of *Candida albicans* with host cells: Virulence factors, host defense, escape strategies, and the microbiota. *J Microbiol* 54, 149–169.

Hogan, D. A. & Kolter, R. (2002). *Pseudomonas-Candida* interactions: an ecological role of virulence factors. *Science* **296**, 2229-2232.

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., Dussault, P. & Nickerson, K. W. (2001). Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67**, 2982–2992.

Houthoofd, K., Braeckman, B. P., Lenaerts, I., Brys, K., De Vreese, A., Van Eygen, S. & Vanfleteren, J. R. (2002). Axenic growth up-regulates mass-specific metabolic rate, stress resistance, and extends life span in *Caenorhabditis elegans*. *Exp Gerontol* **37**, 1371-1378.

Hu, P. J. (2007). Dauer, *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.144.1, http://www.wormbook.org.

Hurley, B. P. & McCormick. B. A. (2008). Multiple roles of phospholipase A_2 during lung infection and inflammation. *Infect Immun* **76**, 2259–2272.

Hutzell, P. A. & Krusberg, L. R. (1982). Fatty Acid Composition of *Caenorhabditis elegans* and *C. briggsae. Comp Biochem Physiol* **73**B, 517-520.

Irazoqui, J. E., Ng, A., Xavier, R. J. & Ausubel, F. M. (2008). Role for beta-catenin and HOX transcription factors in *Caenorhabditis elegans* and mammalian host epithelial-pathogen interactions. *Proc Natl Acad Sci U S A* **105**, 17469–17474.

Irazoqui, J. E., Troemel, E. R., Feinbaum, R. L., Luhachack, L. G., Cezairliyan, B. O. & Ausubel, F. M. (2010). Distinct pathogenesis and host responses during infection of *C. elegans* by *P. aeruginosa* and *S. aureus*. *PLoS Pathog* **6**, e1000982.

Johnson, C. H., Ayyadevara, S., McEwen, J. E. & Shmookler Reis, R. J. (2009). *Histoplasma capsulatum* and *Caenorhabditis elegans*: a simple nematode model for an innate immune response to fungal infection. *Med Mycol* **47**, 808–813.

Johnson, T. E., Mitchell, D. H., Kline, S., Kemal, R. & Foy, J. (1984). Arresting development arrests aging in the nematode *C. elegans*. *Mech Age Dev* **28**, 23-40.

Kahn-Kirby, A. H., Dantzker, J. L. Apicella, A. J. Schafer, W. R. Browse J. *et al.* (2004). Specific polyunsaturated fatty acids drive TRPV-dependent sensory signaling *in vivo*. *Cell* **119**, 889–900.

Kaletta, T. & Hengartner, M. O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov* **5**, 387-398.

Kass, L. R, Brock, D. J, Bloch, K. (1967). Beta-hydroxydecanoyl thioester dehydrase. I. Purification and properties. *J Biol Chem* **242**, 4418–4431.

Katona, I. & Freund, T. F. (2012). Multiple functions of endocannabinoid signaling in the brain. *Annu Rev Neurosci* **35**, 529–558.

Keller, J., Ellieva, A., Ma, D. K., Ju, J. J., Nehk, E., Konkel, A., Falck, J. R., Schunck, W. H. & Menzel, R. (2014). CYP-13A12 of the nematode *Caenorhabditis elegans* is a PUFA-epoxygenase involved in behavioural response to reoxygenation. *Biochem J* **464**, 61–71.

Kerr, J. R., Taylor, G. W., Rutman, A., Hoiby, N., Cole, P. J. & Wilson, R. (1999). *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* **52**, 385-387.

Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M. W. & Ausubel, F. M. (2002). A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. *Science* **297,** 623-626.

Kimble, J. & Hirsh, D. (1979). The post-embryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. *Dev Biol* **70**, 396-417.

Kirienko, N. V. Kirienko, D. R., Larkins-Ford, J., Wählby, C., Ruvkun, G. & Ausubel, F. M. (2013). *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans* iron homeostasis, causing a hypoxic response and death. *Cell Host Microbe* **13**, 406–416.

Kniazeva, M., Crawford, Q. T, Seiber, M., Wang, C. Y. & Han, M. (2004). Monomethyl branched-chain fatty acids play an essential role in *Caenorhabditis elegans* development. *PLoS Biol* **2**, e257.

Kniazeva, M., Sieber, M., McCauley, S., Zhang, K., Watts, J. L. & Han, M. (2003). Suppression of the ELO-2 FA elongation activity results in alterations of the fatty acid composition and multiple physiological defects, including abnormal ultradian rhythms, in *Caenorhabditis elegans*. *Genetics* **163**, 159–69.

König, B., Jaeger, K. E., Sage, A. E., Vasil, M. L. & König, W. (1996). Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect Immun* **64**, 3252-3258.

Kosel, M., Wild, W. Bell, A. Rothe, M. Lindschau, C. *et al.* (2011). Eicosanoid formation by a cytochrome P450 isoform expressed in the pharynx of *Caenorhabditis elegans*. *Biochem J* **435**, 689–700.

Krause, J., Geginat, G. & Tammer, I. (2015). Prostaglandin E_2 from *Candida albicans* stimulates the growth of *Staphylococcus aureus* in mixed biofilms. *PLoS One* **10**, doi: 10.1371/journal.pone.0135404.

Kremmyda, L. S., Tvrzicka, E., Stankova, B. & Zak, A. (2011). Fatty acids as biocompounds: Their role in human metabolism, health and disease-A review. Part 2: Fatty acid physiological roles and applications in human health and disease. *Biomed Pap Med Fac Univ Palacky Olomouc* **155**,195–218.

Krishnamurthy, S., Plaine, A., Albert, J., Prasad, T., Prasad, R. & Ernst. J. F. (2004). Dosage-dependent functions of fatty acid desaturase Ole1p in growth and morphogenesis of *Candida albicans. Microbiology* **150**, 1991–2003.

Kubagawa, H. M., Watts, J. L., Corrigan, C., Edmonds, J. W., Sztul, E. *et al.* (2006). Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment *in vivo*. *Nat Cell Biol* **8**, 1143–48.

Kuhn H. & O'Donnell V. B. (2006). Inflammation and immune regulation by 12/15-lipoxygenase. *Prog Lipid Res* **45**, 334–356.

Kulas, J., Schmidt, C., Rothe, M., Schunck, W. H. & Menzel, R. (2008). Cytochrome P450-dependent metabolism of eicosapentaenoic acid in the nematode *Caenorhabditis elegans*. *Arch Biochem Biophys* **472**, 65–75.

Kurz, C. L. & Ewbank, J. J. (2000). *Caenorhabditis elegans* for the study of host-pathogen interactions. *Trends Microbiol* **8**, 142-144.

Lee, Y. J., Lee, A., Yoo, H. J., Kim, M., Kim, M., Jee, S. H. *et al.* (2018). Effect of weight loss on circulating fatty acid profiles in overweight subjects with high visceral fat area: a 12-week randomized controlled trial. *Nutr J* **17**, 28.

Legendre, C., Mooij, M. J, Adams, C. & O'Gara, F. (2011). Impaired expression of hypoxia-inducible factor-1alpha in cystic fibrosis airway epithelial cells - a role for HIF-1 in the pathophysiology of CF? *J Cyst Fibros* **10**, 286–290.

Leibundgut, M., Maier, T., Jenni, S. & Ban, N. (2008). The multienzyme architecture of eukaryotic fatty acid synthases. *Curr Opin Struct Biol* **18**, 714-725.

Levitin, A. & Whiteway, M. (2007). The effect of prostaglandin E_2 on transcriptional responses of *Candida albicans*. *Microbiol Res* **162**, 201–210.

Lewis, J. A. & Fleming J. T. (1995). Basic culture methods. In *Caenorhabditis elegans,* Modern biological analysis of an organism (ed. Epstein, H.F. & Shakes, D.C). Chapter 1. pp 4-27. Academic Press, California.

Liang, J., Lints, R., Foehr, M. L., Tokarz, R., Yu, L., Emmons, S. W., Liu, J. & Savage-Dunn, C. (2003). The *Caenorhabditis elegans schnurri* homolog sma-9 mediates stage- and cell type-specific responses to DBL-1 BMP-related signaling. *Development* **130**, 6453–6464.

Lindsay, A. K. & Hogan, D. A. (2014). *Candida albicans*: molecular interactions with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Fungal Biol Rev* **28**, 85-96.

Lints, R. & Hall, D. H. (2009). Male reproductive system, general description. In *WormAtlas*. doi:10.3908/wormatlas.2.1, http://www.wormatlas.org.

Louie, A., Baltch, A. L. Smith, R. P. Franke, M. A. Ritz, W. J. Singh, J. K. & Gordon. M. A. (1994). Tumor necrosis factor alpha has a protective role in a murine model of systemic candidiasis. Infect. *Immun* **62**, 2761–2772.

Lu, Y. J., Zhang, Y. M. & Rock, C. O. (2004). Product diversity and regulation of type II fatty acid synthases. *Biochem Cell Biol* **82**, 145–155.

Ma, D. K., Rothe, M., Zheng, S., Bhatla, N., Pender, C. L., Menzel, R. & Horvitz, H. R. (2013). Cytochrome P450 drives a HIF-regulated behavioral response to reoxygenation by *C. elegans*. *Science* **341**, 554–558.

Mahajan-Miklos, S., Tan, M. W., Rahme, L. G. & Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis* model. *Cell* **96**, 47–56.

Mallo, G. V., Kurz, C. L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., Ewbank, J. J. (2002). Inducible antibacterial defense system in *C. elegans*. *Curr Biol* **12**, 1209–1214.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). Molecular cloning: a laboratory manual. (New York: Cold Spring Harbor Laboratory Press), p. 68.

Mansilla, M. C., Cybulski, L. E., Albanesi, D. & de Mendoza, D. (2004). Control of membrane lipid fluidity by molecular thermosensors. *J Bacteriol* **186**, 6681–6688.

Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S. & Lanzo, C. A. (1999). Arachidonic acid oxygenation by COX-1 and COX-2: mechanisms of catalysis and inhibition. *J Biol Chem* **274**, 22903-22906.

Marsh, E. K. & May, R. C. (2012). *Caenorhabditis elegans*, a model organism for investigating immunity. *Appl Environ Microbiol* **78**, 2075-2081.

Mayer, F. L., Wilson, D. & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* **4**,119–128.

McAlester, G., O'Gara, F. & Morrissey, J. P. (2008). Signal-mediated interactions between *Pseudomonas aeruginosa* and *Candida albicans*. *J Med Microbiol* **57**, 563–569.

McLain N., Ascanio R., Baker C., Strohaver R. A. & Dolan J. W. (2000). Undecylenic acid inhibits morphogenesis of *Candida albicans*. *Antimicrob Agents Chemother* **44**, 2873–2875.

McIlroy, A., Caron, G., Blanchard, S., Fremaux, I., Duluc, D., Delneste, Y., Chevailler, A. & Jeannin. P. (2006). Histamine and prostaglandin E up-regulate the production of Th2-attracting chemokines (CCL17 and CCL22) and down-regulate IFN-gamma-induced CXCL10 production by immature human dendritic cells. *Immunology* **117**, 507–516.

Méar, J. B., Kipnis, E., Faure, E., Dessein, R., Schurtz, G., Faure, K. & Guery, B. (2013). *Candida albicans* and *Pseudomonas aeruginosa* interactions: more than an opportunistic criminal association? *Médecine Mal Infect* **43**, 146–151.

Meléndez, A., Tallóczy, Z., Seaman, M., Eskelinen, E. L., Hall, D. H. & Levine, B. (2003). Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. *Science* **301**, 1387-1391.

Miyazaki, M., Jacobson, M. J., Man, W. C., Cohen, P., Asilmaz, E., Friedman, J. M. & Ntambi, J. M. (2003). Identification and characterization of murine SCD4, a novel heart-

specific stearoyl-CoA desaturase isoform regulated by leptin and dietary factors. *J Biol Chem* **278**, 33904–33911.

Mohan, S., Kelly, T. M., Eveland, S. S., Raetz, C. R. & Anderson, M. S. (1994). An *Escherichia coli* gene (*FabZ*) encoding (3R)-hydroxymyristoyl acyl carrier protein dehydrase. Relation to *fabA* and suppression of mutations in lipid A biosynthesis. *J Biol* Chem **269**, 32896-32903.

Morales, D. K. & Hogan, D. A. (2010). *Candida albicans* interactions with bacteria in the context of human health and disease. *PLoS Pathog* **6**, doi: 10.1371/journal.ppat.1000886.

Murakami, M. & Kudo. I. (2002). Phospholipase A_2. *J Biochem* **131**, 285–292.

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H. & Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* **424**, 277–283.

Murzyn, A., Krasowska, A., Stefanowicz, P., Dziadkowiec, D. & Lukaszewicz, M. (2010). Capric acid secreted by *S. boulardii* inhibits *C. albicans* filamentous growth, adhesion and biofilm formation. *PLoS One* **5**, e12050.

Mylonakis, E. & Aballay, A. (2005). Worms and flies as genetically tractable animal models to study host-pathogen interactions. *Infect Immun* **73,** 3833–3841.

Mylonakis, E., Ausubel, F. M., Perfect, J. R., Heitman, J. & Calderwood, S. B. (2002). Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. *Proc Natl Acad Sci U S A* **99**, 15675–15680.

Mylonakis, E., Idnurm, A., Moreno, R., El Khoury, J., Rottman, J. B. *et al.* (2004). *Cryptococcus neoformans* Kin1 protein kinase homologue, identified through a *Caenorhabditis elegans* screen, promotes virulence in mammals. *Mol Microbiol* **54**, 407–419.

Mylonakis, E., Moreno, R., El Khoury, J. B., Idnurm, A., Heitman, J., Calderwood, S. B, *et al.* (2005). *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. *Infect Immun* **73**, 3842–3850.

Nash, E. E., Peters, B. M., Fidel, P. L. & Noverr, M. C. (2015). Morphology-independent virulence of *Candida* species during polymicrobial intra-abdominal infections with *Staphylococcus aureus*. *Infect Immun* **84**, 90–98.

Nash, E. E., Peters, B. M., Palmer, G. E., Fidel, P. L. & Noverr, M. C. (2014). Morphogenesis is not required for *Candida albicans*-*Staphylococcus aureus* intraabdominal infection-mediated dissemination and lethal sepsis. *Infect Immun* **82**, 3426–3435.

Nicholas, W. L., Dougherty, E. C. & Hansen, E. L. (1959). Axenic cultivation of *C. briggsae* (Nematoda: Rhabditidae) with chemically undefined supplements; comparative studies with related nematodes. *Ann N Y Acad Sci* **77**, 218–236.

Nomura, T., Lu, R., Pucci, M. L. & Schuster, V. L. (2004). The two-step model of prostaglandin signal termination: *In vitro* reconstitution with the prostaglandin transporter and prostaglandin 15 dehydrogenase. *Mol Pharmacol* **65**, 973–978.

Noverr, M. C. & Huffnagle. G. B. (2004). Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect Immun* **72**, 6206–6210.

Noverr, M. C., Erb-Downward, J. R. & Huffnagle. G. B. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev* **16**, 517–533.

Noverr, M. C., Phare, S. M., Toews, G. B., Coffey, M. J. & Huffnagle, G. B. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* **69**, 2957-2963.

Ntambi, J. M. (1999). Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J Lipid Res* **40**, 1549–1558.

Obritsch, M. D., Fish, D. N, MacLaren, R. & Jung, R. (2005). Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. *Pharmacotherapy* **25**, 1353–1364.

Odds, F. C., Webster, C. E., Mayuranathan, P. & Simmons, P. D. (1988). Candida concentrations in the vagina and their association with signs and symptoms of vaginal candidosis. *J Med Veterinary Mycology* **26** (5), 277-283.

Okoli, I., Coleman, J. J., Tampakakis, E., An, W. F., Holson, E. *et al*. (2009). Identification of antifungal compounds active against *Candida albicans* using an improved high-throughput *Caenorhabditis elegans* assay. *PLoS One* **4**, e7025.

Olson, S. K., Greenan, G., Desai, A., Muller-Reichert, T. & Oegema, K. (2012). Hierarchical assembly of the eggshell and permeability barrier in *C. elegans*. *J Cell Biol* **198**, 731–748.

Panigrahy, D., Kaipainen, A., Greene, E. R. & Huang, S. (2010). Cytochrome P450-derived eicosanoids: the neglected pathway in cancer. *Cancer Metastasis Rev* **29** (4), 723–735.

Papaioannou, E., Wahjudi, M., Nadal-Jimenez, P., Koch, G., Setroikromo, R., & Quax, W. J. (2009). Quorum-quenching acylase reduces the virulence of *Pseudomonas aeruginosa* in a *Caenorhabditis elegans* infection model. *Antimicrob Agents Chemother* **53**, 4891–4897.

Peleg, A. Y., Tampakakis, E., Fuchs, B. B., Eliopoulos, G. M., Moellering, R. C. *et al.* (2008). Prokaryote-eukaryote interactions identified by using *Caenorhabditis elegans. Proc Natl Acad Sci U S A* **105**, 14585-14590.

Perez, C. L. & Van Gilst, M. R. (2008). A 13C isotope labelling strategy reveals the influence of insulin signaling on lipogenesis in *C. elegans*. *Cell Metab* **8**, 266–274.

Peyou-Ndi, M. M., Watts, J. L. & Browse, J. (2000). Identification and characterization of an animal delta (12) fatty acid desaturase gene by heterologous expression in *Saccharomyces cerevisiae*. *Arch Biochem Biophys* **376**, 399–408.

Powell, J. R. & Ausubel, F. M. (2008). Models of Caenorhabditis elegans infection by bacterial and fungal pathogens. *Methods Mol Biol* **415**, 403–27.

Powell, J. R, Kim, D. H. & Ausubel, F. M. (2009). The G protein-coupled receptor FSHR-1 is required for the *Caenorhabditis elegans* innate immune response. *Proc Natl Acad Sci U S A* **106**, 2782–2787.

Pukkila-Worley, R. & Ausubel, F. M. (2012). Immune defense mechanisms in the *Caenorhabditis elegans* intestinal epithelium. *Curr Opin Immunol* 24, 3–9.

Pukkila-Worley, R., Ausubel, F. M. & Mylonakis, E. (2011). *Candida albicans* infection of *Caenorhabditis elegans* induces antifungal immune defences. *PLoS Pathog* **7** (6), e1002074.

Pukkila-Worley, R., Peleg, A.Y., Tampakakis, E. & Mylonakis, E. (2009). *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. *Eukaryot Cell* **11**, 1750-1758.

Purschke, F. G., Hiller, E., Trick, I. & Rupp, S. (2012). Flexible survival strategies of *Pseudomonas aeruginosa* in biofilms result in increased fitness compared with *Candida albicans*. *Mol Cell Proteomics* **11**, 1652–1669. doi: 10.1074/mcp.M112.017673

Rahme, L., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. G. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899-1902.

Raizen, D. M., Zimmerman, J. E., Maycock, M. H., Ta, U. D., You Y. J. *et al.* (2008). Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* **451**, 569-572.

Rappleye, C. A., Tagawa, A. Le Bot, N. Ahringer, J. & Aroian, R. V. (2003). Involvement of fatty acid pathways and cortical interaction of the pronuclear complex in *Caenorhabditis elegans* embryonic polarity. *BMC Dev Biol* **3**, 8.

Ren, J., Knorr, C., Huang, L. & Brenig, B. (2004). Isolation and molecular characterization of the porcine stearoyl-CoA desaturase gene. *Gene* **340**, 19–30.

Rhome, R. & Del Poeta, M. (2009). Lipid signaling in pathogenic fungi. *Annu Rev Microbiol* **63**, 119–131.

Riddle, D. L. (1988). The dauer larva. In The nematode *Caenorhabditis elegans* (ed. Wood, W. B). Chapter 12. pp 393-412. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Rinzan, F. F. (2009). *Pseudomonas aeruginosa-Candida albicans* interactions from ecological and molecular perspectives. Department of Biology, Georgia State University, Atlanta, USA.

Rosenfeld, I. S., D'Agnolo, G. & Vagelos, P. R. (1973). Synthesis of unsaturated fatty acids and the lesion in fab B mutants. *J Biol Chem* **248**, 2452-2460.

Rowley, A. F., Vogan, C. L., Taylor, G. W. & Clare, A. S. (2005). Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. *J Exp Biol* **208**, 3–14.

Russel, F. G. M., Koenderink, J. B. & Masereeuw, R. (2008). Multidrug resistance protein 4 (MRP4/ABCC4): A versatile efflux transporter for drugs and signalling molecules. *Trends Pharmacol Sci* **29**, 200–207.

Russell, N. J. & Fukunaga, N. (1990). A comparison of thermal adaptation of membrane-lipids in psychrophilic and thermophilic bacteria. *FEMS Microbiol Rev* **75**, 171-182.

Sadamori, S. (1987). Comparative study of lipid composition of *Candida albicans* in the yeast and mycelial forms. *Hiroshima J Med Sci* **36**, 53–59.

Sadikot, R. T., Zeng, H., Azim, A. C., Joo, M., Dey, S. K., Breyer, R. M., Peebles, R. S., Blackwell, T. S. & Christman, J. W. (2007). Bacterial clearance of *Pseudomonas aeruginosa* is enhanced by the inhibition of COX-2. *Eur J Immunol* **37**, 1001–1009.

Sagasti, A., Hisamoto, N., Hyodo, J., Tanaka-Hino, M., Matsumoto, K. & Bargmann, C. I. (2001). The CaMKII UNC-43 activates the MAPKKK NSY-1 to execute a lateral signaling decision required for asymmetric olfactory neuron fates. *Cell* **105**, 221–232.

Saliba, A. M., Nascimento, D. O., Silva, M. C. A., Assis, M. C., Gayer, C. R. M., Raymond, B., Coelho, M. G. P., Marques, E. A., Touqui, L. *et al.* (2005). Eicosanoid-mediated proinflammatory activity of *Pseudomonas aeruginosa* ExoU. *Cell Microbiol* **7**, 1811–1822.

Samuelsson, B., Granstrom, E., Green, K., Hamberg, M. & Hammarstrom, S. (1975). Prostaglandins. *Annu Rev Biochem* **44**, 669–695.

Sapaar, B., Nur, A., Hirota, K., Yumoto, H., Murakami, K., Amoh, T., Matsuo, T. & Ichikawa, T., Miyake, Y. (2014). Effects of extracellular DNA from *Candida albicans* and pneumonia-related pathogens on *Candida* biofilm formation and hyphal transformation. *J Appl Microbiol* **116**, 1531–1542.

Saville, S. P., Lazzell, A. L., Monteagudo, C. & Lopez-Ribot. J. L. (2003). Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot Cell* **2**, 1053–1060.

Schaloske, R. H. & Dennis. E. A. (2006). The phospholipase A_2 superfamily and its group numbering system. *Biochim Biophys Acta* **1761**, 1246-1259.

Schneiter, R. (1999). Brave little yeast, please guide us to Thebes: sphingolipid function in *S. cerevisiae*. *Bioessays* **21**, 1004–1010.

Schuster, V. L. (2002). Prostaglandin transport. *Prostaglandins Other Lipid Mediat* **68**, 633–647.

Schweizer, E. & Hofmann, J. (2004). Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. *Microbiol Mol Biol Rev* **68** (3), 501-517.

Sergeeva, M. G., Gonchar, M. V., Mevkh, A. T. & Varfolomeyev. S. D. (1997). Prostaglandin E_2 biphasic control of lymphocyte proliferation inhibition by picomolar concentrations. *FEBS Lett* **418**, 235–238.

Serhan, C. N. (2002). Endogenous chemical mediators in anti-inflammation and pro-resolution. *Curr Med Chem Anti-Inflammatory Anti-Allergy Agents* **1**, 177–192.

Shaye, D. D. & Greenwald, I. (2011). OrthoList: a compendium of *C. elegans* genes with human orthologs. *PLoS One* **6**, e20085.

Shareck, J. & Belhumeur, P. (2011). Modulation of morphogenesis in *Candida albicans* by various small molecules. *Eukaryot Cell*, ***10*** (8), 1004–1012.

Shibata, Y., Henriksen, R. A., Honda, I., Nakamura, R. M. & Myrvik. Q. N. (2005). Splenic PGE_2-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol* **78**, 1281–1290.

Shiraya, K., Hirata, T., Hatano, R., Nagamori, S., Wiriyasermkul, P., Jutabha, P., Matsubara, M., Muto, S. *et al.* (2010). A novel transporter of SLC22 family specifically transports prostaglandins and co-localizes with 15-hydroxyprostaglandin dehydrogenase in renal proximal tubules. *J Biol Chem* **285**, 22141–22151.

Shirtliff, M. E., Peters, B. M. & Jabra-Rizk, M. A. (2009). Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett* **299**, doi: 10.1111/j.1574-6968.2009.01668.x.

Sifri, C. D., Begun, J. & Ausubel, F. M. (2005). The worm has turned-microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol* **13**, 119–127.

Slack, F. & Ruvkun, G. (1997). Temporal pattern formation by heterochronic genes. *Annu. Rev Genet* **31**, 611-634.

Smith, R. S., Harris, S. G., Phipps, R. & Iglewski, B. (2002). The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-Oxododecanoyl) homoserine lactone contributes to virulence and induces inflammation *in vivo*. *J Bacteriol* **184**, 1132-1139.

Sobel, R. A., Blanchette, B. W., Bhan, A. K. & Colvin. R. B. (1984). The immunopathology of experimental allergic encephalomyelitis. *J Immunol* **132**, 2393-2401.

Soberman, R. J. & Christmas, P. (2003). The organization and consequences of eicosanoid signaling. *J Clin Invest* **111**, 1107–1113.

Sommer, A., Rickert, R., Fischer, P., Steinhart, H., Walter, R. D. & Liebau, E. (2003). A dominant role for extracellular glutathione S-transferase from *Onchocerca volvulus* is the production of prostaglandin D_2. *Infect Immun* **71**, 3603–3606.

Spector, A. A. & Kim, H. Y. (2015). Cytochrome P450 epoxygenase pathway of polyunsaturated fatty acid metabolism. *Biochim Biophys Acta* **1851**, 356–365.

Spychalla, J. P., Kinney, A. J. & Browse, J. (1997). Identification of an animal omega-3 fatty acid desaturase by heterologous expression in *arabidopsis*. *Proc Natl Acad Sci U S A* **94**, 1142–1147.

Stanley-Samuelson, D. W. & Pedibhotla, V. K. (1996). What can we learn from prostaglandins and related eicosanoids in insects? *Insect Biochem Mol Biol* **26**, 223–234.

Stewart, L. C. & Yaffe. M. P. (1991). A role for unsaturated fatty acids in mitochondrial movement and inheritance. *J Cell Biol* **115**, 1249-1257.

Strassmann, G., Patil-Koota, V. Finkelman, F. Fong, M. & Kambayashi. T. (1994). Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E_2. *J Exp Med* **180**, 2365–2370.

Stukey, J. E., McDonough, V. M. & Martin, C. E. (1989). Isolation and characterization of OLEI, a gene affecting fatty acid desaturation from *Saccharomyces cerevisiae*. *J Biol Chem* **264**, 16537-1 6544.

Sulston, J. E. (1976). Post-embryonic development in the ventral cord of *Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci* **275**, 287-297.

Sulston, J. & Hodgkin. J. (1998). Methods, The nematode *C. elegans,* edited by W. B. Wood. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sulston, J. E. & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol* **56**, 110-156.

Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol* **100**, 64-119.

Takahashi, T., Morrow, J. D., Wang, H. & Dey, S. K. (2006). Cyclooxygen-ase-2-derived prostaglandin E_2 directs oocyte maturation by differentially influencing multiple signaling pathways. *J Biol Chem* **281**, 37117–37129.

Tampakakis, E., Peleg, A. Y. & Mylonakis, E. (2009). The interaction of *Candida albicans* with an intestinal pathogen; *Salmonella enterica* serovar *Typhimurium*. *Eukaryot. Cell* **8**, 732–737.

Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G. & Ausubel, F. M. (1999). *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc Natl Acad Sci U S A* **96**, 2408–2413.

Tanaka, T., Ikita, K., Ashida, T., Motoyama, Y., Yamaguchi, Y. & Satouchi, K. (1996). Effects of growth temperature on the fatty acid composition of the free-living nematode *Caenorhabditis elegans*. *Lipids* **31**, 1173–78.

Tehlivets, O., Scheuringer, K. & Kohlwein, S. D. (2007). Fatty acid synthesis and elongation in yeast. *Biochim Biophys Acta* **1771**, 255–270.

Tootle, T. L. & Spradling, A. C (2008). *Drosophila* Pxt: a cyclooxygenase-like facilitator of follicle maturation. *Development* **135**, 839–847.

Trejo-Hernández, A., Andrade-Domínguez, A., Hernández, M. & Encarnación, S. (2014). Interspecies competition triggers virulence and mutability in *Candida albicans–Pseudomonas aeruginosa* mixed biofilms. *ISME J* **8**, doi: 10.1038/ismej.2014.53

Troemel, E. R., Felix, M. A., Whiteman, N. K., Barriere, A. & Ausubel, F. M. (2008). *Microsporidia* are natural intracellular parasites of the nematode *Caenorhabditis elegans*. *PLoS Biol* **6**, e309.

Tsay, J. T., Oh, W., Larson, T. J., Jackowski, S. & Rock, C. O. (1992). Isolation and characterization of the beta-ketoacyl-acyl carrier protein synthase III gene (fabH) from *Escherichia coli* K-12. *J Biol Chem* **267**, 6807– 6814.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs. *Nat New Biol* 231, 232-235.

Vance, R. E., Hong, S., Gronert, K., Serhan, C. N. & Mekalanos, J. J. (2004). The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. *Proc Natl Acad Sci U S A* **101**, 2135–2139.

Villar, C. C., Kashleva, H., Mitchell, A. P. & Dongari-Bagtzoglou, A. (2005). Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. *Infect Immun* **73**, 4588–4595.

Von Ehrenstein, G. & Schierenberg, E. (1980). Cell lineages and development of *Caenorhabditis elegans* and other nematodes. In Nematodes as biological models Vol. I, Behavioral and developmental models (ed. Zuckerman, B. M). Chapter 1. pp 2-68. Academic Press, New York.

Vrablik, T. L. & Watts, J. L. (2012). Emerging roles for specific fatty acids in developmental processes. *Genes Dev* **26**, 631–637.

Vrablik, T. L. & Watts, J. L. (2013). Polyunsaturated fatty acid derived signaling in reproduction and development: insights from *Caenorhabditis elegans* and *Drosophila melanogaster*. *Mol Reprod Dev* **80**, 244–59

Wallis, J. G., Watts, J. L. & Browse, J. (2002). Polyunsaturated fatty acid synthesis: what will they think of next? *Trends Biochem Sci* **27**, 467.

Wang, H. & Dey, S. K. (2005). Lipid signaling in embryo implantation. *Prostaglandins Other Lipid Mediat* **77**, 84–102.

Wang, W. G. Ma, L. Tejada, *et al.* (2004). Rescue of female infertility from the loss of cyclooxygenase-2 by compensatory up-regulation of cyclooxygenase-1 is a function of genetic makeup. *J Biol Chem* **279**, 10649-10658.

Warensjo, E., Riserus, U. & Vessby, B. (2005). Fatty acid composition of serum lipids predicts the development of metabolic syndrome in men. *Diabetologia* **48**, 1999–2005.

Watts, J. L. & Browse, J. (2002). A palmitoyl-CoA-specific delta9 fatty acid desaturase from *Caenorhabditis elegans*. *Biochem Biophys Res Commun* **272**, 263–269.

Watts, J. L. & Browse, J. (2000). Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans. Proc Natl Acad Sci U S A* **99**, 5854–5859.

Welch, J. W. & Burlingame. A. L. (**1973).** Very long chain fatty acids in yeast. *J Bacteriol* **115**, 464-466.

Wenk, M. R. (2010). Lipidomics: New tools and applications. *Cell* **143**, 888–895.

White, S. W., Zheng, J., Zhang, Y. M. & Rock, C. O. (2005). The structural biology of type II fatty acid biosynthesis. *Annu Rev Biochem* **74**, 791–831.

Wolkow, C. A. & Hall, D. H. (2015). Handsobook of *C. elegans* Dauer Anatomy. In *WormAtlas*.

Wood, R. E. (1976). *Pseudomonas*: the compromised host. *Hosp Pract* **11**, 91-100.

Wood, W. B. (1988). Introduction to *C. elegans* biology. In The nematode *Caenorhabditis elegans* (ed. Wood, W. B). Chapter 1. pp 1-16. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Xu, D., Sillaots, S., Davison, J., Hu, W., Jiang, B., Kauffman, S*. et al.* (2009). Chemical genetic profiling and characterization of small-molecule compounds that affect the biosynthesis of unsaturated fatty acids in *Candida albicans*. *J Biol Chem* **284** (29), 19754-19764.

Xu, H., Sobue, T., Thompson, A., Xie, Z., Poon, K., Ricker, A., Cervantes, J., Diaz, P. I. & Dongari-Bagtzoglou, A. (2014). Streptococcal co-infection augments *Candida* pathogenicity by amplifying the mucosal inflammatory response. *Cell Microbiol* **16,** 214–231.

Xu, X., Teck Ho Lee, R., Fang, H., Wang, Y., Li, R., Zou, H., Zhu, Y. & Wang, Y. (2008). Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host Microbe* **4**, 28–39.

Yano, K., Yamada, T., Banno, Y., Sekiya, T. & Nozawa, Y. (1982). Modification of lipid composition in a dimorphic fungus, *Candida albicans*, during the yeast cell to hypha transformation. *Jpn J Med Mycol* **23**, 159–165.

Zaborin, A. *et al.* (2009). Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proc Natl Acad Sci U S A* **106**, 6327–6332.

Zhang, H., Abraham, N., Khan, L. A. & Gobel. V. (2015). RNAibased biosynthetic pathway screens to identify *in vivo* functions of non-nucleic acid-based metabolites such as lipids. *Nat Protoc* **10**, 681–700.

Zhang, Y., Adams, I. P. & Ratledge, C. (2007). Malic enzyme: the controlling activity for lipid production? Overexpression of malic enzyme in *Mucor circinelloides* leads to a 2.5-fold increase in lipid accumulation. *Microbiology* **153**, 2013–2025.

Zhang, Y. M., Marrakchi, H. & Rock, C. O. (2002). The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. *J Biol Chem* **277**, 15558–15565.

Zhang, H., Wang, P. & Qi, Q. (2006). Molecular effect of FadD on the regulation and metabolism of fatty acid in *Escherichia coli*. *FEMS Microbiol Lett* **259**, 249–253.

Zheng, J. & Greenway, F. L. (2012). *Caenorhabditis elegans* as a model for obesity research. *Int J Obes* **36**, 186–194.

Zhu, H. & Han. M. (2014). Exploring developmental and physiological functions of fatty acid and lipid variants through worm and fly genetics. *Annu Rev Genet* **48,** 119–148.

Zhu, K., Choi, K. H., Schweizer, H. P., Rock, C. O. & Zhang, Y. M. (2006). Two aerobic pathways for the formation of unsaturated fatty acids in *Pseudomonas aeruginosa*. *Molec Microbiol* **60**, 260–273.

Zugasti, O. & Ewbank, J. J. (2009). Neuroimmune regulation of antimicrobial peptide expression by a noncanonical TGF-beta signaling pathway in *Caenorhabditis elegans* epidermis. *Nat Immunol* **10**, 249–256.

Zurier. R. B., Sun, Y. P., George, K. L. *et al.* (2009). Ajulemic acid, a synthetic cannabinoid, increases formation of the endogenous proresolving and anti-inflammatory eicosanoid, lipoxin A_4. *FASEB J* **23**, 1503–1509.

CHAPTER 2

Establishment of *C. elegans* infection model

2.1 Abstract

The discovery of substantial cohesion between the pathogenicity of microorganisms in mammals and simple non-vertebrate host models, including the nematode *Caenorhabditis elegans*, has created the foundation in order to develop high throughput genetic analysis to help study the virulence factors of microbial pathogens in live animal models. The free-living nematode *C. elegans* is a widely used model system in the studies of immunology, genetics and also host-pathogen interactions, due to its many traits, such as simple growth conditions, rapid growth rates, short generation time, simple body structure and transparent nature. In this study, we used *C. elegans* as a host to study the polymicrobial interactions between two pathogens that are ecologically associated and clinically troublesome: the yeast, *Candida albicans* and bacterium, *Pseudomonas aeruginosa.* Previous research indicates significant interaction between these two microorganisms *in vitro*, as well as between the individual pathogens and the host during infection. The main objective of this specific study is to establish the effect of co-infection of the nematode, *C. elegans* by laboratory and clinical strains of *P. aeruginosa* and *C. albicans* on survival of the nematode. In order to do this, we successfully cultivated and monitored the life cycle of *C. elegans glp-4; sek-1* hermaphrodite at the temperature of 15 °C. Thereafter *C. elegans* was successfully infected with the two pathogens separately and in combination. This study indicated that these polymicrobial interactions are more pathogenic to *C. elegans* than monomicrobial infections. These findings present a promising extension to the ongoing research in the study of monomicrobial pathogenesis using *C. elegans* and also provides new information in terms of a whole animal model system used in studying the multifaceted dynamics of a polymicrobial infection.

Keywords: *Caenorhabditis elegans*, *Candida albicans*, *Pseudomonas aeruginosa*, pathogen, co-infection, monomicrobial, polymicrobial, virulence, pathogenesis

2.2 Introduction

The ubiquitous soil living nematode *Caenorhabditis elegans,* has become a widely used host model in the study of variety of research questions in the field of immunology, genetics, and host-pathogen interactions. Interestingly, this *C. elegans* infection model has numerous technical advantages compared to mammalian infection models. In such that investigations of mammalian pathogenesis is complicated by long reproductive cycles, difficult to handle and complexity of mammalian internal organs (Breger *et al.*, 2007). However, *C elegans* has ease of handling, simplicity of equipment, relative low cost, simple growth conditions, self-fertile (hermaphrodite producing approximately 300 progeny), small brood sizes and no ethical considerations (Breger *et al.*, 2007; Everman *et al.*, 2015). Moreover, the nematode *C. elegans* has a short reproductive cycle of about 2-3 weeks (Brenner, 1974). This life cycle consists of the embryonic stage, larva 1 to larva 4 (L1-L4) and adulthood (Brenner, 1974). Briefly, after the nematode hatches from an egg, it passes through four developmental stages, L1 to L4 larvae and finally to a fully matured adult stage (Brenner, 1974). The nematode can also survive some of the harsh environmental conditions such as starvation, by entering a growth arrested stage called dauer larvae stage, in which it usually survives for about two to three months (Cassada and Russell, 1975). In addition, the nematode transparent, and the simple and streamlined body structure offers a simplified *in vivo* model system that is able to mimic pathogenic and physiological mechanisms occurring during the time of infection, moreover, it also enables microscopic visualization of internal events (Brenner, 1974; Troemel *et al.*, 2006; Pukkila-Worley *et al.*, 2009; Akira, 2009; Everman *et al.*, 2015). For instance, the nematode intestine has been used to readily recognise pathogen-specific virulence factors interacting with epithelial surfaces (Everman *et al.*, 2015). Interestingly, intestinal epithelium of *C. elegans* share similar structure, morphology, and also function (including to serve as a first defence against invading microbial pathogens) with human intestinal epithelial cells (Troemel *et al.*, 2006; Akira, 2009; Everman *et al.*, 2015).

Thus, such a model system will assist in advancing our current knowledge of microbial pathogenesis within the realistic environment that enables existence of polymicrobial species. Thus far, its usage has focused on the investigations of monomicrobial infections, whereby a wide range of human medically relevant pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, *Candida albicans* and *Enterococcus faecalis* have been characterized pathogenic towards *C. elegans* (Garsin *et al.*, 2001; Breger *et al.*, 2007; Pukkila-Worley *et al.*, 2009; Irazoqui *et al.*, 2010; Durai *et al.*, 2011; Everman *et al.*, 2015). However, humans are often inhabited or co-infected with polymicrobial pathogens, and such interactions can trigger virulence capability of either organism. For example, most studies

describe the antagonistic interaction between *C. albicans* and *P. aeruginosa in vitro* (Hogan and Kolter, 2002; Rinzan, 2009; Shirtliff *et al.*, 2009; Méar *et al.*, 2013; Lindsay and Hogan, 2014; Xu *et al.*, 2014, Fourie *et al.*, 2016). Here researchers discovered that during the polymicrobial biofilms formation, *P. aeruginosa* can inhibit hyphal formation of *C. albicans*, through secretion of numerous virulence factors, which includes production of phenazine pigment called pyocyanin (Kerr *et al.*, 1999; Brand *et al.*, 2008). At the same time, *C. albicans* secretes quorum sensing molecule, farnesol, which reduces *Pseudomonas* quinolone signal with downstream reduction in pyocyanin production (Cugini *et al.*, 2007). Moreover, production of ethanol in *C. albicans* has also been shown to trigger formation of biofilms and to inhibit motility of *P. aeruginosa* (Chen *et al.*, 2014).

Regardless of the abundance of polymicrobial interactions in nature, at present there is a devastating scarcity of *in vivo* infection model systems that can functionally explore the virulence mechanisms of polymicrobial species. Moreover, there are challenges existing with cultivating mocroorganisms growing in polymicrobial interactions for pathogenesis studies, thus there is a desperate requirement for a facile, genetically tractable infection model. Although a model system for the co-infection of *C. elegans* by *C. albicans* and *P. aeruginosa* has been established (Peleg *et al.*, 2008), little is known about the interaction specifically between these pathogens in *C. elegans* infection model. Therefore, in this present study, we sought to expand the usage of *C. elegans* infection models to study the co-infection by *C. albicans* and *P. aeruginosa*, and also their virulence potential in *C. elegans*.

2.3 Materials and methods

2.3.1 Monitoring of *Caenorhabditis elegans* life cycle

2.3.1.1 Strains used

Caenorhabditis elegans glp-4; sek-1 hermaphrodites, obtained from the *Caenorhabditis* Genetic Center, College of Biological Sciences, University of Minnesota, were propagated on Nematode Growth Medium (NGM) (2.5 g/L peptone, 3 g/L sodium chloride, 17 g/L agar) spotted with *Escherichia coli* OP50 (a uracil-requiring mutant of *E. coli*). The rationale behind the use of *C. elegans glp-4*; *sek-1* mutant as a replacement for wild type nematodes, is due to the fact that *C. elegans* wild type is capable of producing many offspring which can confound killing assays, this can be challenging when separating adults from progeny. Moreover larvae can hatch inside the nematode, killing the nematode through a process called matricidal killing, which is not directly related to pathogen pathogenicity (Breger *et al.*, 2007; Peleg *et al.*, 2008; Pukkila-Worley *et al.*, 2009). In contrast to wild types, *glp-4* mutants cannot produce gonads

or progeny at 25 °C, making it beneficial for these studies, and mutant nematodes are able to survive longer than normal wild type nematodes (Miyata *et al.*, 2008). *Caenorhabditis elegans sek-1* nematodes are immunocompromised, allowing them to become infected with various pathogens, since *SEK-1* encodes a conserved mitogen-activated protein kinase (MAPKK) involved in the innate immune response (Kim *et al.*, 2002). Stocks of *E. coli* OP50 were kept at -80 °C, and during experiments, the frozen aliquots were thawed then grown in Luria-Bertani (LB) broth (10 g/L sodium chloride, 5 g/L yeast extract; 10 g/L tryptone,). A 24 h fresh bacterial culture was spotted on NGM agar followed by incubation at 37 °C overnight. *Caenorhabditis elegans* nematodes were transferred to new NGM agar plates seeded with lawn of *E. coli* OP50 every 6 days. All *C. elegans* Stocks were kept at 15 °C until further use.

2.3.1.2 Monitoring of life cycle at 15 °C

About 10 to 15 reproductively active synchronized *C. elegans* adult hermaphrodites were moved to fresh NGM agar plate thereafter incubated at 15 °C to allow the nematode to have time to lay eggs. After laying of eggs, all adult nematodes were removed from plate. Then the eggs remaining on the agar plates were allowed to hatch, then go through all stages of life cycle (L1 larvae-adult), and the time needed for each developmental stage to occur was monitored at 15 °C. Thereafter approximately 20 young adult nematodes were moved to a fresh NGM agar plate with *E.coli* OP50 lawn. This is followed by incubation of NGM agar plates with nematodes at 15 °C to allow nematodes to propagate and feed on all the bacterial food. Once all the bacterial food has been depleted, the newly hatched nematodes undergo growth arrest entering the dauer larvae stage. All manipulations were done at room temperature (20 ± 1 °C) (Porta-de-la-Riva *et al.*, 2012).

2.3.2 *Candida albicans* infection model

2.3.2.1 Strains used

Candida albicans SC5314 reference strain and two clinical strains, *C. albicans* Ho6 and Ho153, isolated from tracheal aspirate patients acquired from the University of the Free State Yeast Culture Collection, were used. Yeast strains were preserved on yeast extract-peptone-dextrose (YPD) agar (5 g/L peptone, 3 g/L yeast extract, 10 g/L glucose, 16 g/L agar) at 30 °C. The *C. elegans glp-4*; *sek-1* strain was used as an infection to perform all experiments. The nematodes were propagated on new NGM plates with *E. coli* OP50 lawns using technique previously described by Brenner, 1974. In all experiments we used *E. coli* OP50 as our control.

2.3.2.2 *C. albicans-C. elegans* liquid medium pathogenesis assay

A previously described protocol by Breger and co-workers (2007) was modified for establishment of *C. elegans* infected with *C. albicans* in a liquid medium pathogenesis assay. For each strain, freshly grown *C. albicans* cells were inoculated into 5 ml of YPD broth then incubated at 37 °C overnight. Thereafter, a 100 µl aliquot of each yeast strain was evenly spread into a 10 mm square lawn on a large (100 mm x 15 mm) brain-heart infusion agar plate (BHI) (7.8 g/L brain extract, 9.7 g/L heart extract, 2.5 g/L disodium phosphate, 2.0 g/L dextrose, 15 g/L agar), then incubated at 37 °C overnight. Synchronized L4 or adult *C. elegans* nematodes grown at 15 °C were washed from two large plates containing *E. coli* OP50 with a sterile M9 buffer (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 5 g/L NaCl, 0.25 g/L $MgSO_4.7H_2O$).

Bacterial food source, which may skew the infection process, was removed from the samples via sucrose floatation prior to nematode collection (Jenkins, 1964). Briefly, using a 15 ml conical centrifuge tube, equal volumes of the washed nematodes preparation and the stock of 60 % sucrose solution were mixed to yield an approximate sucrose concentration of 30 %, and a final fluid volume of 7 to 10 ml. The samples were kept cold to inhibit thrashing of nematodes. Thereafter, the samples were spinned in a cold centrifuge for 5 min at a high speed. Floating nematodes were aspirated using a transfer pipette and expelled into a clean conical centrifuge tube. Furthermore, there was a repeated washing of harvested nematodes with distilled water and centrifugation.

Thereafter, roughly 400 to 500 washed nematode were placed on the centre of *C. albicans* lawns grown on BHI agar. The BHI agar plates with infected nematodes and also nematodes feeding on *E. coli* OP50 as our control, were incubated at 25 °C for 4 h. After incubation, nematodes were carefully washed off the BHI agar plates with 6 ml of sterile M9 buffer and transferred to 15 ml conical tube. Nematodes were thoroughly washed with M9 buffer four times. Any microbial contaminants, which may confound the infection process, was removed via sucrose floatation during the washing step (Jenkins, 1964). About sixty to seventy nematodes were transferred into 2 ml of liquid medium (80 % M9 buffer, 20 % BHI) in a single well of a six-well tissue culture plate. Nematodes were daily monitored by scoring them as either alive, dead or dead with hyphal formation. If nematodes did not show any movement in response to picking mechanical stimulation, they were considered dead thus removed from liquid medium assay and stored at -80 °C until further use.

2.3.3 *Pseudomonas aeruginosa* infection model

2.3.3.1 Strains used

Pseudomonas aeruginosa PAO1 reference strain and two clinical strains, *P. aeruginosa* Iso6 and Iso20, isolated from tracheal aspirate and provided by Department of Medical Microbiology at the Health Sciences faculty of University of the Free State, were used. All bacterial strains were maintained in LB broth (10 g/L sodium chloride, 5 g/L yeast extract, 10 g/L tryptone) at 37 °C. The *C. elegans glp-4*; *sek-1* strain was used as an infection model to perform all experiments. Nematodes were propagated on new NGM plates containing *E. coli* OP50 lawns through methods previously described by Brenner, 1974. In all experiments we used *E. coli* OP50 as our control.

2.3.3.2 *P. aeruginosa*-*C. elegans* liquid medium pathogenesis assay

To assess effects of *P. aeruginosa* infection on *C. elegans*, we made use of the liquid killing medium assay, following a similar protocol that was previously reported by Breger *et al.* (2007). For each strain, freshly grown cells of *P. aeruginosa* were inoculated respectively into 5 ml of LB broth medium followed by incubation at 37 °C overnight. An aliquot of 100 µl of bacterial suspension was evenly spread onto a 10 mm square lawn on a large (100 mm x 15 mm) BHI agar plate, then incubated at 37 °C overnight. Synchronized L4 or adult *C. elegans* nematodes, growing on two large NGM agar plates seeded with *E. coli* OP50 were washed off carefully with a sterile M9 buffer. Thereafter, roughly 400 to 500 washed nematode were placed on the centre of *P. aeruginosa* lawns grown on BHI agar. The BHI agar plates with infected nematodes and also nematodes feeding on *E. coli* OP50 as our control, were incubated at 25 °C for 4 h. After incubation period, nematodes were carefully washed off the BHI agar plates with 6 ml of sterile M9 buffer and transferred into 15 ml conical tube. Importantly, great care was taken when transferring the nematodes into the conical tube to minimize the transfer of bacteria. Nematodes were thoroughly washed with M9 buffer four times. Bacterial contaminants, which may confound the infection process, was removed via sucrose floatation during washing steps (Jenkins, 1964). About sixty to seventy nematodes were transferred into 2 ml of liquid medium (80 % M9 buffer, 20 % BHI) in a single well of a six-well tissue culture plate. Nematodes were daily monitored by scoring them as either alive or dead. If nematodes did not show any movement in response to picking mechanical stimulation, they were considered dead thus removed from the liquid medium assay and kept at -80 °C until further use.

2.3.4 *Candida albicans-Pseudomonas aeruginosa* co-infection model

The methodology used for the *C. elegans*-*P. aeruginosa*-*C. albicans* liquid medium assay was based on the methods described previously by Breger and co-workers (2007). Freshly grown strains of *C. albicans* were each inoculated into 5 ml of YPD broth medium then incubated at 37 °C overnight. Similarly, strains of *P. aeruginosa* were each inoculated into 5 ml of LB broth and incubated at 37 °C overnight. Lawns of either *C. albicans* or *P. aeruginosa* were prepared separately by plating 100 µl of the yeast or bacterial strains as 10 mm square lawns on a large (100 mm x 15 mm) BHI agar plate, then incubated at 37 °C for 24 h. Synchronized L4 or adult *C. elegans glp-4*; *sek-1* nematodes, growing on two large NGM agar plates seeded with *E. coli* OP50 were washed off carefully with a sterile M9 buffer. Thereafter, approximately 400 to 500 washed nematode were placed on the centre of *C. albicans* lawns growing on BHI agar. The BHI agar plates with infected nematodes and also nematodes feeding on *E. coli* OP50 as our control, were incubated at 25 °C for 2 h. After the incubation period, nematodes were carefully washed off the BHI agar plates with 6 ml of sterile M9 buffer and transferred to15 ml conical tube. Nematodes were thoroughly washed four times with M9 buffer. Any microbial contaminants, which may confound the infection process, was removed via sucrose floatation during the washing step (Jenkins, 1964). Thereafter, washed nematodes were added to the centre of the *P. aeruginosa* lawns then incubated at 25 °C for further 2 h. After incubation period, nematodes were carefully washed off the BHI agar plates with 6 ml of sterile M9 buffer and transferred to 15 ml conical tube. Importantly great care was taken to minimize the transfer of microbes into the conical tube. Nematodes were thoroughly washed with M9 buffer four times. About sixty to seventy nematodes were transferred into 2 ml of liquid medium (80 % M9 buffer, 20 % BHI) in a single well of a six-well tissue culture plate. Nematodes were daily monitored by scoring them as either alive, dead or dead with hyphae piercing the cuticle. If nematodes did not show any movement in response to picking mechanical stimulation, they were considered dead thus removed from the liquid medium assay and stored at -80 °C until further use.

2.3.5 Visualisation of hyphae formation by *Candida albicans*

2.3.5.1 Transmission electron microscopy

Caenorhabditis elegans were infected with either single species of *C. albicans* or in combination with *P. aeruginosa* as described above. Dead infected nematodes samples were prepared respectively for transmission electron microscopy (TEM) based on the protocol by Swart *et al.* (2010). Briefly, the first step is the fixation with 3 % glutardialdehyde (Merck, Darmstadt, Germany) buffered with 0.1 M sodium phosphate buffer (pH 7) for 3 h. This then

is followed by a second fixation with a buffered solution of 1 % osmium tetroxide (Merck, Darmstadt, Germany) for another 1 h. Each of the fixation steps were followed with rinsing of the nematodes with the same sodium phosphate buffer for 5 min, twice after both the glutardialdehyde fixation and after the osmium tetroxide fixation steps. The nematodes were then dehydrated with acetone in the following series of concentrations; 50 %, 70 %, 95 %, with each step for 20 min and twice in 100 % acetone for 1 h. After the dehydration process, the nematodes were embedded accordingly in epoxy resin followed by polymerization for 8 h at 70 ºC in special moulds. All the embedded nematode samples were sliced into 60 nm thick sections by a Leica ultracut UM7 microtome by means of a glass knife. After the sections were mounted on copper grids, then they were subsequently double stained using uranyl acetate (Merck, Darmstadt, Germany) (10 min) followed by lead citrate (Merck, Darmstadt, Germany) (5 min), and after each staining there was rinsing. The sections were viewed using a TEM [FEI (Phillips) CM 100, Netherlands]. For each observation, we evaluated about 10 cross-sections, and chose the respective representative images.

2.3.5.2 Fluorescence microscopy

Caenorhabditis elegans were infected with single species *C. albicans* or in combination with *P. aeruginosa* PAO1 reference strain as described above. However, the strains of *C. albicans* used in this experiment were as follows: *C. albicans* M2194 (which fluoresces only when hyphae are present) and *C. albicans* M137 (which fluoresces constitutively). These strains were kindly provided by Prof. Bernhard Hube, Microbial Pathogenicity Mechanism, Leibniz-institut für Naturstoff-Forschung und Infektionsbiologie (Table 1). Dead nematodes were examined using a fluorescent inverted Olympus CKX53 microscope**.**

Table 1. *Candida albicans* strains

Strain number	Strain name	Genotype	Reference
M2194	SC5314 + pECE-1GFP	ECE1/ece1::(P_ECE1-GFP-T_ACT1	Moyes *et al.*, 2016
M137	CAI4 + P143	rps1::(P_ACT1-GFP URA3)	Fradin *et al.*, 2005

2.3.6 Statistical analyses

Each stage specific time and *C. elegans* pathogenesis assay presented was repeated in triplicate on separate occasions and calculated the mean and standard deviation. A student *t*-test was carried out in order to determine the statistically significant differences between data sets. Moreover, the *p* value of ≤ 0.05 was considered significant.

2.4 Results and discussion

2.4.1 Life cycle of *C. elegans*

The greatest advantage of using *C. elegans* as a biological model, is its short life cycle of about 2-3 weeks (Brenner, 1974). After *C. elegans* hatches from an egg, it passes through four larval stages (L1-L4) just before moulting into mature reproductive adults (Brenner, 1974). At the end of each larva stage, the nematode undergoes moulting, where the stage-specific cuticle is shed off and the new one develops (Raizen *et al*., 2008). The nematode develops at different rates depending on various temperatures, showing a rapid life cycle at higher temperature than at lower temperatures (Brenner, 1974; Porta-de-la-Riva *et al*., 2012). For instance, a typical life cycle takes about 90 hours at 15 °C, and about 45 hours when grown at 25 °C (Porta-de-la-Riva *et al*., 2012). Thus, studies on different developing rates at different temperatures is required in order to perform subsequent experiments. In our present study we determined the duration of the different stages at 15 °C in order to conduct further experiments.

Figure 1 shows the respective results of *C. elegans* life cycle at 15 °C. We observed that it takes 24 h for eggs to hatch to L1 larvae stage. From the L1 to L2 larvae stage it took *C. elegans* 8 h, while from L2 to L3 larvae stage it took 16 h. For L3 to reach L4 larvae stage, it took 8 h. Furthermore, from L4 larvae stage to adulthood it took 16 h. An increase in size of nematodes occurred between each stage, as it develops from L1 to L4 larvae and to matured adults. The adults took 24 h to lay eggs. When nematodes were allowed to consume all available bacterial food, they entered the growth arrested dauer larvae stage. This was seen after 2 weeks. The dauer larvae are highly stress-resistant and survive longer than other developmental stages of *C. elegans* (Cassada and Russell, 1975). Moreover, we could morphologically distinguish dauer larvae from other developmental stages, as they are thinner and denser than normal stages. In addition, they were motionless, unless disturbed. Their oral orifice was plugged, preventing the nematode from feeding. Another behaviour that distinguish dauer larvae is that they usually clustered together into a droplet of condensation. After 3 months, we supplied the dauer larvae nematodes with food and they bypassed the L2 and L3

larvae stage and entered directly into L4 larvae, where they proceeded to develop normally and could survive for 2 more weeks.

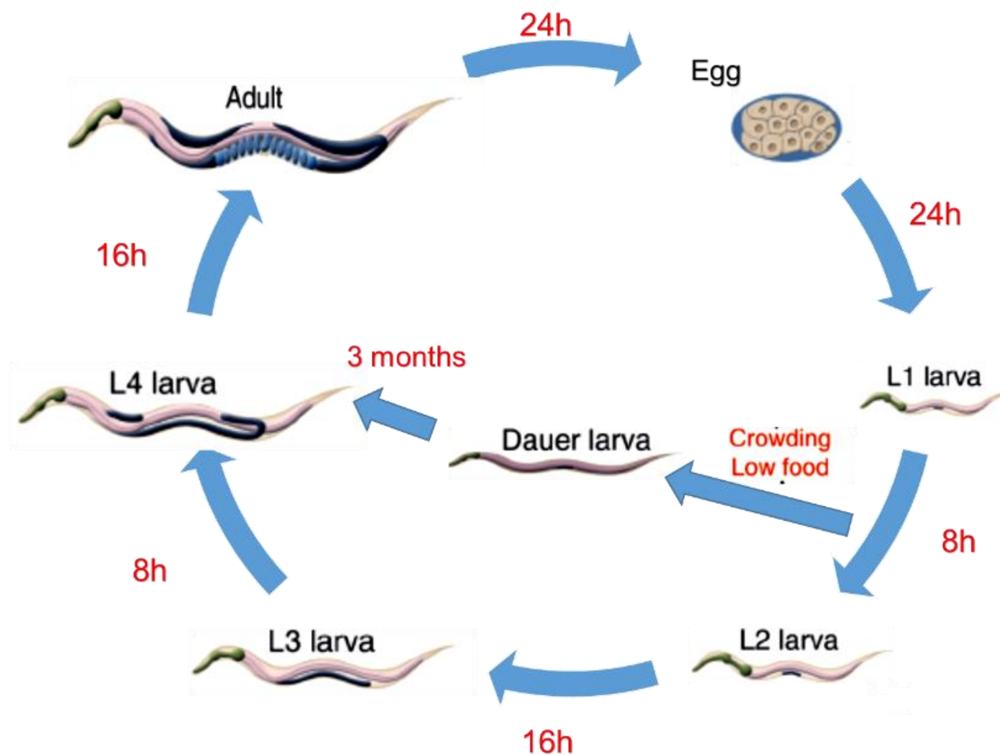


Figure 1. The typical life cycle of *C. elegans* at 15 °C for approximately 4 days. After completion of embryogenesis, L1 larvae hatch out of the eggshell and begin the process of post-embryonic development. The nematode continues developing through L2-L3 larval stages until reaching adulthood. Prior to the L2 larva stage, unfavourable conditions triggers the nematode to enter dauer larval stage. The amount of time spent at each stage is indicated along the arrows.

2.4.2 *Candida albicans* infection model

So far, there is a gap that exists in the studies of pathogen-pathogen interactions due to scarcity of realistic *in vivo* models. However, *C. elegans* is illustrated as a convenient biological model in order to study monomicrobial pathogenesis (Couillault and Ewbank, 2002; Balla and Troemel, 2013). The simple body structure and transparent nature of the nematode gives a simplified *in vivo* model capable of mimicking the physiological and pathogenic mechanisms that takes place during infection (Brenner, 1974). Therefore in this present study, we illustrate

that this nematode can readily serve as a suitable infection model to study *C. albicans* pathogenesis.

After infecting L4 or adult *C. elegans* nematodes with each of the *C. albicans* strains, we characterized these nematodes as either live or dead. We observed that the live nematodes maintained a sinusoidal shape and could move their body while pumping their pharyngeal muscles. However, as the dead nematodes became bloated with *C. albicans* cells they appeared rod shaped or straightened and rigid, stretching and lengthening their body. In addition, there was lack of mobility in dead nematodes. We also did not observe any hyphal formation in all *C. albicans* strains. Figure 2 indicates the killing of L4 and adult *C. elegans* by the different *C. albicans* strains. For both L4 larvae and adult nematodes, the survival of *C. albicans* infected nematodes were significantly reduced compared to control nematodes fed with *E. coli* OP50 ($p \leq 0.05$). In both cases it took seven days for all *C. albicans* strains to kill 100 % of the nematodes. The virulence of *C. albicans* towards *C. elegans* was visually evident after two days of infection. *Candida albicans* SC5314 killed 50 % of the L4 larvae between two and three days, while *C. albicans* Ho6 and *C. albicans* Ho153 killed 50 % within five days (Figure 2A). We observed a similar trend with the *C. elegans* adult nematode (Figure 2B), where *C. albicans* SC5314 killed 50 % of the adult nematode within three days, while *C. albicans* Ho6 and *C. albicans* Ho153 killed 50 % within five days. Due to these differences in rate of killing, we considered *C. albicans* SC5314 to be more virulent than *C. albicans* Ho6 and *C. albicans* Ho153.

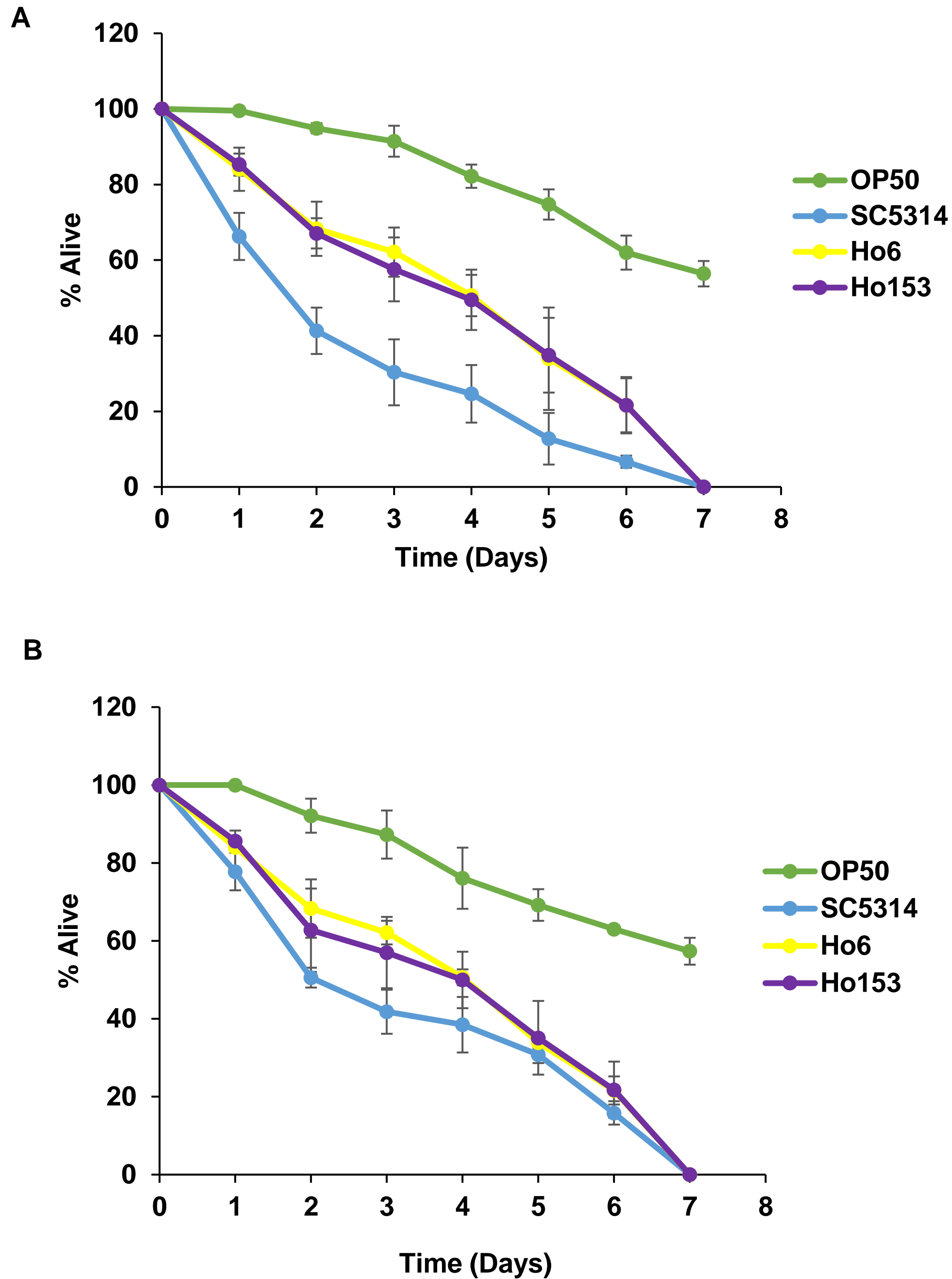


Figure 2. (A). *Candida albicans* killing L4 larvae *C. elegans*. (B). *Candida albicans* killing adult *C. elegans*. The survival of nematodes were significantly reduced when exposed to *C. albicans* compared to *E. coli* OP50 control ($p \leq 0.05$). The *C. albicans* reference strain SC5314 was more virulent to *C. elegans* than two *C. albicans* clinical strains (Ho6 and Ho153).

2.4.3 *Pseudomonas aeruginosa* infection model

The *C. elegans* host model is considered to be of great importance in the field of bacterial pathogenesis. Thus it has emerged as a well-known convenient host-pathogen model, pertaining studies that revealed that there exists some overlap between bacterial virulence factors employed upon infection of vertebrate and invertebrate hosts (Aballay and Ausubel, 2002; Ewbank, 2002; Sifri *et al.*, 2005). Therefore, to further expand the use of this model to study virulence in bacteria, we developed a killing assay using *P. aeruginosa* and L4 larvae or adult *C. elegans* nematodes. The capability of *P. aeruginosa* reference strain PAO1 to kill *C. elegans* was compared to the clinical strains Iso6 and Iso20. Figure 3 indicates that for both L4 larvae and adult nematodes, the longevity of *P. aeruginosa* infected nematodes were significantly reduced compared to uninfected nematodes ($p \leq 0.05$). The pathogenicity was visually evident after two days of infection. *Pseudomonas aeruginosa* PAO1 killed 50 % of the L4 larvae within four days, while *P. aeruginosa* Iso6 killed 50 % within two days and *P. aeruginosa* Iso20 killed 50 % within three days (Figure 3A). However in terms of the adult nematodes, we observed a slightly different trend (Figure 3B), where *P. aeruginosa* PAO1 and Iso6 killed 50 % of the adult worms between one and two days, while *P. aeruginosa* Iso20 killed 50 % between two and three days. We therefore considered *P. aeruginosa* PAO1 to be more virulent than *P. aeruginosa* Iso6 and *P. aeruginosa* Iso20, with *P. aeruginosa* Iso20, being the least virulent of the three strains.

Previous studies showed that the nematode can be successfully used as an infection host used for numerous studies of reference strain *P. aeruginosa* PAO1 (Darby *et al.*, 1999; Gallagher and Manoil, 2001). Extensive studies focusing on the effect of *P. aeruginosa* infection on *C. elegans* have resulted in the discovery of four different infection models of which differ slightly depending on the strain used, different medium as well as incubation conditions (Tan *et al.*, 1999; Darby *et al.*, 1999; Gallagher and Manoil, 2001; Sifri *et al.*, 2005; Begun *et al.*, 2005). For instance, *P. aeruginosa* can either kill the nematodes in slow infectious process or rapidly, through toxin-mediated mechanisms (Tan *et al.*, 1999; Darby *et al.*, 1999). Darby and co-workers, (1999) described a rapid, paralytic killing mechanism that highly depend on production of neurotoxin, hydrogen cyanide, which triggers *P. aeruginosa* PAO1 strain to cause lethal paralyzes and kill *C. elegans* within hours (Gallagher and Manoil, 2001). Furthermore, Kirienko and co-workers (2013) showed that in liquid medium killing assay, *P. aeruginosa* is known to produce siderophore pyoverdine which induce a hypoxic response which is then followed by death of the nematode. Thus, we can speculate that this might be a reason for our results of *P. aeruginosa* PAO1 showing more virulence compared to other strains investigated. It will be important to extent our findings by closely investigating the virulence factors, such as pyoverdine, of *P. aeruginosa*.

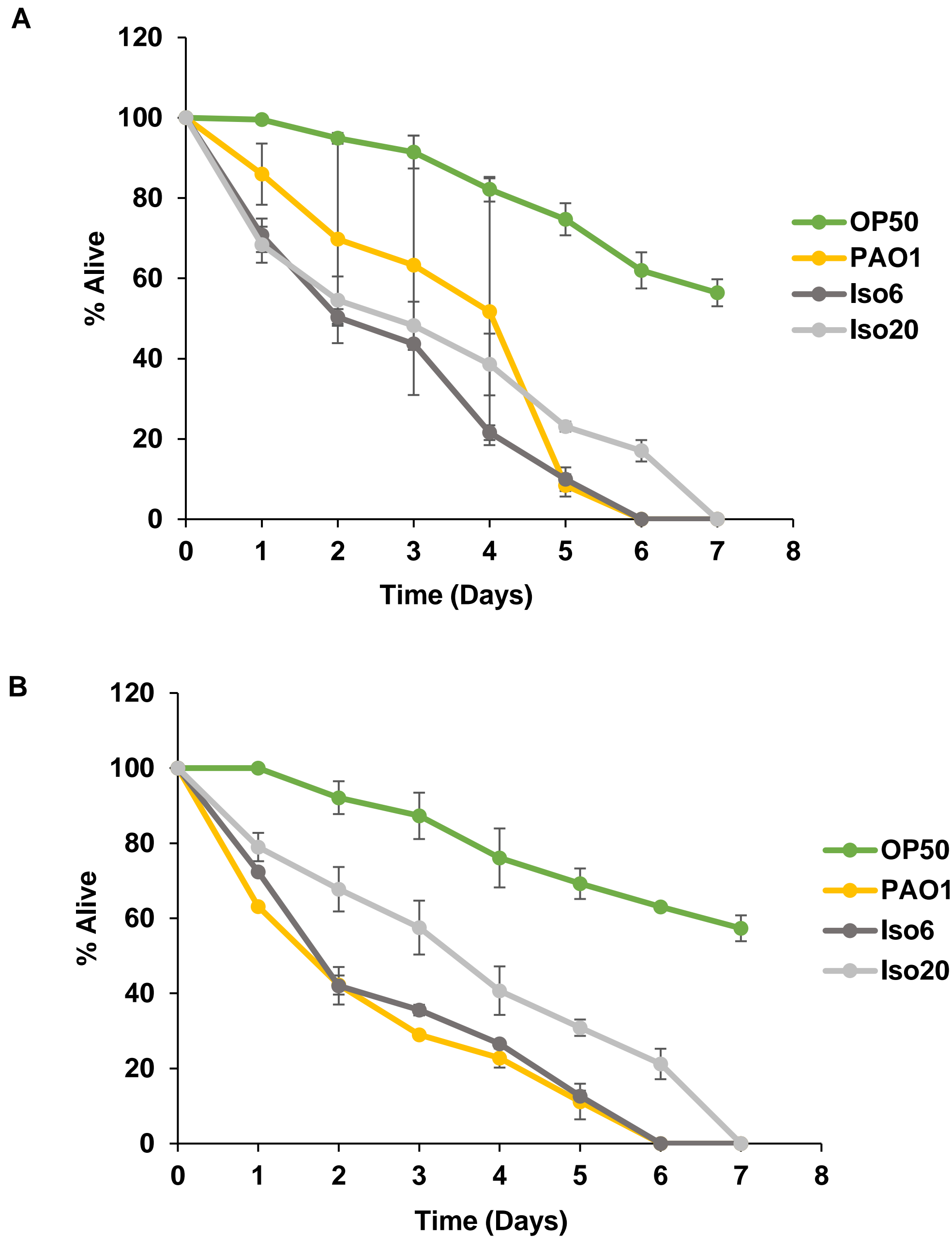


Figure 3. (A) *Pseudomonas aeruginosa* killing L4 larvae *C. elegans*. (B) *Pseudomonas aeruginosa* killing adult *C. elegans*. The survival of nematodes were significantly reduced when exposed to *Pseudomonas aeruginosa* ($p \leq 0.05$). The *Pseudomonas aeruginosa* reference strain PAO1 was more virulent to *C. elegans* than two *Pseudomonas aeruginosa* clinical strains (Iso6 and Iso20).

2.4.4 *Candida albicans* and *Pseudomonas aeruginosa* co-infection model

The opportunistic bacterium, *Pseudomonas aeruginosa* is an emerging, problematic multidrug resistant human pathogen and similar to the fungus, *C. albicans*, has a tendency to cause infection in immunocompromised individuals (Pfaller and Diekema, 2004; Driscoll *et al.*, 2007). Both microbes have been found to often share similar ecological niches within hosts, including, vascular and urinary catheters, bronchial airways and patient wounds (Richard *et al.*, 1999; Rosenthal *et al.*, 2006). Taking into consideration the increasing importance of *C. albicans* and *P. aeruginosa* towards morbidity and mortality of most hospitalized patients, in our present study we sought to further define their pathogenicity using the host model *C. elegans*.

As depicted in figure 4, we observed a significant reduction in the survival of *C. elegans* when infected with *C. albicans* and *P. aeruginosa* polymicrobial strains compared to *E. coli* OP50 control strain. Moreover, although *C. albicans* and *P. aeruginosa* can independently kill the nematode, however polymicrobial infection of both these pathogens, significantly reduced their survival compared with monomicrobial infection (Figure 2 and Figure 3). Most co-infection combinations killed 100 % of L4 larvae within four days. However, all the combinations that included *C. albicans* Ho6 were less virulent and took five days to kill 100 % of the L4 larvae (Figure 4A). Surprisingly, the combination of the two most virulent strains in the monomicrobial infections (*C. albicans* SC5314 and *P. aeruginosa* PAO1) was not the outmost virulent combination, killing 50 % of the L4 larvae only after 3 days, while, the combination *C. albicans* SC5314 and *P. aeruginosa* Iso20 killed 50 % of the larvae within one day. We observed a similar trend with adult nematodes (Figure 4B), where it took four days for most co-infection combinations to kill 100 % of the adult nematodes, except for combinations *C. albicans* Ho6 and *P. aeruginosa* Iso6, *C. albicans* Ho6 and *P. aeruginosa* Iso20 and also *C. albicans* Ho153 and *P. aeruginosa* PAO1 which took five days to kill 100 % of the adult nematodes. All co-infection combinations killed 50 % of the adult nematodes within three days, while combination *C. albicans* SC5314 and *P. aeruginosa* Iso20 again killed 50 % of the adult nematodes within one day.

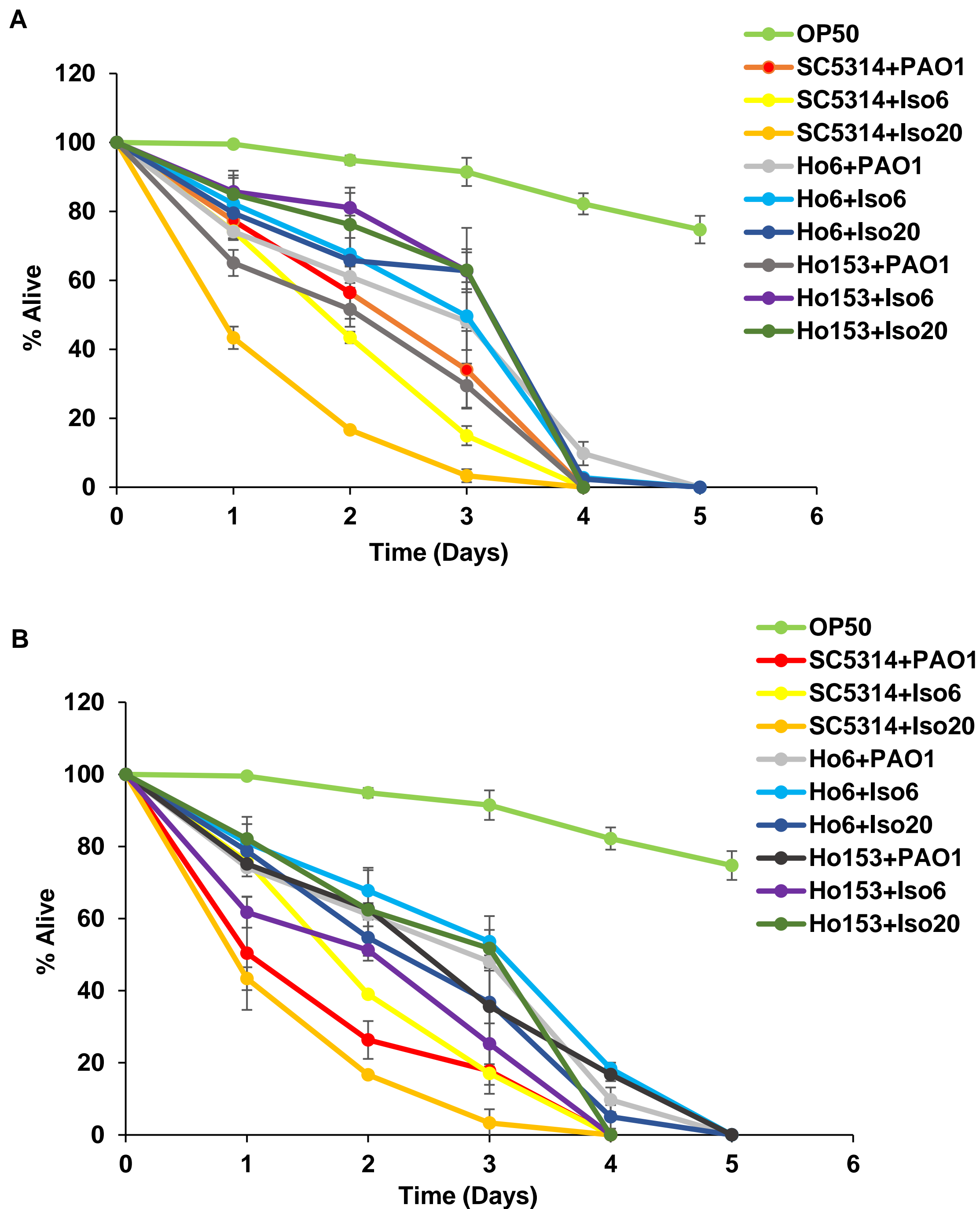


Figure 4. (A) *Candida albicans* and *Pseudomonas aeruginosa* co-infection on *C. elegans* L4 larvae over time. (B) *Candida albicans* and *Pseudomonas aeruginosa* co-infection on adult nematodes over time. SC5314 + Iso20 co-infection were most virulent combinations, while Ho6 + PAO1 co-infection were least virulent.

These were unexpected results and in contrast to literature. For instance, Peleg *et al*. (2008), specifically used *C. elegans* to evaluate the polymicrobial interaction of *C. albicans* and *Acinetobacter baumannii*. An antagonistic relationship between the two pathogens was described, where *A. baumannii* inhibited some of the significant virulence factors of *C. albicans,* including hyphal formation and biofilm formation, resulting in reduced *C. albicans* pathogenicity. This was interesting since single species of *C. albicans* and *A. baumannii* independently killed *C. elegans* at a rapid rate, however infection with both pathogens caused nematodes to survive longer compared with single species *C. albicans* infection (Peleg *et al.,* 2008). Given all this, Peleg and co-workers (2008) assumed that *A. baumannii* either significantly reduces or slows the degree of *C. albicans* hyphal formation in *C. elegans* intestines, therefore significantly reducing nematode mortality. Moreover, Cruz *et al*. (2013) also supported the antagonistic observations by investigating the interaction of *C. albicans* and bacterium, *Enterococcus faecalis*. The monomicrobial infection with either *C. albicans* or *E. faecalis* was able to significantly reduce the nematode survival, however, polymicrobial infection with *C. albicans* and *E. faecalis* prevented preventing hyphal morphogenesis by *C. albicans,* thus caused a much lesser tissue damage to *C. elegans*. Interestingly, when *C. albicans* grows with *E. faecalis*, it seemed to protect the bacterium from cell death (Cruz *et al*., 2013).

This discrepancy between our results and literature can be explained by the fact that we never observed hyphal formation by *C. albicans* either during mono- or polymicrobial infections. *Candida albicans* yeast cells are able to transition to hyphae form and this hyphal form is an important virulence factor in mammals (Lo *et al*., 1997; Braun and Johnson, 1997; Saville *et al*., 2003). In previous studies, Breger *et al*. (2007) showed that *C. albicans* can vigorously form invasive hyphae in the *C. elegans* intestines, which fully penetrated the nematode cuticle. However mutants that cannot form hyphae seemed to be avirulent, implying that hyphal morphogenesis might be one of the mechanism of nematode killing (Breger *et al*., 2007). Pukkila-Worley and co-worker (2009) also found that more than half of the nematodes infected with the reference strain *C. albicans* SC5314 died within 48 h and the dead nematode showed hyphae piercing the nematode cuticle. Thereafter, there was a second phase that followed this rapid initial decline in nematode survival, in this second phase the nematodes died slower than the initial phase. In these latter nematodes, no hyphae was observed in either the intestine nor piercing through the infected nematodes cuticle (Pukkila-Worley *et al*., 2009).

In order to confirm that *C. albicans* in our study does not produce hyphae, we examined *C. elegans* infected with *C. albicans* as well as combination of both *C. albicans* SC5314 and *P. aeruginosa* PAO1, using both light microscope and transmission electron microscopy (TEM) (Figure 5) and observed a striking lack of hyphal formation in both cases.

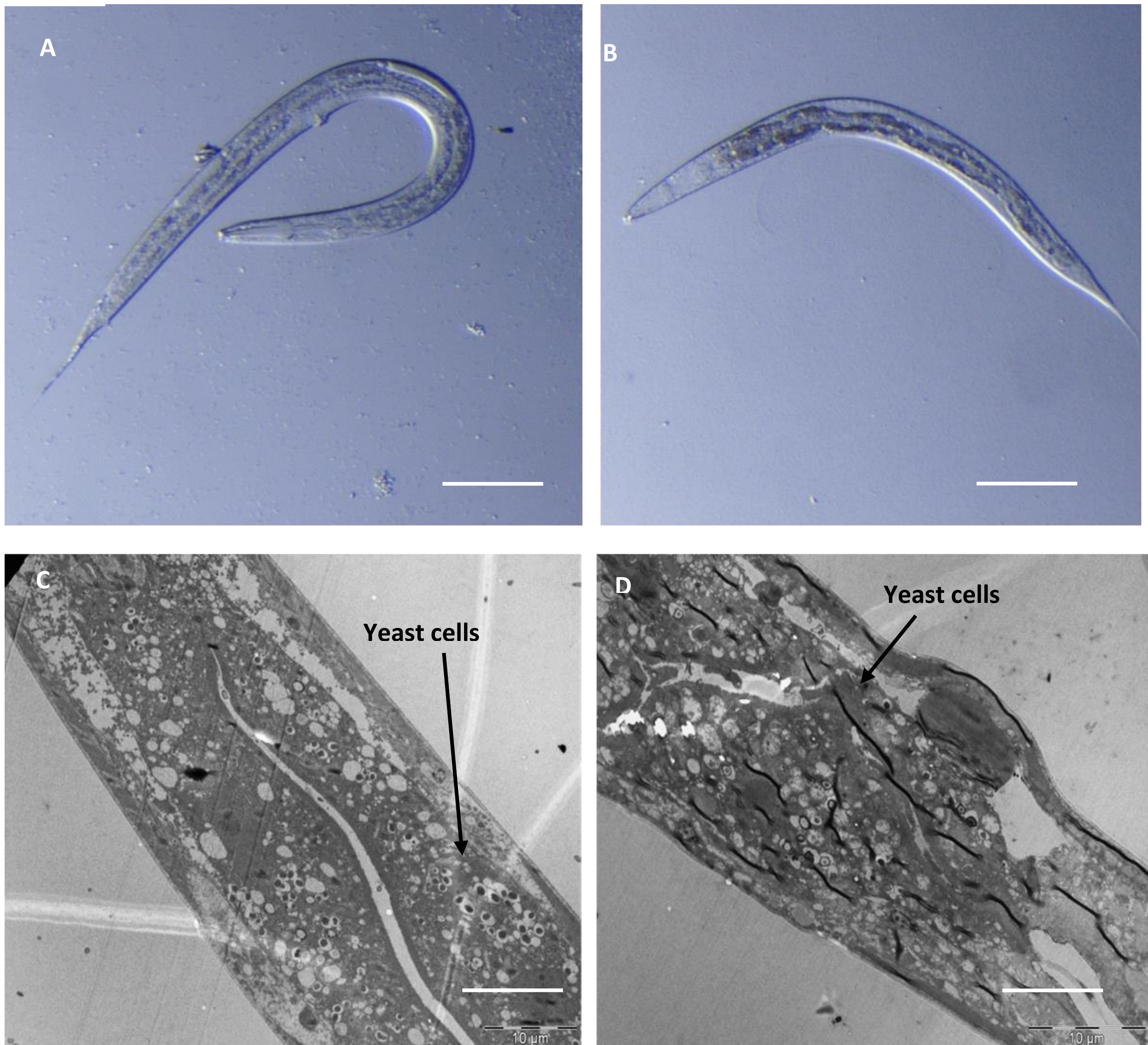


Figure 5. Lack of hyphal formation by *Candida albicans* SC5314 in *Caenorhabditis elegans*. (A) Light micrograph of nematode infected with only *C. albicans* SC5314 strain. Note absence of hyphae piercing the cuticle. (B) Light micrograph of nematode infected with *C. albicans* SC5314 and *Pseudomonas aeruginosa* PAO1 strains. The scale bar of 200 µm. (C) Transmission electron micrograph of nematode infected with only *C. albicans* SC5314 strain. (D) Transmission electron micrograph of nematode infected with *C. albicans* SC5314 and *P. aeruginosa* PAO1 strains. The scale bar of 10 µm.

We also used *C. albicans* M2194, which fluoresces under when hyphae are produced and *C. albicans* M137, a constitutively fluorescent strain. From our results we observed no fluorescence in monomicrobial infections with *C. albicans* M2194 (Figure 6B) and polymicrobial infections with *C. albicans* M2194 and *P. aeruginosa* PAO1 (data not shown). In addition, as seen in Figure 6D, *C. albicans* M137 did fluoresce, clearly indicating only the yeast cells inside the nematode without any hyphal formation. Taken together all these observations from our current study, we can speculate that the observed killing of *C. elegans* by all *C. albicans* strains occurred through a process called yeast-dependent and not through hyphal formation. This yeast-dependent process was also seen in studies done by Pukkila-Worley and co-workers, (2009) using two uncommon human pathogenic fungi (*Debaryomyces hansenii* and *Candida lusitaniae*) family of *C. albicans*, which are incapable of forming hyphae. Although infection with both these pathogens did not form any hyphae in *C. elegans*, they were still able to kill the nematode, however slower than hyphae producing *C. albicans*. Therefore the observed features suggested that *D. hansenii and C. lusitaniae* killed *C. elegans* through a yeast-dependent process (Pukkila-Worley *et al*., 2009), similar to our observations.

The relevance of this observation can also be seen in other infection models. Roux and colleagues (2009) used immunocompetent rats to evaluate the relationship between *C. albicans* colonisation and the existence of *P. aeruginosa* pneumonia in lungs. They found that *C. albicans* enhanced infection of *P. aeruginosa* by impairing macrophage function, causing a significant increase in the occurrence of *P. aeruginosa* pneumonia. Moreover they likely observed any hyphal form of *C. albicans* in the rat lungs (Roux *et al*., 2009). Tan *et al*. (2016), further extended these findings by evaluating *C. albicans*-*A. baumannii* co-infection in a rat model. Their results suggested that colonization of *C. albicans* in the respiratory tract of rats assisted in the development of severe *A. baumannii* infection (Tan *et al*., 2016). In addition, more studies were described in mice models, investigating the virulence of co-infection of *C. albicans* and *Staphylococcus aureus* (Carlson, 1983b). From the monomicrobial infection of *S. aureus* alone, no cells could be identified in peripheral sites like in the spleen, blood, or pancreas, and the mice was able to survive (Carlson, 1983b). However, after administration of a similar dose of *S. aureus* coupled with heat-inactivated *C. albicans* or with a sublethal dose of *C. albicans*, *S. aureus* was identified in abdominal organs and all samples of blood and majority of the mice were dead (Carlson, 1983b). These results speculated that *C. albicans* protects *S. aureus* in the peritoneal cavity thereafter enhances its virulence by enabling *S. aureus* to disseminate throughout to the peripheral tissues (this is a process not observed in the monomicrobial infection with *S. aureus*) (Carlson, 1983b; Carlson and Johnson, 1985). Moreover, similar observations were discovered by the same researchers

when using the Gram-positive bacterium, *E. faecalis* and also for another Gram-negative bacterium *Serratia marcescens* (Carlson, 1983a).

Taken together, it is clear that yeast-bacterial polymicrobial interactions can either display synergistic or antagonistic interactions, depending on either the *in vitro* or *in vivo* studies. Also that it is possible for co-infections to be more virulent compared to single infections. Thus from our observations we can speculate that *C. albicans* and *P. aeruginosa* interactions indicate complex, synergistic yeast-bacterial interactions in *C. elegans* infection model.

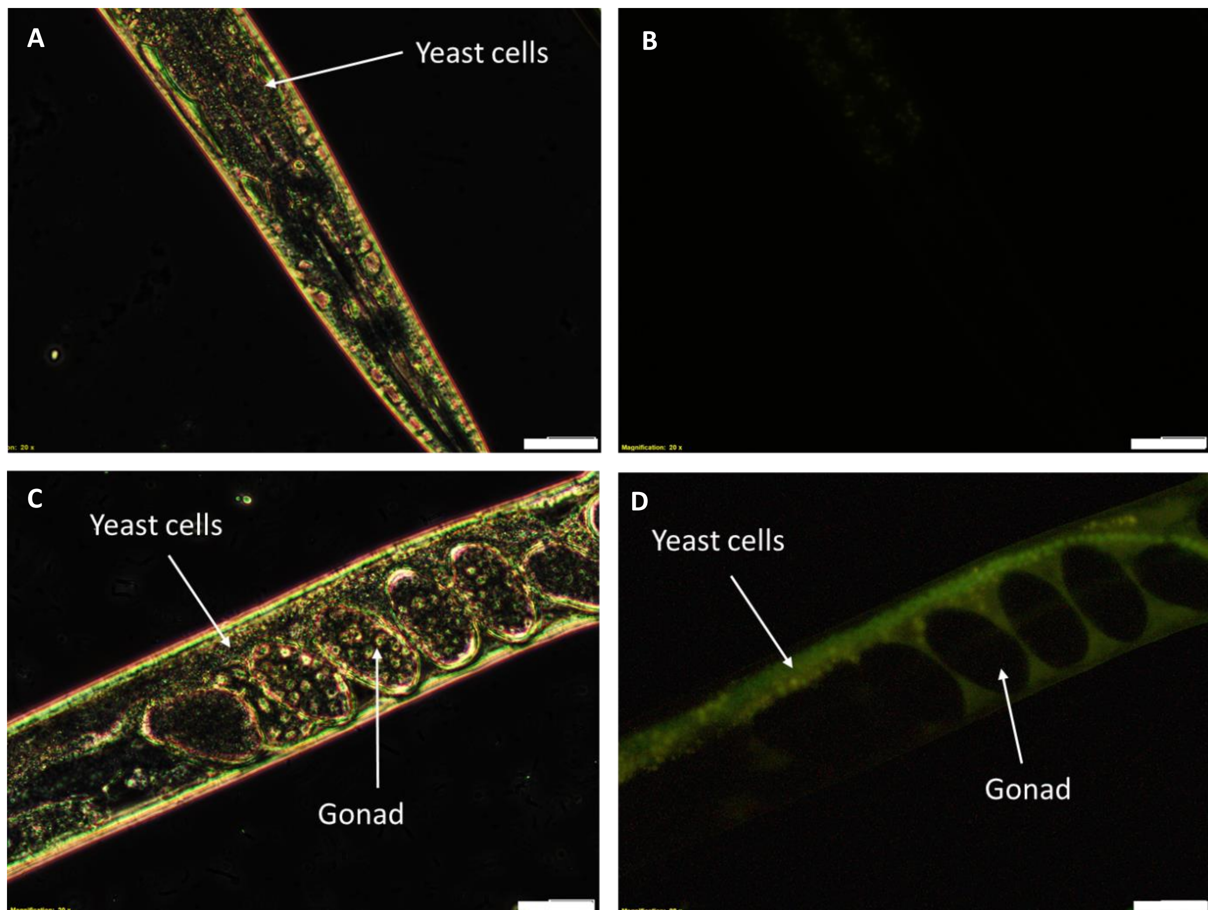


Figure 6. (A) Light microscope and (B) fluorescent micrograph of *C. elegans* infected with *C. albicans* M2194 showing no fluorescence. No hyphal formation. (C) Light microscope and (D) fluorescent micrograph of *C. elegans* infected with *C. albicans* M137. Importantly, no hyphal formation was observed. Only the yeast cells of *C. albicans* fluoresced inside the intestines of nematode. The scale bar of 20 µm.

2.5 Conclusions

It is true that medically important interactions between pathogenic bacteria and fungi are common in most settings. These pathogenic microbial interactions are highly complex, and the type of interaction that occurs usually depends on a wide range of environmental, pathogen and host factors. Thus developing appropriate *in vitro* and *in vivo* models to fully characterize these pathogenic microbial interactions and their virulence mechanisms is important for our understanding of their role in human disease. Given the significance of the yeast, *C. albicans*, to human health with its mutual cohabitation with the bacterium *P. aeruginosa* (Richard *et al*., 1999; Rosenthal *et al*., 2006), we used *C. elegans* as an infection model to help identify and study interactions between these two pathogens. Our monomicrobial infection results demonstrated that for either pathogen, the reference strains were more virulent than the clinical strains. In contrast, the polymicrobial infections showed that combination of both *C. albicans* and *P. aeruginosa* had a positive impact on each other's virulence leading to extremely high mortality of *C. elegans*. These findings present an extension toward ongoing research of monomicrobial and polymicrobial infection in *C. elegans*.

Having confirmed that in our study we hardly observed any evident changes in *C. albicans* morphology *in vivo*, formation, we concluded that hyphal formation may not be an important virulence factor required to induce pathogenicity by *C. albicans*. According to literature, the capability of *C. albicans* to transition morphologically between yeast and hyphal form has been considered as a major virulence factor of this pathogen, accompanied tissues penetration evading immune defences, thus causing cell damage, moreover it triggers the upregulation of virulence factors not directly functional in *C. albicans* morphogenesis (Braun and Johnson, 1997; Lo *et al*., 1997; Saville *et al*., 2003). However, studies by Pukkila-Worley *et al*. (2009) showed that *C. albicans* yeast form is virulent enough to also cause infection and death in *C. elegans*. Thus also our results further emphasise the fact that lack of hyphal formation is not essentially required for pathogenicity. For future research, it will be crucial to investigate growth conditions that can induce hyphal formation by *C. albicans* in *C. elegans*. This might help to get results in *C. elegans* model that are more reflective of the morphological and physiological changes that normally occur in infected patients. Moreover, establishing *C. elegans* as an infection model in order to study polymicrobial infections, particularly *C. albicans* and *P. aeruginosa,* enables further characterization of virulence mechanisms employed by these pathogens, this also help fill the gaps in the ongoing research towards therapeutic and prevention strategies.

2.6 Reference

Aballay, A. & Ausubel. F. M. (2002). *Caenorhabditis elegans* as a host for the study of host-pathogen interactions. *Curr Opin Microbiol* **5**, 97–101.

Akira, S. (2009). Pathogen recognition by innate immunity and its signaling. *Proc Jpn Acad Ser B Phys Biol Sci* **85**, 143–156.

Balla, K. M. & Troemel, E. R. (2013). *Caenorhabditis elegans* as a model for intracellular pathogen infection. *Cell Microbiol* **15**, 1313-1322.

Begun, J., Sifri, C. D., Goldman, S., Calderwood, S. B. & Ausubel, F. M. (2005). *Staphylococcus aureus* virulence factors identified by using a high-throughput *Caenorhabditis elegans*-killing model. *Infect Immun* **73**, 872–877.

Brand, A., Barnes, J. D., Mackenzie, K. S., Odds, F. C. & Gow, N. A. R. (2008). Cell wall glycans and soluble factors determine the interactions between the hyphae of *Candida albicans* and *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **287**, 48–55.

Braun, B. R. & Johnson, A. D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science* **277**, 105–109.

Breger, J., Fuchs, B. B., Aperis, G., Moy, T. I., Ausubel, F. M. & Mylonakis, E. (2007). Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS Pathog* **3**, e18 doi:10.1371/journal.ppat.0030018

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Carlson, E. (1983a). Enhancement by *Candida albicans* of *Staphylococcus aureus*, *Serratia marcescens*, and *Streptococcus faecalis* in the establishment of infection in mice. *Infect Immun* **39**, 193–197.

Carlson, E. (1983b). Effect of strain of *Staphylococcus aureus* on synergism with *Candida albicans* resulting in mouse mortality and morbidity. *Infect Immun* **42**, 285–292.

Carlson, E. & Johnson, G. (1985). Protection by *Candida albicans* of *Staphylococcus aureus* in the establishment of dual infection in mice. *Infect Immun* **50**, 655–659.

Cassada, R. C. & Russell, R. L. (1975). The dauer larva, a postembryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev Biol* **46**, 326-342.

Chen, A. I., Dolben, E. F., Okegbe, C., Harty, C. E., Golub, Y., Thao, S., Ha, D. G., Willger, S. D., Toole, G. A. O. *et al.* (2014). *Candida albicans* ethanol stimulates *Pseudomonas*

aeruginosa WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog* **10**, doi: 10.1371/journal.ppat.1004480.

Couillault, C. & Ewbank, J. J. (2002). Diverse bacteria are pathogens of *Caenorhabditis* elegans. *Infect Immun* **70**, 4705-4707.

Cruz, M. R., Graham, C. E., Gagliano, B. C., Lorenz, M. C. & Garsin, D. A. (2013). *Enterococcus faecalis* inhibits hyphal morphogenesis and virulence of *Candida albicans*. *Infect Immun* **81**, 189–200.

Cugini, C., Calfee, M. W., Farrow, J. M. III., Morales, D. K., Pesci, E. C. & Hogan, D. A. (2007). Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. *Mol Microbiol* **65**, 896–906.

Darby, C., Cosma, C. L., Thomas, J. H. & Manoil. C. (1999). Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 15202–15207.

Driscoll, J. A., Brody, S. L. & Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* **67**, 351–368.

Durai, S., Pandian, S. K. & Balamurugan, K. (2011). Establishment of a *Caenorhabditis elegans* infection model for *Vibrio alginolyticus*. *J Basic Microbiol* **51**, 243–252.

Everman, J. L., Ziaie, N. R., Bechler, J. & Bermudez, L. E. (2015). Establishing *Caenorhabditis elegans* as a model for *Mycobacterium avium* subspecies *hominissuis* infection and intestinal colonization. *Biol Open* **4** (10), 1330-1335.

Ewbank, J. J (2002). Tackling both sides of the host-pathogen equation with *Caenorhabditis elegans*. *Microbes Infect* **4**, 247–256.

Fourie, R., Ells, R., Swart, C. W., Sebolai, O. M., Albertyn, J. & Pohl, C. H. (2016). *Candida albicans* and *Pseudomonas aeruginosa* interaction, with focus on the role of eicosanoids. *Front Physiol* **7**, 64.

Fradin, C., De Groot, P., MacCallum, D. *et al.* (2005). Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* **56**, 397–415.

Gallagher, L.A. & Manoil, C. (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* **183**, 6207–6214.

Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., Calderwood, S. B. & Ausubel, F. M. (2001). A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci U S A* **98**, 10892-10897.

Hogan, D. A. & Kolter, R. (2002). *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* **296**, 2229-2232.

Irazoqui, J. E., Troemel, E. R., Feinbaum, R. L., Luhachack, L. G., Cezairliyan, B. O. & Ausubel, F. M. (2010). Distinct pathogenesis and host responses during infection of *C. elegans* by *P. aeruginosa* and *S. aureus*. *PLoS Pathog* **6**, e1000982.

Jenkins, W. R. (1964). A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Dis Rep* **48**, 692.

Kerr, J. R., Taylor, G. W., Rutman, A., Hoiby, N., Cole, P. J. & Wilson, R. (1999). *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* **52**, 385-387.

Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M. W. & Ausubel, F. M. (2002). A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. *Science* **297**, 623-626.

Kirienko, N. V. Kirienko, D. R., Larkins-Ford, J., Wählby, C., Ruvkun, G. & Ausubel, F. M. (2013). *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans* iron homeostasis, causing a hypoxic response and death. *Cell Host Microbe* **13**, 406–416.

Lindsay, A. K. & Hogan, D. A. (2014). *Candida albicans*: molecular interactions with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Fungal Biol Rev* **28**, 85-96.

Lo, H. J., Kohler, J. R., Di Domenico, B., Loebenberg, D., Cacciapuoti A, *et al.* (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939–949.

Méar, J. B., Kipnis, E., Faure, E., Dessein, R., Schurtz, G., Faure, K. *et al.* (2013). *Candida albicans* and *Pseudomonas aeruginosa* interactions: more than an opportunistic criminal association? *Médecine Mal Infect* **43**,146-151.

Miyata, S., Begun, J. Troemel, E. R. & Ausubel, F. M. (2008). DAF-16-dependent suppression of immunity during reproduction in *Caenorhabditis elegans*. *Genetics* **178**, 903–918.

Moyes, D. L., Wilson, D., Richardson, J. P, Mogavero, S., Tang, S. X, Wernecke, J. *et al.* (2016). Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature* **7** (7597), 64-68.

Peleg, A.Y., Tampakakis, E., Fuchs, B. B., Eliopoulos, G. M., Moellering, R. C. *et al.* (2008). Prokaryote-eukaryote interactions identified by using *Caenorhabditis elegans. Proc Natl Acad Sci U S A* **105**, 14585-14590.

Pfaller, M. A. & Diekema, D. J. (2004). Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. *Clin Microbiol Infect* **10** (1), 11-23.

Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A. & Cerón, J. (2012). Basic *Caenorhabditis elegans* Methods: Synchronization and Observation. *J Vis Exp* **64**, e4019 10.3791/4019, doi: 10.3791-4019.

Pukkila-Worley, R., Peleg, A.Y., Tampakakis, E. & Mylonakis, E. (2009). *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. *Eukaryotic Cell* **11**, 1750-1758.

Raizen, D. M., Zimmerman, J. E., Maycock, M. H., Ta, U. D., You, Y. J., Sundaram, M. V. & Pack, A. I. (2008). Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* **451** (7178), 569-572.

Richards, M. J., Edwards, J. R., Culver, D. H. & Gaynes, R. P. (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med* **27**, 887–892.

Rinzan, F. F. (2009). *Pseudomonas aeruginosa-Candida albicans* interactions from ecological and molecular perspectives. Department of Biology, Georgia State University, Atlanta, USA.

Rosenthal, V. D. Maki, D. G., Salomao, R., Moreno, C. A., Mehta, Y., Higuera, F. *et al.* (2006). Device-associated nosocomial infections in 55 intensive care units of 8 developing countries. *Ann Intern Med* **145**, 582–591.

Roux, D., Gaudry, S., Dreyfuss, D., El-Benna, J., de Prost, N., Denamur, E., Saumon, G. & Ricard, J. (2009). *Candida albicans* impairs macrophage function and facilitates *Pseudomonas aeruginosa* pneumonia in rat. *Crit Care Med* **37**, 1062–1067.

Saville, S. P., Lazzell, A. L., Monteagudo, C. & Lopez-Ribot, J. L. (2003). Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot Cell* **2**, 1053–1060.

Shirtliff, M. E., Peters, B. M. & Jabra-Rizk, M. A. (2009). Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett* **299**, doi: 10.1111/j.1574-6968.2009.01668.x.

Sifri, C. D., Begun, J. & Ausubel, F. M. (2005). The worm has turned-microbial virulence modelled in *Caenorhabditis elegans*. *Trends Microbiol* **13**, 119–127.

Swart, C. W., Swart, H. C., Coetsee, E., Pohl, C. H., Van Wyk, P. W. J. & Kock, J. L. F. (2010). 3-D architecture and element composition of fluconazole treated yeast asci. *Sci Res Essays* **5** (22), 3411-3417.

Tan, X., Chen, R., Zhu, S., Wang, H., Yan, D., Zhang, X. & Mylonakis, E. (2016). *Candida albicans* airway colonization facilitates subsequent *Acinetobacter baumannii* pneumonia in a rat model. *Antimicrob Agents Chemother* **60** (6), 3348–3354.

Tan, M. W., Mahajan-Miklos, S. & Ausubel, F. M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proc Natl Acad Sci U S A* **96**, 715–720.

Troemel, E. R., Chu, S. W., Reinke, V., Lee, S. S., Ausubel, F. M. & Kim, D. H. (2006). MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. *PLoS Genet* **2,** e183.

Xu, L., Wang, F., Shen, Y., Hou, H., Liu, W., Liu, C. *et al.* (2014). *Pseudomonas aeruginosa* inhibits the growth of pathogenic fungi: *in vitro* and *in vivo* studies. *Exp Ther Med* **7**, 1516–1520.

CHAPTER 3

Investigating the possible cause for observed differences in virulence

3.1 Abstract

A fundamental question in pathogenicity is what causes virulence in pathogens. One of the most important causes of virulence is involvement of virulence factors. *Pseudomonas aeruginosa* is amongst the well-studied opportunistic pathogens that cause serious infections, as it affects, amongst others, patients suffering from cystic fibrosis or other various chronic lung diseases. In most cases the pathogen causes chronic infection as a result of it being able to produce biofilms in these patients, and also it's alerting resistance to antibiotic treatment. The virulence of *P. aeruginosa* is normally ascribed to production of various virulence factors, which includes pyoverdine, pyocyanin, proteases, such as elastase and alkaline protease, phospholipases such as exotoxin A and phospholipase C. Likewise, in most individuals, *C. albicans* usually resides as a harmless lifelong commensal pathogen in humans, nonetheless under circumstances where the host is immunocompromised, it causes infections ranging from superficial to life-threatening systemic infections. At present several factors with their functions have been discovered, known to be associated to the virulence potential of this pathogenic yeast. Amongst them are molecules capable of mediating host cells adhesion and invasion, such as, morphogenesis, secretion of hydrolases, thigmotropism, biofilm formation and other fitness attributes. Our knowledge of when and how most virulence factors contribute to infection has drastically increased within the past years. Moreover, some mechanisms underlying these virulence factors have recently been discovered. The main objective of this present study is to investigate the virulence factors responsible for the observed differences in virulence caused by *C. albicans* and *P. aeruginosa* strains that are used to establish *Caenorhabditis elegans* infection model described in the previous chapter. We have investigated a panel of important *in vitro* virulence factors in these species. Associations were assessed between pyocyanin and pyoverdine production, secretion of hydrolytic enzymes and microbial burden. The results suggest that observed differences in virulence of *C. albicans* and *P. aeruginosa* strains during mono-microbial infections may be due to production of various virulence factors. However, for the polymicrobial infections the virulence of the different combinations is proportional to its ability to colonise *C. elegans*.

Keywords: Pathogenicity, *Candida albicans, Pseudomonas aeruginosa,* virulence factors, pyocyanin, pyoverdine, hydrolytic enzymes, microbial burden, *Caenorhabditis elegans*

3.2 Introduction

A better understanding of the pathogenic consequences of most bacterial-fungal polymicrobial interactions has come predominately from microbial pathogenicity studies using infection models. However, evaluating the changes in virulence in a bacterial-fungal co-infection context may be a complex task and depends on several factors. A common Gram negative bacterium, *Pseudomonas aeruginosa,* is known as an opportunistic human pathogen that is familiar to every hospital and intensive care unit (van 't Wout *et al.*, 2015; Dzvova *et al.*, 2017). This bacterium is the frequent cause of nosocomial infections, many of which are responsible for substantial mortality due to recurring antibiotic resistant isolates (Sadikot *et al.*, 2005; van 't Wout *et al.*, 2015; Dzvova *et al.*, 2017). Furthermore, its survival in the host is sustained by the secretion of various virulence factors and toxins, including pyoverdine, pyocyanin, proteases, (e.g. alkaline protease and elastase), and also phospholipases (e.g. exotoxin A and phospholipase C) respectively (Sadikot *et al.*, 2005; Rada and Leto, 2013; van 't Wout *et al.*, 2015; Dzvova *et al.*, 2017). Of all the toxins mentioned, pyocyanin, a blue phenazine compound, is an important virulence factor which is a redox active substance, and is toxic to numerous bacteria and fungi by being able to cause damage to mammalian cells (Hassett *et al.*, 1992). Pyocyanin can also interfere with critical host defence mechanisms and contributes to the symptoms associated with *Pseudomonas*-associated lung diseases (Denning *et al.*, 1998; Sadikot *et al.*, 2005; van 't Wout *et al.*, 2015).

Candida albicans is one of the leading opportunistic human pathogen that can cause superficial infections (e.g. oral or vaginal candidiasis), and also life-threatening systemic infections (Mayer *et al.*, 2013). During both superficial and systemic infection, *C. albicans* depend on wide range of virulence factors, assisting it to colonise within the microbiota in the commensal phase and to also invade host tissue during infection (Mayer *et al.*, 2013). A couple of traits, including extracellular secretion of hydrolytic enzymes (lipases, phospholipases and proteases) which enable the breakdown of host cells and tissue invasion, expression of adhesins and invasins, which enable binding to host cells and on the cell surface, the morphological transition (yeast to hyphal transition and white to opaque transition), thigmotropism (contact sensing), and formation of biofilms, are considered as virulence factors of *C. albicans* (Calderone and Fonzi, 2001; Mayer *et al.*, 2013; Fourie *et al.*, 2016).

We investigated selected virulence factors that might potentially influence the infection of *P. aeruginosa* and *C. albicans*. The thought is that such virulence factors could be used by themselves or as adjuncts that would boost the pathogenicity of *C. albicans* and *P. aeruginosa* leading to observed virulence. Therefore, the aim of this study is to test particular bacterial or fungal factors in pathogenesis *in vitro*, by investigating the most virulent strain in comparison

to the least virulent strain obtained from the results of the established *C. elegans* infection model. Another common determinant of virulence in infection models is the change in the pathogens microbial burden. To this end we also investigated the microbial burden of *C. elegans* during mono- and polymicrobial infections by the most and least virulent strains and combinations.

3.3 Materials and methods

3.3.1 Strains used

Pseudomonas aeruginosa evaluated in this present study were PAO1 (most virulent strain) and Iso20 (least virulent strain), and *Candida albicans* strains were SC5314 (most virulent strain) and Ho6 (least virulent strain) reported in the previous chapter. The most virulent combination was *C. albicans* SC5314 and *P. aeruginosa* Iso20 and the least virulent combination was *C. albicans* Ho6 and *P. aeruginosa* PAO1. Bacterial strains were cultivated on Luria-Bertani (LB) agar (20 g/L agar, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride), while yeast strains were cultivated on yeast extract-peptone-dextrose (YPD) agar (10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 16 g/L agar) and incubated at 37 °C for 24 h. *Caenorhabditis elegans glp-4; sek-1*, was obtained from the *Caenorhabditis* Genetic Center, College of Biological Sciences, University of Minnesota and propagated on Nematode Growth Medium (NGM) agar plates (3 g/L sodium chloride, 2.5 g/L peptone, 17 g/L agar) spotted with *Escherichia coli* OP50 (a uracil-requiring mutant of *E. coli*). Stocks of *E. coli* OP50 were stored at -80 °C, and before usage, frozen aliquots were allowed to thaw then grown in LB broth. A 24 h fresh bacterial culture was spotted on the NGM agar medium and plates were incubated at 37 °C for 24 h. *Caenorhabditis elegans* nematodes were transferred to new NGM agar plates seeded with *E. coli* OP50 every 6 days. All stocks of *C. elegans* were kept at 15 °C until further use.

3.3.2 *In vitro* virulence factors of *Pseudomonas aeruginosa*

3.3.2.1 King A assay

Freshly grown *P. aeruginosa* strains were each inoculated into LB broth, followed by incubation at 37 °C for 24 h. The bacterial cultures were adjusted to optical density (OD_{600}) 0.1 in 5 ml LB broth and streaked onto King A agar (15 g/L agar, 1.4 g/L magnesium chloride, 20 g/L peptone, 10 g/L potassium sulphate) plates, which were then incubated at 37 °C for 24 h. King A agar plates were observed for a colour change. The presence of a green or blue colour was interpreted as indicating the production of pyocyanin. The presence of a yellow

pigment only was interpreted as indicating the production of pyoverdin, or fluorescein (King, *et al.*, 1954).

3.3.2.2 King B assay

Each strain of *P. aeruginosa* were inoculated into LB broth, followed by incubation at 37 °C for 24 h. Thereafter, the bacterial cultures were adjusted to optical density (OD_{600}) of 0.1 in 5 ml LB broth and streaked onto King B agar (20 g/L peptone, 10 ml glycerol, 1.5 g/L dipotassium phosphate, 15 g/L agar, 11.5 g/L magnesium sulphate) plates, and then incubated at 37 °C for 24 h. King B plates were observed under ultraviolet light at 360 nm for fluorescence. Presence of a yellow fluorescent pigment was interpreted as indicating the production of pyoverdin, or fluorescein (described by King *et al.*, 1954, modified according to the recommendations of the US Pharmacopoeia).

3.3.2.3 Quantitative pyocyanin assay

A 24 h culture of *P. aeruginosa* strains were inoculated into LB broth, followed by incubation at 37 °C for 24 h. The bacterial cultures were adjusted to optical density (OD_{600}) 0.1 in 5 ml LB broth medium. Pyocyanin was extracted from the supernatants of cultures grown then measured by the method described previously by Essar *et al.* (1990). A 5 ml volume of supernatant was extracted with 3 ml chloroform and shaken for a few minutes until the pyocyanin is diffused, which turns the solvent blue. The chloroform layer was then transferred to a new clean test tube and re-extracted with 1 ml 0.2 M HCl in order to produce pink to deep red solution, therefore this colour change confirms the presence of pyocyanin. After centrifugation (600 x *g* at 4 °C for 10 min), concentrations of pyocyanin were determined based on measurement of A_{520} and expressed as µg pyocyanin produced per ml culture supernatant, calculated using the following formula: A_{520} x 17.072 (Essar *et al.*, 1990; Saha *et al.*, 2008). All experiments were done in triplicate and the average and standard deviation were calculated respectively. A student *t*-test was carried out in order to determine the statistical significant difference between data sets. Moreover, the *p* value of ≤ 0.05 was considered significant.

3.3.2.4 Swarming motility assay

Freshly grown *P. aeruginosa* strains were inoculated into LB broth medium then incubated at 24 h at 37 °C. Bacterial suspensions were adjusted to OD_{600} 0.1 in 5 ml phosphate-buffered saline (PBS). A volume of 3 µl of bacterial suspensions were spotted on each swarming agar (0.5 % blood agar base, 0.25% beef extract, 0.19 % yeast extract, 0.4 % peptone) plate, then incubated at 30 °C for 24 h. Thereafter, presence of swarming motility was interpreted as a rapid migration over the agar surface in a coordinated manner. (Rashid and Kornberg, 2000; Krishnan *et al.*, 2012).

3.3.2.5 Hydrolytic enzyme activity assay

Freshly grown 24 h *P. aeruginosa* strains on LB agar medium were grown in LB broth for 24 h. Cells were centrifuged at 4000 x *g* at 4 °C for 1 min and carefully washed three times with sterile PBS then diluted to OD_{600nm} of 0.01. To test the proteinase activity, a volume of 3 µl of the standard bacterial suspensions were spotted onto LB agar plates supplemented with 0.2 % Bovine Serum Albumin (BSA) (Roche, USA) (Ells *et al.*, 2014). To test for phospholipase activity, 3 µl of the standard cell suspensions were spotted onto Sabouraud 4 % dextrose agar (SDA) (0.005 M calcium chloride, 1 M sodium chloride, and 8 % sterile egg yolk) (Price *et al.*, 1982; Kouker and Jaeger, 1987; Ells *et al.*, 2014). To test for lipase activity, 3 µl of the standard cell suspensions were spotted onto tributyrin agar plates (3 g/L yeast extract, 5 g/L peptone, 12 g/L agar) (Sigma-Aldrich, USA) (Samaranayake *et al.*, 2005; Ells *et al.*, 2014). All plates were incubated at 37 °C for 5 days to observe the halo around spotted cell suspensions and micrographs were taken. The activity of hydrolytic enzymes was calculated based on the ratio of colony diameter to colony total diameter plus halo (Pz). The Pz value of 1 indicated no enzyme activity, while < 1 indicated that a strain secretes the enzyme (Price *et al.*, 1982; Ells *et al.*, 2014). All experiments were done in triplicate, and calculated the average and standard deviation respectively. A student *t*-test was carried out in order to determine the statistically significant differences between data sets. Moreover, the p value of ≤ 0.05 was considered significant.

3.3.3 *In vitro* virulence factors of *Candida albicans*

3.3.3.1 Hydrolytic enzyme activity assay

A previously described protocol for hydrolytic enzyme activity assay by Price *et al.* (1982) was modified. Briefly, *Candida albicans* SC5314 and Ho6 stains were each cultivated on YPD agar plates at 37 °C overnight. A loop full of each colony was inoculated onto Yeast Nitrogen

glucose (10 g/L glucose, 6.7 g/L Yeast Nitrogen Base (YNB) without ammonium sulphate and amino acids (Difco, USA)) agar plates for 24 h. Yeast cells were then centrifuged at 4000 x *g* at 4 °C for 1 min, then washed three times with PBS and diluted to OD_{600nm} of 0.01. To test for proteinases, a volume of 3 µl of the standard yeast suspensions were spotted onto YNB-glucose agar plates supplemented with 0.2 % BSA (Price *et al.*, 1982; Ells *et al.*, 2014). To test for phospholipase activity, 3 µl of the standard cell suspension was spotted onto SDA (0.005 M calcium chloride, 1 M sodium chloride, and 8 % sterile egg yolk) (Price *et al.*, 1982; Kouker and Jaeger, 1987; Ells *et al.*, 2014). To test for lipase activity, 3 µl of the standard cell suspensions were spotted onto tributyrin agar plates (Samaranayake *et al.*, 2005; Ells *et al.*, 2014). All plates were incubated at 37 °C for 5 days to observe the halo around the spotted cell suspensions and micrographs were taken. The activity of hydrolytic enzymes was determined by calculating the Pz value. The Pz value of 1 indicated no enzyme activity, while < 1 indicated that a strain secretes the enzyme (Price *et al.*, 1982; Ells *et al.*, 2014). All experiments were done in triplicate, and calculated the average and standard deviation respectively. A student *t*-test was carried out in order to determine the statistically significant differences between data sets. Moreover, the p value of ≤ 0.05 was considered significant.

3.3.4 Microbial burden

The technique used to establish *C. elegans* liquid medium infection model was according to the technique described previously by Breger and co-workers (2007) and detailed in the previous chapter. The number of *C. albicans* and *P. aeruginosa* colony forming units (CFUs) per *C. elegans* was quantified according to the protocol by Garsin *et al.* (2001). After infection, nematodes were scored daily into two categories: alive and dead. Dead nematodes were counted and transferred into a 2 ml plastic tube and were mechanically disrupted by using a pipette. Microscopic analysis was done to confirm successful breaking of infected *C. elegans*. Dilution series was done on all samples. The dilution range of 1×10^5 to 1×10^8 was plated on either LB agar (*P. aeruginosa*), or YPD agar (*C. albicans*) then incubated at 37 °C. Daily plate counts were performed and CFUs calculated. All experiments were done in triplicate and the average and standard deviation were calculated respectively. A student *t*-test was carried out in order to determine the statistically significant differences between data sets. Moreover, the p value of ≤ 0.05 was considered significant.

3.4 Results and discussions

3.4.1 *Pseudomonas aeruginosa in vitro* virulence factors

In our previous results, after establishing *C. elegans* infection models, it was observed that *P. aeruginosa* PAO1 was the most virulent isolate while *P. aeruginosa* Iso20 was the least virulent isolate to the nematode. Therefore, the *in vitro* production of selected virulence factors of these two strains were evaluated in order to gain more insight on the intrinsic differences in their virulence potential. Table 1 shows the summary of results obtained from the *in vitro* studies.

Table 1. Virulence factors of *Pseudomonas aeruginosa.*

Strain	King A assay (Pyocyanin)	King B assay (Pyoverdine)	Quantitative pyocyanin assay	Swarming motility
PAO1	Yellow-Green pigment	Yellow-Green pigment	0.28 µg/ml (± 0.07)	Positive
Iso20	No pigment	No pigment	0.64 µg/ml (± 0.06)	Negative

Firstly we investigated pyocyanin production by *P. aeruginosa* strains on King A medium. According to literature, King A medium can enhance *P. aeruginosa* to the produce pyocyanin (King *et al.*, 1954; Hassett *et al.*, 1992). This can be observed by a blue pigment that diffuses into the culture medium. The production of pyocyanin differs depending on the strains of *P. aeruginosa* and culture growth conditions. When the pigment is not the usual blue colour, it may be due to the production of two or more coloured substances. For instance, even though King A medium specifically enhances production of a blue pigment, it is possible that other pigments such as brown (pyomelanin), green (pyoverdine), or red (pyorubin) pigments can appear masking the pyocyanin (Leonard, 1924; King *et al.*, 1954; Wilson and Miles, 1964). In our study, on King A agar medium, strain PAO1 produced a yellow-green pigment, indicating pyoverdine production. In contrast, strain Iso20 grew only as white to cream colonies and did not produce any visible pigment. However, it was found that both strains produced low concentrations of pyocyanin when grown in liquid media, with strain Iso20 producing 0.64

μg/ml (± 0.06), and *P. aeruginosa* PAO1 producing significantly less pyocyanin at only 0.28 μg/ml (± 0.07) ($p = 0.002$).

King B agar allows the production of pyoverdine, a primary siderophore that fluoresces a yellow-green pigment under ultraviolet light (UV) in certain strains of *Pseudomonas* (King *et al.*, 1954). The medium is mostly used in water analysis, to detect and differentiate *P. aeruginosa*, which produces yellow-green pigment while other species of *Pseudomonas* do not. Importantly, King B agar favours the production of pyoverdine while inhibiting the production of pyocyanin (King *et al.*, 1954). This is because dipotassium phosphate used in this medium trigger an increase in the concentration of phosphorus contributed by the peptone and stimulates the pyoverdine production, while inhibiting the pyocyanin production (King *et al.*, 1954). Moreover magnesium sulphate in the medium contributes the cations necessary for pyoverdine production (King *et al.*, 1954). Thus in our current study, under UV light at 360 nm, *P. aeruginosa* PAO1 fluoresced yellow-green on King B agar, while *P. aeruginosa* Iso20 was non-fluorescent, confirming the pyoverdine production seen with King A agar.

Another virulence factor investigated is swarming motility, which is known as a rapid and coordinated migration of a population of bacterial species across a semisolid surface (Henrichsen, 1972). Most of the researchers have shifted their focus into the direction of swarming motility phenomenon, is since it is suspected to be highly associated with biofilm formation of *P. aeruginosa*. Usually the ability to display swarming motility is evaluated using swarm plate in which microorganisms are spot-inoculated at the centre of swarming agar plate, thereafter incubated overnight (Krishnan *et al.*, 2012). The diameter of the resulting "motility zone" formed by migrating bacteria is then measured. Thus the aim of this study was based on investigating the formation of swarming motility phenotype by *P. aeruginosa* PAO1 and Iso20 strains. On the swarming agar, strain PAO1 showed swarming motility while no motility was observed for *P. aeruginosa* Iso20 (Table 1).

Taken together, all these observed data clearly suggested that there are multiple virulence factors of *P. aeruginosa* that may cause virulence, including pyoverdine, pyocyanin and swarming motility. Thus it was observed that both strains produced pyocyanin, however only strain PAO1 produced pyoverdine. This pyoverdine production by *P. aeruginosa* was also observed in liquid killing assay, where it induced hypoxic response, thereafter killing *C. elegans* (Kirienko *et al.*, 2013). Thus, we can speculate that the difference in pyoverdine production between the two strains might be a reason for the observed difference in virulence of the two strains. It was also evident that *P. aeruginosa* PAO1 showed swarming motility while strain Iso20 did not. This may be due to the fact that swarming motility is often co-regulated with toxin secretion (Allison *et al.*, 1992; Givskov *et al.*, 1995).

3.4.2 Hydrolytic enzyme activity assay for *Pseudomonas aeruginosa*

Next, for us to more broadly explore the involvement of other potential virulence factors, we performed *in vitro* enzymatic tests for the expression of lipase, phospholipase and protease activities by *P. aeruginosa* PAO1 and Iso20. Both strains were found to be positive for the production of protease and lipase, while no production of phospholipase (Pz = 1) was observed (Figure 1). Although *P. aeruginosa* PAO1 had a slightly higher lipase activity [Pz = 0.2 (± 0.01)] than strain Iso20 [Pz = 0.28 (± 0.01)], this difference was not statistically significant (p = 0.38). We also discovered no significant difference in the level of protease activity between the two strains.

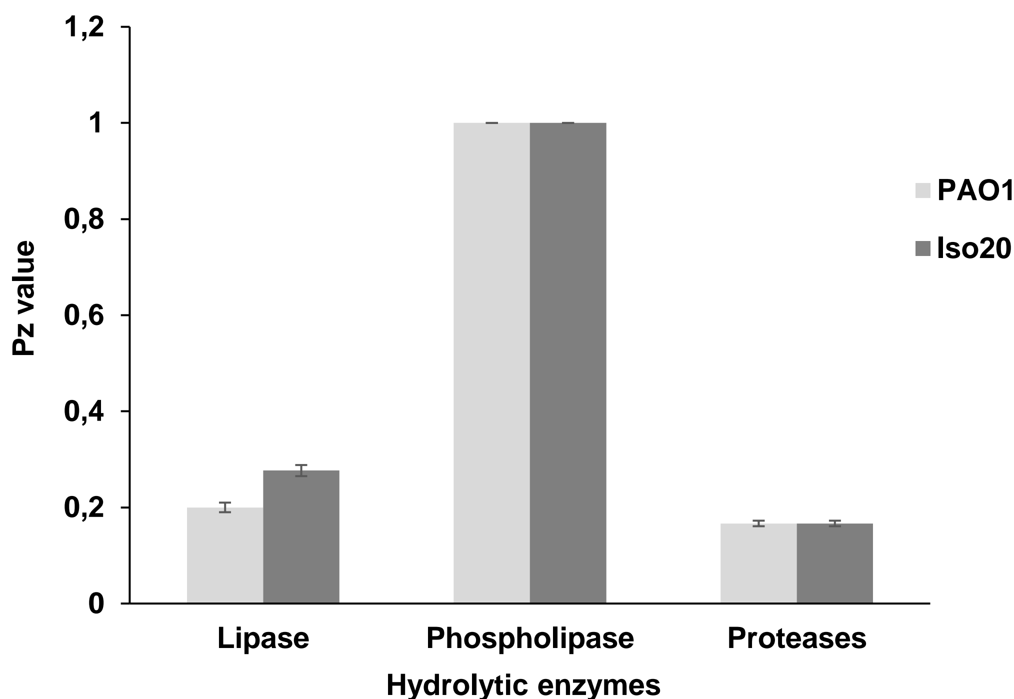


Figure 1. Hydrolytic enzyme activity assay for *Pseudomonas aeruginosa*. Plates were incubated at 37 °C for 5 days to test for secretion of proteases, phospholipases and lipases. The Pz value of 1 indicated no enzyme production, while < 1 indicated that a strain secretes the enzyme. Both PAO1 and Iso20 strains secreted lipases and proteases. However, no secretion of phospholipases was observed in both PAO1 and Iso20 strains. The data is presented as the average and standard deviation of biological triplicates.

3.4.3 Hydrolytic enzyme activity assay for *Candida albicans*

Candida albicans is a well-known pathogenic microorganism that secretes various virulence factors including the production of extracellular hydrolytic enzymes (Schaller *et al.*, 2005). These secreted extracellular hydrolytic enzymes are implicated in enhancing the efficiency of extracellular nutrient acquisition (Naglik *et al.*, 2003). Thus extracellular hydrolytic enzymes, specifically phospholipases and proteases, are thought to play a vital role in *C. albicans* overgrowth because these enable adherence to host cell surface, tissue penetration and host tissue invasion (Hube and Naglik, 2002; Schaller *et al.*, 2005).

In the previous chapter, *Candida albicans* SC5314 and Ho6 strains showed difference in virulence towards *C. elegans* during infection. In this current study we determined if the virulence observed in both the SC5314 and Ho6 strains could be influenced by secretion of proteases, phospholipases and lipases. Both *C. albicans* strains could produce phospholipase and protease, however no lipase activity was detected ($Pz = 1$) (Figure 2). Slight differences were observed in the protease and phospholipase activities of the two strains, with strain SC5314 displaying higher protease and lower phospholipase activity than strain Ho6, however, these differences were not statistically significant ($p > 0.05$).

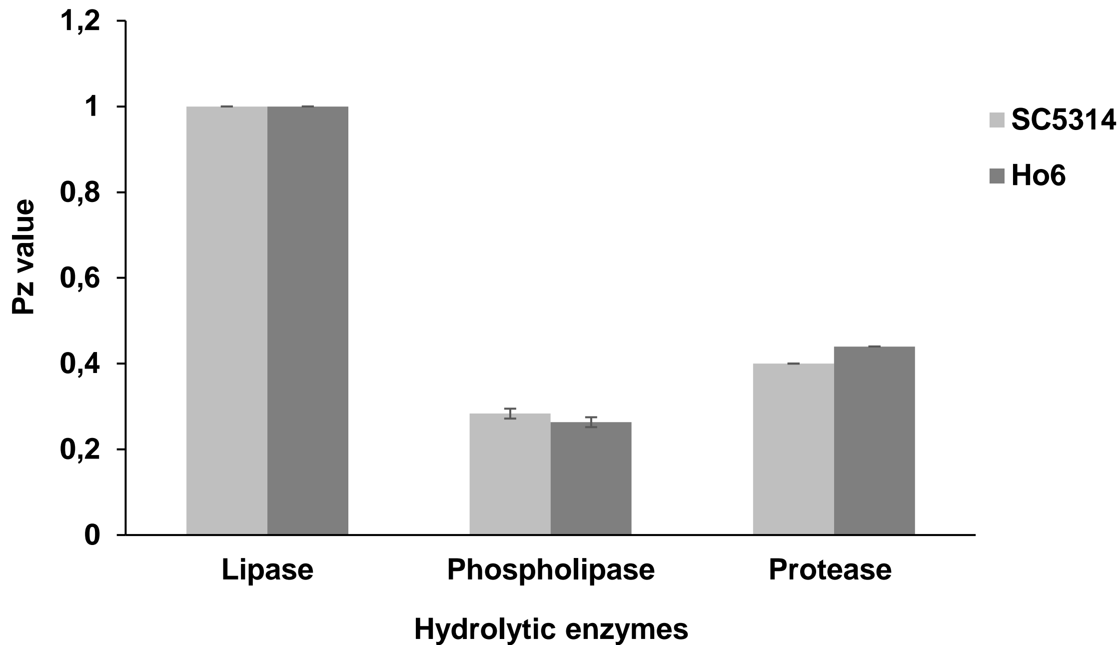


Figure 2. Hydrolytic enzyme activity assay for *Candida albicans*. Plates were incubated at 37 °C for 5 days to test for secretion of proteases, phospholipases and lipases. The Pz value of 1 indicated no enzyme production, while < 1 indicated that the strain secretes the enzyme. Lower Pz value means high enzymatic production while high Pz value mean lower enzymatic production. Both SC5314 and Ho6 strains produced phospholipases and proteases enzymes. No secretion of lipases was observed in both strains. The data is presented as the average and standard deviation of biological triplicates.

These results support the speculated reports in literature, that proteinase and phospholipases are the two most important families of *C. albicans* extracellular enzymes, amongst those associated with virulence (Schaller *et al.*, 2005). The secreted aspartyl proteinase (SAPs) degrades most human proteins (Schaller *et al.*, 2005). At present, the proteolytic activity is attributed to a multigene SAP family of *C. albicans,* which comprises ten members, *SAP1-10*. For example, *SAP1-8* are mostly secreted then released to the surrounding medium, while *SAP9* and *SAP10* remain bound to the cell surface (Naglik *et al.*, 2003; Taylor *et al.*, 2005; Albrecht *et al.*, 2006). Moreover, *SAP1-3* can cause damage to reconstituted human epithelium *in vitro*, and also contribute to virulence in a mouse model of systemic infection (Hube *et al.*, 1997; Schaller *et al.*, 1999). Naglik and co-workers (2003) reported that expression of SAP family genes is related to the co-regulation of other virulence factors, which includes adherence, biofilm formation, filamentation, phenotypic switching, in addition to tissue invasion, penetration, nutrition and interaction with the host's immune system. For instance,

discovery and characterization of the white to opaque switching revealed that this phenotypic switching have an effect on several cellular characteristics, such as proteinase production (Naglik *et al.*, 2003). As a result, a connection between SAP secretion and this switching was then defined in WO-1 *C. albicans* strains (Naglik *et al.*, 2003). At present, in WO-1 strain, *SAP1* is the only proteinase that is strictly under regulation of phenotypic switching, where it is highly expressed only in opaque cells (Naglik *et al.*, 2003). The *SAP8* is another *SAP* gene that is differentially expressed during phenotypic switching, since transcripts were detected in the opaque phenotype but not the white phenotype (Biswas *et al.*, 2007).

Several studies show that phospholipases are also important *C. albicans* pathogenicity determinants having a significant function of causing damage to host cell membranes by destroying phospholipids, therefore causing cell lysis and facilitating tissue invasion (Biswas *et al.*, 2007). Four classes of phospholipases (A, B, C, and D) were previously reported in *C. albicans* on the basis of the ester bond they cleave (Calderone and Fonzi, 2001; Biswas *et al.*, 2007). Niewerth and Korting (2001) showed that amongst the four phospholipase (PLA, PLB, PLC and PLD), only PLB1 is involved in causing virulence in the candidiasis animal model. Moreover, Mavor and co-workers (2005) reported that only the five members of class B (PLB1-5) are extracellular, contributing to virulence via disruption of host membranes.

3.4.4 Microbial burden

We next addressed the issue whether more virulent strains of *P. aeruginosa*, *C. albicans* or combinations could be correlated to higher microbial burdens in *C. elegans*. During infection, it appeared that the pathogens persisted inside the nematodes, multiplied to cause colonisation and later reached the level whereby they killed *C. elegans*. In our results of single infection, the most virulent strain of *C. albicans* (SC5314) or *P. aeruginosa* (PAO1) resulted in lower microbial burden than the least virulent strains (Ho6 or Iso20) (Figure 3). From this it is clear that the virulence observed in killing of nematodes was not due to number of cells but rather specific virulence factors of the different strains. Previous studies showed that expression of certain virulence factors *in vitro* can be correlated to pathogenicity *in vivo* (Ghannoum, 2000; Naglik *et al.*, 2003; Ells *et al.*, 2014). Ibrahim and co-workers (1995) discovered that patients infected with *C. albicans* produced high levels of phospholipase and germ tubes *in vitro* compared to commensal strains. This *in vitro* extracellular phospholipase levels was correlated with mortality when using a murine infection model (Ibrahim *et al.*, 1995). However, Koga-Ito and co-workers (2006), observed no significant difference in the *in vitro* phospholipases levels between commensal strains and patients infected by *C. albicans*, but observed a significant difference in the level of *in vitro* proteases. Thus, when we attempt to

correlate our *in vitro* results regarding *C. albicans* and *P. aeruginosa* factors that may contribute towards virulence and killing of *C. elegans*, it may be speculated that virulence factors of *P. aeruginosa* PAO1 (the most virulent strain), such as, pyoverdine and swarming motility may be responsible for the increased virulence of this strain, compared to strain Iso20 strain (Table 1). Kirienko *et al.* (2013) also showed the important role of this toxin in liquid killing assay, where it induces hypoxic response followed by killing of *C. elegans*. We observed that although some of the virulence factors of *C. albicans*, such as secretion of proteases and phospholipase, known to contribute to virulence, the data we obtained regarding virulence factors of the two *C. albicans* strains was insufficient to explain the difference in virulence between them.

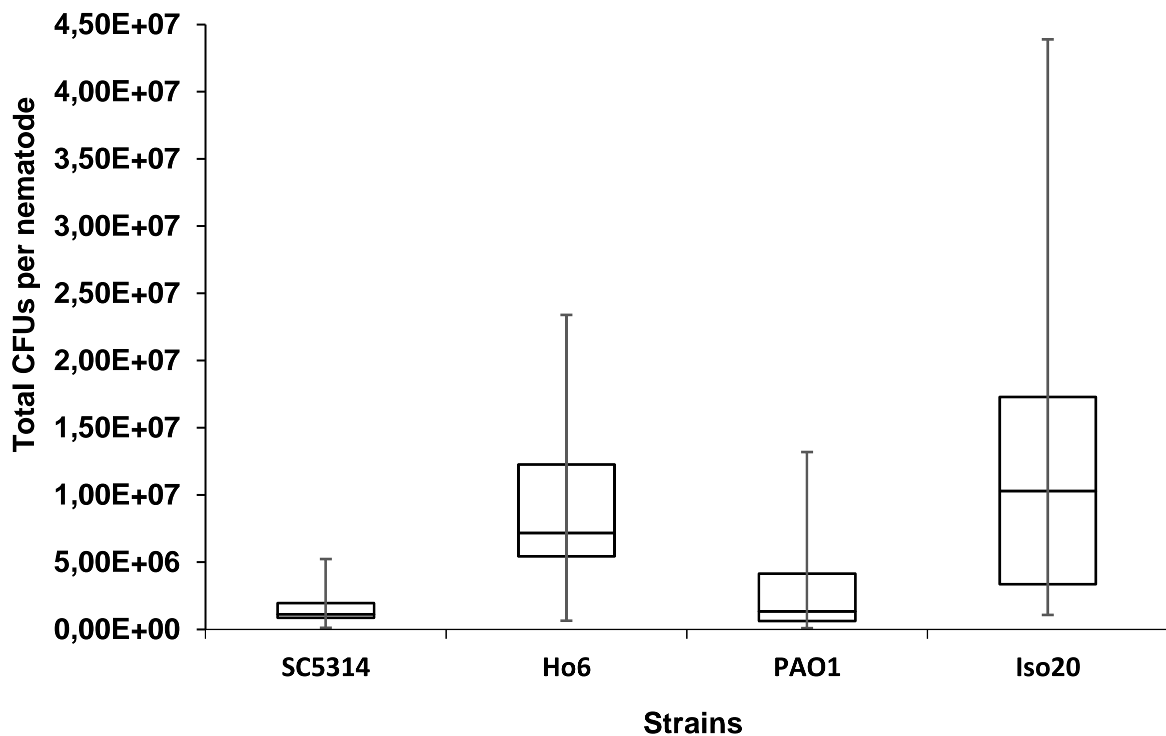


Figure 3. Total colony forming units (CFUs) per *C. elegans* nematode of single infection of *C. albicans* (SC5314 or Ho6) and *P. aeruginosa* (PAO1 or Iso20).

Unexpectedly, when we determined the microbial burden of co-infections, this involvement of virulence factors was not evident. The total microbial burden of the most virulent combination (*C. albicans* SC5314 and *P. aeruginosa* Iso20) was higher than for the least virulent combination (*C. albicans* Ho6 and *P. aeruginosa* PAO1) (Figure 4).

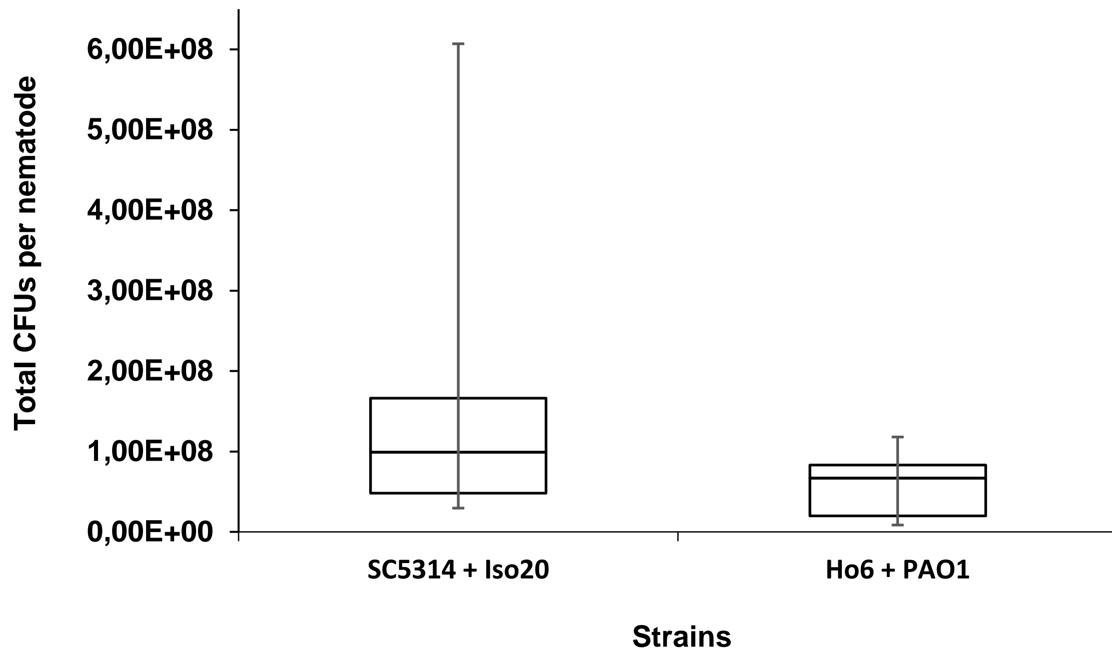


Figure 4. Total colony forming units (CFUs) per *C. elegans* nematode after co-infection of *C. albicans* and *P. aeruginosa* (SC5314 + Iso20 and Ho6 + PAO1).

When comparing the effect of co-infection on the microbial burden of specific pathogens (Figure 5), we see that for both *P. aeruginosa* strains, co-infection resulted in an increased microbial burden (Figure 5A). This was especially evident for strain Iso20. This result was not unexpected, since it is known that co-incubation of *P. aeruginosa* with *C. albicans in vitro* biofilms, increases the CFUs of *P. aeruginosa*. Interestingly, when the microbial burden of *C. albicans* in the co-infections were determined, the expected antagonistic influence of *P. aeruginosa* was not observed (Figure 5B). However, contrary to the *in vitro* findings, showing that *C. albicans* is killed by *P. aeruginosa* (Fourie *et al.*, 2016), co-infection also increased the microbial burden of *C. albicans* SC5314 and did not influence the microbial burden of strain Ho6. This different co-stimulatory response may explain the observed difference in virulence of the two combinations.

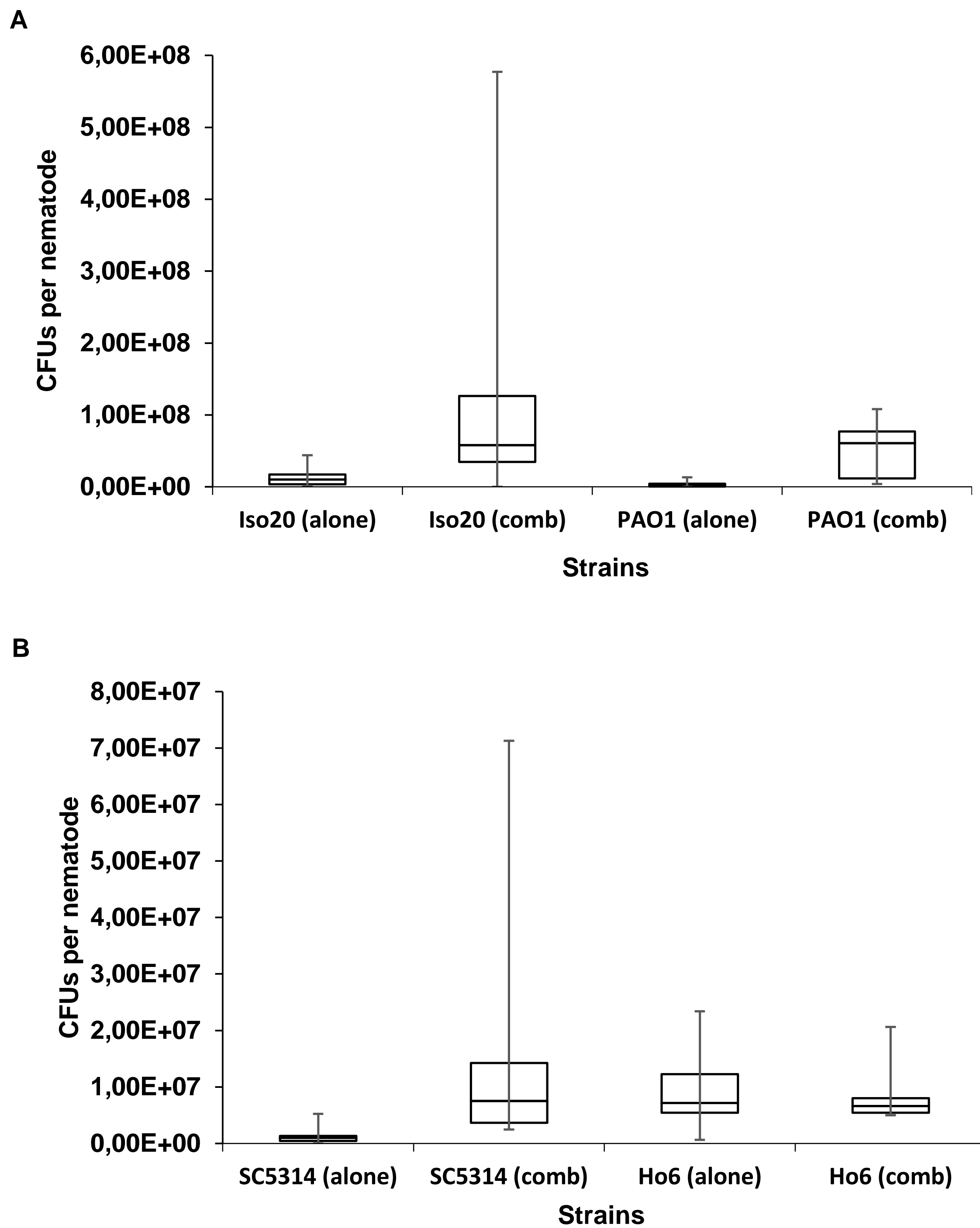


Figure 21. Colony forming units (CFUs) of *P. aeruginosa* (A) and *C. albicans* (B) per *C. elegans* nematode. Monomicrobial infections are referred as “alone”, while co-infections are referred as “comb”.

We also speculate that co-infection in this model may influence expression of virulence factors by the pathogenic strains, which in turn may influence the interaction between the pathogens. The result of this is that virulence of co-infection is strongly influenced by microbial burden and that this is dependent on the specific strains in the co-infection. Co-infection with a yeast and a bacterium may also influence the immune response of the host. Pukkila-Worley and co-workers (2011) found that *C. elegans* has different and distinct immune responses to *P. aeruginosa* and to *C. albicans* respectively, and that the nematode selectively represses antibacterial immune effectors when *C. albicans* is present. However, these studies were, performed using monomicrobial infections and the immune response to such a combined infection is currently unknown.

3.5 Conclusions

In summary, our recent investigations was to further broaden our understanding of the *C. albicans* and *P. aeruginosa* virulence factors which contribute to pathogenicity and killing in *C. elegans*. Secreted virulence factors of *P. aeruginosa* PAO1 strain, such as pyoverdine as well as swarming motility may account for this strains observed higher virulence compared to Iso20. However, there are many other virulence factors produced by *P. aeruginosa* that were not studied and it should be noted that they might also contribute to observed differences in virulence. Additionally, relative contribution of these virulence factors remains to be investigated. In this study we also observed that several virulence factors of *C. albicans* that are produced *in vitro*, such as secretion of proteases and phospholipase, and although these contribute to virulence in different infection model, the data we obtained were not sufficient to assign the difference in virulence to a specific secreted enzyme. Other researchers found that the ability of *C. albicans* to form hyphae in *C. elegans* is an important determinant to virulence in this nematode system (Pukkila-Worley *et al.*, 2011), however, since *C. albicans* did not form hyphae in our study, this was not a contributing factor and not included in the study of *C. albicans* virulence factors. This does not exclude the possibility that other virulence factors may contribute to the observed differences in virulence of the two *C. albicans* strains. This may be further studied using deletion mutants that lack the ability to produce selected virulence factors. Surprisingly, mortality in co-infections correlated with increased microbial burden of pathogens in *C. elegans*. This highlights the complexities when dealing with polymicrobial infections, where one has to study the interactions between pathogens, but also the interactions with the host to gain the full picture. Acquiring full insight on the virulence mechanisms used by *C. albicans* and *P. aeruginosa* during infection is important for development of new therapies and diagnostics.

3.6 References

Albrecht, A., Felk, A., Pichova, I., Naglik, J. R., Schaller, M., de Groot, P. *et al.* (2006). Glycosylphosphatidylinositol anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host pathogen interactions. *J Biol Chem* **281**, 688-94.

Allison. C., Lai, H. C. & Hughes, C. (1992). Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis. Mol Microbiol* **6**, 1583–1591.

Biswas, S., Van Dijck, P. & Datta, A. (2007). Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol Rev* **71**, 348-376.

Breger, J., Fuchs, B. B., Aperis, G., Moy, T. I., Ausubel, F. M. & Mylonakis, E. (2007). Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS Pathog* **3**, e18 doi:10.1371/journal.ppat.0030018.

Calderone, R. A. & Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. *Trends Microbiol* **9**, 327-35.

Denning. G. M., Railsback, M. A., Rasmussen, G. T., Cox, C. D. & Britigan, B. E. (1998). *Pseudomonas* pyocyanine alters calcium signaling in human airway epithelial cells. *Am J Physiol* **274**, 893-900.

Dzvova, N., Colmer-Hamood, J. A., Griswold, J. A. & Hamood A. N. (2017). Isolation and characterization of HepP: a virulence-related *Pseudomonas aeruginosa* heparinise. *BMC Microbiol* **17**, 233.

Ells, R., Kilian, W., Hugo, A., Albertyn, J., Kock, J. L. F. & Pohl, C.H. (2014). Virulence of South African *Candida albicans* strains isolated from different clinical samples. *Med Mycology* **52**, 246-253.

Essar, D. W., Eberly, L., Hadero, A. & Crawford, I. P. (1990). Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol* **172**, 884–900.

Fourie, R., Ells, R., Swart, C. W., Sebolai, O. M., Albertyn, J. & Pohl, C. H. (2016). *Candida albicans* and *Pseudomonas aeruginosa* interaction, with focus on the role of eicosanoids. *Front Physiol* **7**, 64.

Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V. *et al.* (2001). A simple model host for identifying gram-positive virulence factors. *Proc Natl Acad Sci U S A* **98**, 10892–10897.

Ghannoum, M. A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* **13**, 122–143.

Givskov, M., Eberl, L., Christiansen, G., Benedik, M. J. & Molin, S. (1995). Induction of phospholipase- and flagellar synthesis in *Serratia liquefaciens* is controlled by expression of the flagellar master operon *flhD*. *Mol Microbiol* **15**, 445-454.

Hassett, D. J., Charniga, L., Bean, K., Ohman, D. E. & Cohen, M. S. (1992). Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defences, and demonstration of a manganese cofactored superoxide dismutase. *Infect Immun* **60**, 328–336.

Henrichsen, J. (1972). Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36**, 478-503.

Hube, B. & Naglik, J. (2002). Extracellular hydrolases. In *Candida* and Candidiasis (ed. Calderone, R. A). Chapter 5. pp 107-122. ASM Press, Washington, DC.

Hube, B., Sanglard, D., Odds, F. C., Hess, D., Monod, M., Schäfer, W. *et al.* (1997). Disruption of each of the secreted aspartyl proteinase genes *SAP1, SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. *Infect Immun* **65**, 3529-38.

Ibrahim, A. S., Mirbod, F., Filler, S. G. *et al.* (1995). Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect Immun* **63**, 1993–1998.

King, E. O., Ward, M. K. & Raney. D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**, 301-307.

Kirienko, N. V. Kirienko, D. R., Larkins-Ford, J., Wählby, C., Ruvkun, G. & Ausubel, F. M. (2013). *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans* iron homeostasis, causing a hypoxic response and death. *Cell Host Microbe* **13**, 406–416.

Koga-Ito, C. Y., Lyon, J. P., Vidotto, V. & de Resende, M. A. (2006). Virulence factors and antifungal susceptibility of *Candida albicans* isolates from oral candidiasis patients and control individuals. *Mycopathologia* **161**, 219–223.

Kouker, G. & Jaeger, K. E. (1987). Specific and sensitive plate assay for bacterial lipases. *Applied Environmental Microbiol* **53**, 211-213.

Krishnan, T., Yin, W. F. & Chan, K. G. (2012). Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzygium aromaticum*) bud extract. *Sensors* **12** (4), 4016-4030.

Leonard, V. (1924). Pyorubrin, a new pigment produced by *Bacillus pyocyaneus*. *Amer J Hyg* **4**, 404.

Mavor, A. L., Thewes, S. & Hube, B. (2005). Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes. *Curr Drug Targets* **6**, 863-74.

Mayer, F. L., Wilson, D. & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* **4**,119–128.

Naglik, J. R., Challacombe, S. J. & Hube, B. (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* **67**, 400-428.

Niewerth, M. & Korting, H. C. (2001). Phospholipases of *Candida albicans. Mycoses* **44**, 361-367.

Price, M. F., Wilkinson, I. D. & Gentry, L. O. (1982). Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia* **20**, 7-14.

Pukkila-Worley, R., Ausubel, F. M., Mylonakis, E. (2011). *Candida albicans* infection of *Caenorhabditis elegans* induces antifungal immune defences. *PLoS Pathog* **7** (6), e1002074

Rada, B. & Leto, T. L. (2013). Pyocyanin effects on respiratory epithelium: relevance in *Pseudomonas aeruginosa* airway infections. *Trends Microbiol* **21** (2), 73–81.

Rashid, M. H. & A. Kornberg. (2000). Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **97**, 4885–4890.

Sadikot, R. T., Blackwell, T. S., Christman, J. W. & Prince, A. S. (2005). Pathogen–host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* **171** (11), 1209–1223.

Saha, S., Thavasi, R. & Jayalakshmi, S. (2008). Phenazine pigments from *Pseudomonas aeruginosa* and their application as antibacterial agent and food colourants. *Res J Microbiol* **3**, 122-128.

Samaranayake, Y. H., Dassanayake, R. S., Jayatilake, J. A., Cheung, B. P., Yau, J. Y., Yeung, K. W. *et al.* (2005). Phospholipase B enzyme expression is not associated with other

virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. *J Med Microbiol* **54**, 583-93.

Schaller, M., Borelli, C., Korting, H. C. & Hube, B. (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**, 365-77.

Schaller, M., Korting, H. C., Schäfer, W., Bastert, J., Chen, W. & Hube B. (1999). Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol Microbiol* **34**, 169-80.

Taylor, B. N., Hannemann, H., Sehnal, M., Biesemeier, A., Schweizer, A., Röllinghoff, M. *et al.* (2005). Induction of *SAP7* correlates with virulence in an intravenous infection model of candidiasis but not in a vaginal infection model in mice. *Infect Immun* **73**, 7061-3.

van 't Wout, E. F. A., van Schadewijk, A., van Boxtel, R., Dalton, L. E., Clarke, H. J., Tommassen, J. *et al.* (2015). Virulence factors of *Pseudomonas aeruginosa* induce both the unfolded protein and integrated stress responses in airway epithelial cells. *PLoS Pathog* **11** (6), e1004946.

Wilson, G. S. & Miles, A. A. (1964). Topley and Wilson's Principles of bacteriology and immunity, 5th ed., London, p. 639.

CHAPTER 4

Influence of infection on fatty acid composition and prostaglandin E_2 production

4.1 Abstract

Caenorhabditis elegans is becoming more recognised as an important model for the study of lipid metabolism. As such, *C. elegans* offers an opportunity to understand how mechanisms as diverse as behaviour and metabolism are intertwined to ultimately determine an organism's fat content. For instance, *C. elegans* synthesise a wide range of polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA, 20:4n6) and eicosapentaenoic acid (EPA, 20:5n3), from saturated fatty acids obtained from their *Escherichia coli* OP50 diet. Here, we use the infection of *C. elegans* by a human fungal pathogen, *Candida albicans,* and human Gram negative bacterial pathogen, *Pseudomonas aeruginosa,* and as an experimental system to investigate the influence of infection on fatty acid composition and prostaglandin production. Thus the total lipids of infected *C. elegans* were extracted and the fatty acids were converted to their corresponding methyl esters (FAMEs) then analysed by gas chromatography (GC). In addition, prostaglandin E_2 (PGE_2) was extracted from infected nematodes and analysed by liquid chromatography mass spectrometry (LC-MS/MS). Using the *C. elegans* infection model, we observed changes in fatty acid profiles of the major unsubstituted long chain fatty acids. However, no correlation was found between the virulence of infection and the change in fatty acids of the nematodes. We did not detect any PGE_2 in nematodes with monomicrobial infections, but were able to detect PGE_2 in *C. elegans* when infected with the most virulent polymicrobial infection. Since *C. elegans* does not synthesise PGE_2, this indicates that infecting pathogens are able to produce PGE_2 in the host. The role of this PGE_2 in the observed differences in virulence still needs to be further investigated.

Key word: *Caenorhabditis elegans, Candida albicans*, fatty acids, gas chromatography, lipid metabolism, liquid chromatography mass spectrometry, prostaglandin E_2, *Pseudomonas aeruginosa,*

4.2 Introduction

Although lipids have multiple functions in immunity, currently there is relatively little evidence that exists on specific manipulation of the lipid metabolism in response to infection. An attractive animal model for studies of lipid function is the nematode *Caenorhabditis elegans*. This popular model organism has a simple anatomy, well-annotated genome, and its well-understood developmental programs, moreover, it is easy and inexpensive to maintain in the laboratory, has a short lifespan, and ease of genetic analysis allow for studies of diverse biological processes, including those related to human nutrition and disease (Corsi *et al.*, 2015). In the laboratory, the nematodes can be cultivated on petri dish plates with *Escherichia coli* lawns, which provide nutrients, such as carbohydrates, proteins, and saturated and monounsaturated fatty acids derived from digestion of bacterial membranes (Brooks *et al.*, 2009). The nematode can synthesise all the essential fatty acids *de novo*, moreover it has a wide range of fatty acid desaturases and elongases that synthesise long chain polyunsaturated fatty acids, such as arachidonic acid (AA, 20:4n6) and eicosapentaenoic acid (EPA, 20:5n3) (Watts, 2008, 2016).

Lipids are also involved in fungal growth and also pathogenicity (Noverr *et al.*, 2003; Erb-Downward and Huffnagle, 2006; Shea *et al.*, 2006; Rhome and Del Poeta, 2009). For instance, human fungal pathogen *Candida albicans,* which is often not found alone in a host, but in concert with other microorganisms such as bacteria (Diaz *et al.*, 2014; Lindsay and Hogan, 2014), may be influenced by immunomodulatory fatty acid metabolites produced by both the host and bacterial co-colonisers. One such bacterium is *Pseudomonas aeruginosa*, which is often isolated together with *C. albicans* (Bragonzi *et al.*, 2009). During infection, *C. albicans* and *P. aeruginosa* are known to stimulate several host cells to release AA (Castro *et al.*, 1994; Agard *et al.*, 2013). The host makes use of this AA to produce immunomodulatory eicosanoids such as prostaglandins, leukotrienes, thromboxanes and lipoxins (Dennis and Norris, 2015). Importantly, these eicosanoids regulate the balance of inflammation during infection, and can either cause inflammation or resolution of the inflammatory response. Moreover, in *C. albicans*, lipid molecules that includes fatty acids and eicosanoids are reported to control yeast to hyphal transition. In addition, this fungus has been reported to also produce immunomodulatory lipids, such as 3,18-dihydroxeicosatetraenoic acid (3,18-diHETE) and prostaglandins from exogenous or host derived AA (Deva *et al.*, 2000; Erb-Downward and Noverr, 2007; Fourie *et al.*, 2016). Interestedly, *P. aeruginosa* can also metabolise exogenous AA, producing prostaglandins and hydroxyeicosatetraenoic acids (Lamacka and Sajbidor, 1995; Vance *et al.*, 2004; Fourie *et al.*, 2016). Interestingly, these eicosanoids may have adverse roles in the dynamic of pathogen-pathogen interaction and pathogen-host interaction,

moreover they can have an effect not only on the dynamics of this polymicrobial infection but also on the survival of host during infection (Fourie *et al.*, 2016).

Although there has been intensive studies done on the function of lipid mediators between monomicrobial pathogenic species and hosts, little evidence still exists concerning their function in polymicrobial infection of *C. albicans* and *P. aeruginosa* and towards the host. Therefore this permits investigations for us to gain full insight regarding the *C. albicans* and *P. aeruginosa* polymicrobial interaction in context of prostaglandin, and also role of these prostaglandins in a host, such as *C. elegans*, during co-infection. This could give valuable insight into the role of PGE_2 during polymicrobial infection by these microorganisms.

4.3 Materials and methods

4.3.1 Strains used

Caenorhabditis elegans glp-4; sek-1 , was obtained from the *Caenorhabditis* Genetic Center, College of Biological Sciences, University of Minnesota and propagated on Nematode Growth Medium (NGM) agar plates (2.5 g/L peptone, 17 g/L agar, 3 g/L sodium chloride) spotted with *Escherichia coli* OP50 (a uracil-requiring mutant of *E. coli*). Stocks of *E. coli* OP50 were stored at -80 °C, and before usage, frozen aliquots were thawed then cultured in Luria-Bertani (LB) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) broth medium. A 24 h fresh bacterial culture was spotted on the NGM agar medium plates to make a lawn thereafter incubated at 37 °C overnight. Nematodes were transfered to new NGM agar plates seeded with *E. coli* OP50 every 6 days and stocks kept at 15 °C. *Pseudomonas aeruginosa* evaluated in this study were PAO1 (most virulent strain) and Iso20 (least virulent strain), and *Candida albicans* strains were SC5314 (most virulent strain) and Ho6 (least virulent strain). Bacterial strains were grown on LB agar, while yeast strains were grown on yeast extract-peptone-dextrose (YPD) agar (5 g/L peptone, 3 g/L yeast extract, 10 g/L glucose, 16 g/L agar) and incubated at 37 °C. Nematodes that only feed on *E. coli* OP50 were regarded as experimental control throughout all the experiments.

4.3.2 *C. albicans-P. aeruginosa-C. elegans* liquid medium pathogenesis assay

The methodology used for the *C. elegans-P. aeruginosa-C. albicans* liquid medium assay was according to the protocol described previously by Breger *et al.*, 2007. Freshly grown *C. albicans* strains were each inoculated into 5 ml of YPD broth medium then incubated at 37 °C overnight. Similarly, *P. aeruginosa* strains were each inoculated into 5 ml of LB broth medium then incubated at 37 °C overnight. For single species infections, lawns of either *C. albicans* or

P. aeruginosa were prepared separately by plating 100 µl of the yeast or bacterial strains into 10cm square lawns on brain-heart infusion agar (BHI) (2.5 g/L disodium phosphate, 7.8 g/L brain extract, 2.0 g/L dextrose, 9.7 g/L heart extract, 15 g/L agar) plates, then incubated at 37 °C for 24 h. Synchronized L4 *C. elegans glp-4*; *sek-1* nematodes, grown at 15 °C, were carefully washed from 250 NGM agar plates containing a lawn of *E. coli* OP50, with a sterile M9 buffer (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.25 g/L $MgSO_4.7H_2O$, 5 g/L NaCl). Nematodes were further washed four times with M9 buffer then centrifuged at 700 rpm for 5 minutes in 30 % sucrose solution, to separate nematodes from bacterial food source and other possible contaminants. Furthermore, nematodes that floated in sucrose solution were washed with M9 buffer, and centrifuged at 2000 rpm for 2 minutes to form a pellet. Washed nematodes were then placed at the center of the *C. albicans or P. aeruginosa* lawns and incubated at 25 °C for 4 h. For the polymicrobial infections, the nematodes were first allowed to feed on *C. albicans* lawns for 2 h, washed then transferred to *P. aeruginosa* lawns for an additional 2 h.

Nematodes were then carefully washed into 50 ml conical tube using 20 ml of sterile M9 buffer and washed four times with sterile M9 buffer. Nematodes were then transferred into a single well of a six-well tissue culture plate containing 4 ml of liquid medium (80 % M9, 20 % BHI) and incubated at 25 °C for 24 h. Thereafter, the nematodes were collected from liquid medium and transferred into 50 ml conical tube, then carefully washed with 20 ml of M9 buffer. Bacterial and yeast which may skew the *C. elegans* lipid profiles was removed via sucrose floatation prior to nematode collection (Jenkins, 1964). Collected nematodes were then frozen immediately at −80 °C until lipid extraction.

4.3.3 Cultivation of microbes for lipid analysis

Freshly grown *C. albicans* SC5314 and Ho6 strains were inoculated into YPD broth, while *P. aeruginosa* PAO1 and Iso20 strains were inoculated into LB broth medium then incubated at 37 °C overnight. The yeast and bacteria suspensions were adjusted to OD_{600} 0.1 in 5 ml LB broth. A volume of 3 µl of yeast or bacterial suspensions were inoculated on BHI agar plates and microbial lawn was made. Petri dish plates were incubated at 37 °C for 24 h. After incubation, the individual lawns were scraped off and washed four times with sterile PBS. Supernatant and cells were collected in 50 ml conical tubes and cells were centrifuged (5750 *g* at 4 °C for 5 minutes, Eppendorf, Germany), then frozen immediately at −80 °C until lipid extraction.

4.3.4 Influence of infection on nematode fatty acid composition

4.3.4.1 Extraction of fatty acids

Fatty acids extraction of the infected nematodes as well as the microbes alone were carried out according to the methodology by Folch *et al.* (1957). Briefly, frozen nematodes or microbial cells were freeze dried and dry biomass weighed. Nematodes or microbial cells were ground and total lipids extracted overnight with chloroform: methanol (2:1 v/v). Thereafter, the extract was filtered, washed with distilled water and dried under vacuum. The weight of the total lipids in each sample were determined and lipids were stored at -80 °C for further usage.

4.3.4.2 Gas chromatography analysis

Fatty acids were transesterified to form methyl esters (FAMEs) using 0.5 N NaOH and 14 % boron trifluoride in methanol (Slover and Lanza, 1979; Hur *et al.*, 2004; Diaz *et al.*, 2005). Fatty acid methyl esters were quantified using a Varian 430 flame ionization gas chromatography (GC), with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 µm film thicknesses). All the analysis were performed using an initial isothermic period (40 °C for 2 minutes). Thereafter, the temperature was increased at a rate of 4 °C/minute to 230 °C. This was followed by an isothermic period of 230 °C for 10 minutes. The FAMEs were then dissolved in *n*-hexane (1 µl) then injected into the column using a Varian CP 8400 Autosampler. The injection port and detector were both maintained at a constant temperature of 250 °C. The hydrogen, at 45 psi, served as the carrier gas, while nitrogen served as the make-up gas. Finally the chromatograms were recorded using the Galaxy Chromatography Software. The FAME samples were identified by comparing the retention times of authentic standards (Supelco 37 Component Fame Mix 47885-U, Sigma-Aldrich). All other reagents and solvents that were used were of analytical grade and obtained from Merck Chemicals. The unsaturation index was calculated by characterizing each fatty acid in a group based on the double bond number and multiplied each group by its double bond number.

4.3.5 Influence of infection on nematode prostaglandin E_2 production

4.3.5.1 Extraction of prostaglandin E_2

Prostaglandins were extracted using a modified protocol by Ells *et al.* (2013). Briefly, frozen nematodes were weighed, allowed to thaw on ice and resuspended in 1 N citric acid (160 µl) with 1 mM ethylenediaminetetraacetic acid (EDTA). A volume of 20 µl (10 %) butylated hydroxytoluene (BHT, Sigma-Aldrich) was added as antioxidant to combat degradation of

PGE_2 and vortexed to mix thoroughly. Washed ceramic beads (hydrochloric acid, 3 h, pre-cleaned with deionized water; overnight in oven 100 °C) (600 µl) were added to the nematode suspension. The nematode suspension was disrupted with Beadbug microtube homogenizer for 30 seconds followed by cooling on ice for 30 seconds, this was repeated for 10 rounds. After breaking, PGE_2 were extracted with 2 ml hexane: acetate (1:1 v/v), centrifuged (at 1878 *g* for 10 minutes, 4 °C) to remove cellular debris, and also the organic phase was removed and transferred into a different tube. This previous step was repeated three times and the organic phase was pooled and evaporated under stream of N_2 in pre-washed glass vials. Samples were stored at -80 °C until use.

4.3.5.2 Detection of prostaglandin E_2 by LC-MS/MS

Samples were resuspended into 500 µl of formic acid. In addition, 5 ng PGE_2 (Sigma-Aldrich) was added to each sample to evaluate efficiency of extraction and to identify retention time of authentic PGE_2. For detection of PGE_2 in samples, the method proposed by Brose *et al.*, 2011 was used. In this method, 20 µl of each sample was separated on a C18 (XDB-C18, 1.8 µm, 4.6 x 50 mm, Agilent) column at a flow rate of 300 µl/min using 10 mM ammonium acetate (mobile phase A) and methanol/10 mM ammonium acetate (mobile phase B). The column was equilibrated and loaded at 5 % B, increasing to 10 % B over 4 minutes, 95 % B for 5 minutes, followed by re-equilibration at 5 % for a total runtime of 15 minutes. Eluting analytes were ionised in negative electrospray mode with a 4500 V ion spray voltage and 500 °C heater temperature to evaporate excess solvent, 50 psi nebuliser gas, 50 psi heater gas and 25 psi curtain gas. Samples were analysed using a 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer (AB Sciex) and Shimadzu UFLC stack with LC-20AB binary pump and SIL-20A HT autosampler as front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software. The targeted analysis for the extracted PGE_2 described above used 5 MRM transitions: 351.1 > 315.2; 351.1 > 271.2; 351.1 > 333.3; 351.1 > 189.0; 351.1 > 235.1.

4.3.6 Statistical analysis

All experiments were executed in triplicate and repeated four times. The average and standard deviation was calculated. The student *t*-test was carried out to determine statistically significant differences between data sets. A p value of ≤ 0.05 was considered significant.

4.4 Results and discussions

4.4.1 Fatty acids composition

The free living nematode *Caenorhabditis elegans* is a proven model organism for lipid metabolism research. *Caenorhabditis elegans* is able to synthesize all the necessary long chain fatty acids from the bacterial food source (*E. coli* OP50), enabling studies on the manipulation of lipid synthesis and content in this nematode (Watts and Browse, 2002; Ashrafi *et al.*, 2003; Kniazeva *et al.*, 2004; Van Gilst *et al.*, 2005). The biosynthesis of these fatty acids in *C. elegans* is catalysed by several enzymatic activities of elongase and desaturase, encoded by *fat-1*, *fat-2*, *fat-3*, *fat-4*, and *elo-1* respectively (Figure 1) (Watts and Browse, 2002; Ashrafi *et al.*, 2003; Kniazeva *et al.*, 2004; Van Gilst *et al.*, 2005).The FAT-5 desaturase catalyse palmitic acid (16:0), producing palmitoleic acid (16:1n7), further elongated to cis-vaccenic acid (18:1n7) (Watts and Browse 2000). The FAT-6 and FAT-7 desaturases mainly act on stearic acid (18:0), producing oleic acid (18:1n9) (Watts and Browse 2000). Unlike in mammals, in *C. elegans* 18:1n9 can be further desaturated and elongated to form polyunsaturated fatty acids, thus 18:1n9 only accumulates to a small degree in *C. elegans* membranes and neutral lipids, even though it is a much more significant component of mammalian membranes (Wallis *et al.*, 2002).

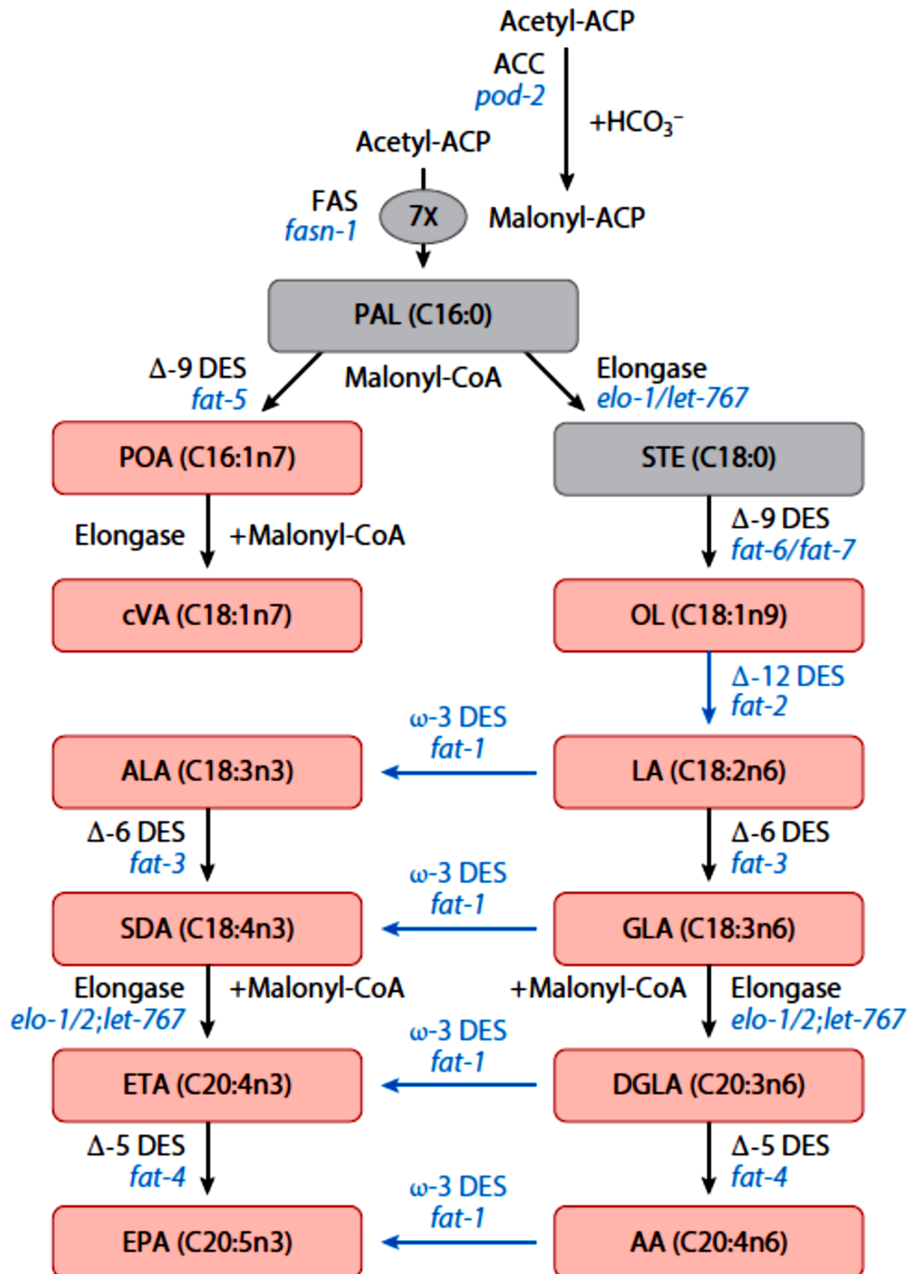


Figure 1. *Caenorhabditis elegans* Unsaturated fatty acid biosynthetic pathway. Abbreviations: AA, arachidonic acid; ACC, acetyl-CoA carboxylase; ACP, acyl carrier proteins; ACS, acyl-CoA synthase; ALA, α-linolenic acid; BCKAD, branched-chain α-keto acid dehydrogenase complex; cVA, cis-vaccenic acid; C5ISO, isovaleric acid; C13ISO, 11-methyldodecanoic acid; C15ISO, 13-methyltetradecanoic acid; C17ISO, 15-methylhexadecanoic acid; DES, desaturase; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; FAS, fatty acid synthase; GLA, γ-linolenic acid; KAR, 3-ketoacyl-CoA reductase; LA, linolenic acid; OL, oleic acid; PAL, palmitic acid; POA, palmitoleic acid; PUFA, polyunsaturated fatty acid; SDA, stearidonic acid; STE, stearic acid (Zhu and Han, 2014).

Firstly, from the *C. elegans* fed on control *E. coli* OP50, twenty-three different fatty acids were identified, and the results of the quantitative analyses of FAMEs derived from the lipids of *C. elegans* are shown in Table 1. The identified fatty acids ranged from 12 to 22 carbons in length, and 35 % were saturated, while 65 % were unsaturated.

Table 1. Fatty acid composition of *C. elegans* grown on *E. coli* OP50.

Common name	Formula
Lauric	12:0
Myristic	14:0
Pentadecylic	15:0
Palmitic	16:0
Palmitoleic	16:1n9
Margaric	17:0
Stearic acid	18:0
Oleic	18:1n9
Vaccenic	18:1n7
Nonoadecanoic	19:0
Linoleic	18:2n6
Arachidic	20:0
γ-Linolenic	18:3n3
α-Linolenic	18:3n3
Octadecatetraenoic	18:4n3
Eicosadienoic	20:2n6
Eicosatrienoic	20:3n6
Erucic	22:1n13
Eicosatrienoic	20:3n3
Arachidonic	20:4n6
Docosadienoic	22:2n6
Eicosopentaenoic	20:5n3
Docosapentaenoic	22:5n3

We then only focused on the major unsubstituted long chain fatty acids (LCFAs) (Figure 2), i.e. myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1n9), margaric acid (17:0), stearic acid (18:0), oleic acid (18:1n9), vaccenic acid (18:1n7), linoleic acid (18:2n6), nonadecanoic acid (19:0), eicosopentaenoic acid (20:5n3) and erucic acid (22:1n13). Margaric acid (17:0) was the predominant fatty acid, with an average of 24 % of the total major fatty acids (Figure 2).

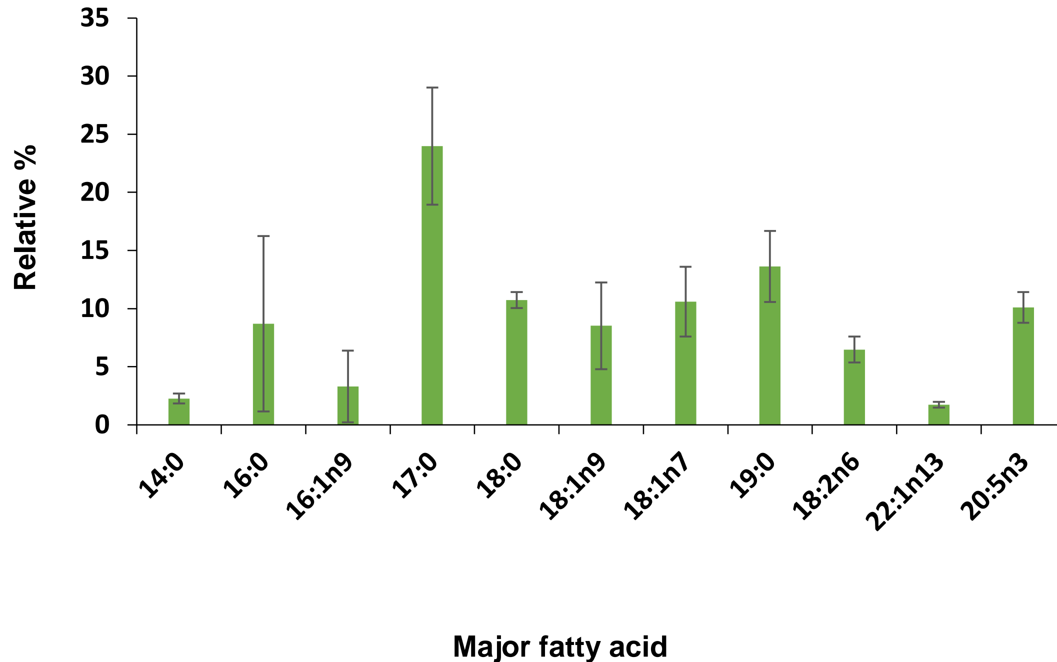


Figure 2. Major unsubstituted LCFAs in *C. elegans* on *E. coli* OP50 as control. Data sets represent the average of three independent experiments and error bars represent the standard deviations.

According to literature, the *E. coli* OP50 bacterial food source of *C. elegans* contain saturated fatty acids (SFAs) and monounsaturated fatty acid (MUFAs), including 16:1n9 and 18:1n11, however not 18:1n9, in their membrane. As nematodes feed on these bacterial strain, these fatty acids can be incorporate into their lipids. In the study recently done by Henry *et al.* (2016), about 28 different fatty acids were discovered, with a length ranging from 12 to 22 carbons, and majority of those consisting of 12 to 17 carbons were saturated, with the exception of 16:1 and 17:1. However, unlike with our current study, stearic acid (18:0) was the predominant

saturated fatty acid, with an average of 6.5 % of total fatty acids. They obtained other saturated acids, such as lauric (12:0), 13:0, 14:0, pentadecylic (15:0), 16:0, 17:0, arachidic (20:0) and 22:0. Interestingly, 65 % of the identified fatty acids were unsaturated comprising 18 to 20 carbon atoms, and monounsaturated acids accounted for 35.6 % of the total fatty acids. Moreover they identified 16:1, 18:1, and 20:1. The fatty acid 18:1 contained close to 62 % of the monounsaturated fatty acids. Single forms of 18:2, 20:2, 20:3, and 20:5 were identified, as well as two isomers each of 18:3 and 20:4. Other numerous fatty acid components were discovered in trace amounts and were identified as 12:0, *iso*- 14:0, 16:1, and 22:0 (Henry *et al*., 2016). Their results supported the results we obtained in our studies, whereby from the *C. elegans* feeding on *E. coli* OP50, twenty-three different fatty acids were identified (Table 1). The identified fatty acids ranged from 12 to 22 carbons in length, and 35 % were saturated, while 65 % were unsaturated and most of this fatty acids were also identified by Henry and co-workers (2016).

Although lipids are known to play multiple roles in immunity, relatively little evidence exists for the specific manipulation of the lipid metabolism in response to infection. Thus we aimed to investigate the effect of infection on the abundance of major unsubstituted LCFAs as well as the unsaturation index of nematode lipids. After infecting the nematodes with monomicrobial species, we observed that *C. albicans* Ho6 and *P. aeruginosa* Iso20 were the least virulent strains in terms of killing *C. elegans*, however they had fatty acid composition very similar to *C. albicans* SC5314 and *P. aeruginosa* PAO1 most virulent strains, thus providing a useful control for changes due to nutritional differences between *E. coli*, *C. albicans* and *P. aeruginosa*. All nematodes infected with monomicrobial species decreased the unsaturation index of the nematode lipids (Figure 3). However, the decrease in unsaturation index for the infection with *C. albicans* Ho6 was not statistically significant [$P = 0.22$ (± 10.45)]. Co-infection changed the unsaturation index, with the combination of *C. albicans* Ho6 and *P. aeruginosa* PAO1 also decreasing the unsaturation index [$P = 0.0003$ (± 1.31)]. Surprisingly, co-infection with C*. albicans* SC5314 and *P. aeruginosa* Iso20 significantly increasing the unsaturation index [$P = 0.018$ (± 7.30)] (Figure 3).

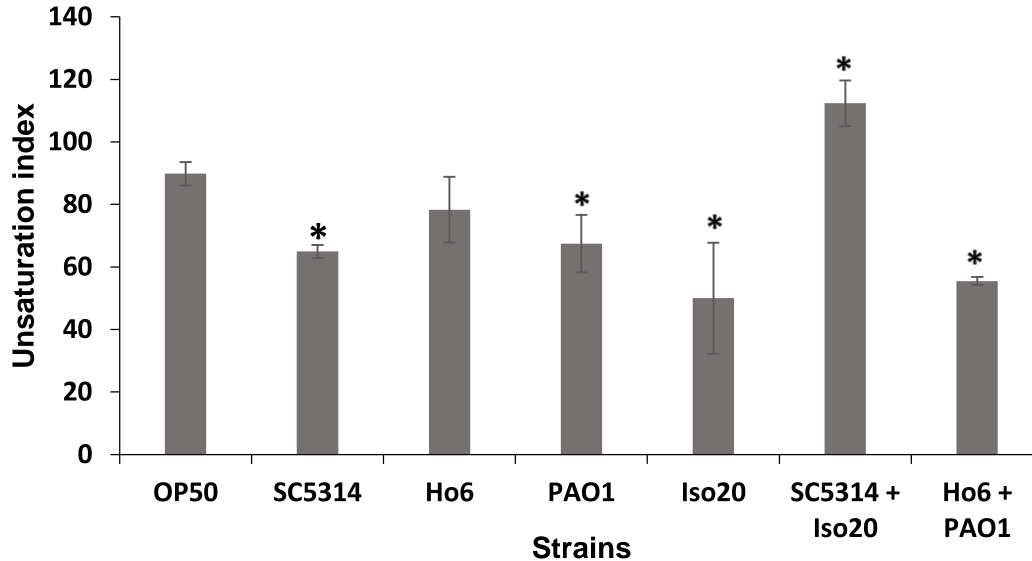
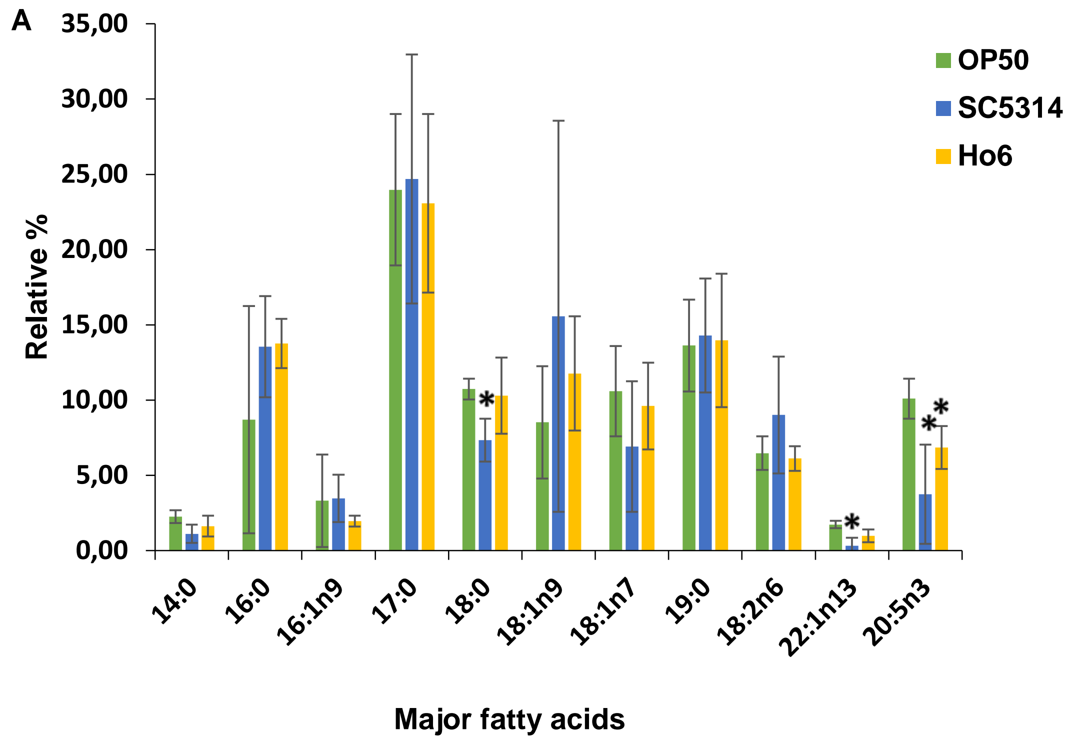


Figure 3. Unsaturation index of major unsubstituted LCFAs in *C. elegans* infected with *C. albicans*, *P. aeruginosa* and co-infection compared to uninfected *C. elegans* on *E. coli*. Error bars represent the standard deviations and asterisk (*) indicate a significant difference from *E. coli* OP50 with P values <0.05; Student's *t*-test.

The change in unsaturation index of nematodes upon infection with *C. albicans* SC5314 was found to be due to significant decreases in the relative percentage 22:1n9 and especially 20:5n3 (Figure 4A). Similarly, the decrease in unsaturation index after infection with both strains of *P. aeruginosa* can be ascribed to significant decreases in the relative percentage of 22:1n9 as well as the polyunsaturated fatty acids, 18:2n6 and 20:5n3 (Figure 4B). Interestingly, infection with *P. aeruginosa* PAO1 showed a significant increase in the relative percentage of 18:1n7. Nandakumar and Tan (2008) showed similar trend, where nematodes infected with both *P. aeruginosa* PA14 and an avirulent mutant, PA14DgacA, showed an increase in relative percentage of 20:5n3, and had more than twice the 18:1n7 content compared to control (*E. coli* OP50-1) nematodes. They argued that the higher levels of 18:1n7 in the infected nematode is most likely due to nutritional differences between *P. aeruginosa* and *E. coli* rather than differences in virulence. Thus they concluded that *P. aeruginosa* PA14 and PA14DgacA are similar in fatty acid content, but are highly divergent from *E. coli* OP50-1 (Nandakumar and Tan, 2008). Furthermore, in our study, no significant differences were observed between the fatty acid profiles (including the relative percentage 18:1) of control nematodes and those infected with the least virulent *P. aeruginosa* Iso20 strain (Figure 4B). However, in the case of the more virulent *P. aeruginosa* PAO1, a higher relative percentage 18:1 is seen. This may indicate that, in contrast to the speculation by Nandakumar and Tan

(2008), the infection associate changes in 18:1 content of infected nematode lipids may not only be due to the fatty acids nutritional differences available from *E. coli* and *P. aeruginosa.*

Moreover, in the present study infection by either *P. aeruginosa* PAO1 or Iso20 resulted in a significant decrease in the relative percentage 18:0 in addition to the mentioned decrease in 18:2 and 20:5. Similar results were obtained by Nandakumar and Tan, 2008, where nematodes infected with the virulent strain *P. aeruginosa* PA14 had significantly decreased levels of 18:0, 18:1, 18:2, 18:3n3 and 18:3n6 compared to nematodes exposed to *E. coli* or the attenuated PA14DgacA strains. These changes in fatty acids content also corresponded with their studies of gene expression revealing infection-induced changes in gene expression. They observed that three of the genes up-regulated in response to infection, *fat6, fat2* and *fat3*, are involved in the synthesis of fatty acids listed above, potentially indicating a feedback loop, where decreases in LCFA levels during infection could induce increased expression of corresponding biosynthetic genes. The infection-specific decreases in fatty acid levels led to their hypothesis that these LCFAs may be involved in immunity against pathogens (Nandakumar and Tan, 2008). For future research, it will be of interest to look at this changes in gene expressions.



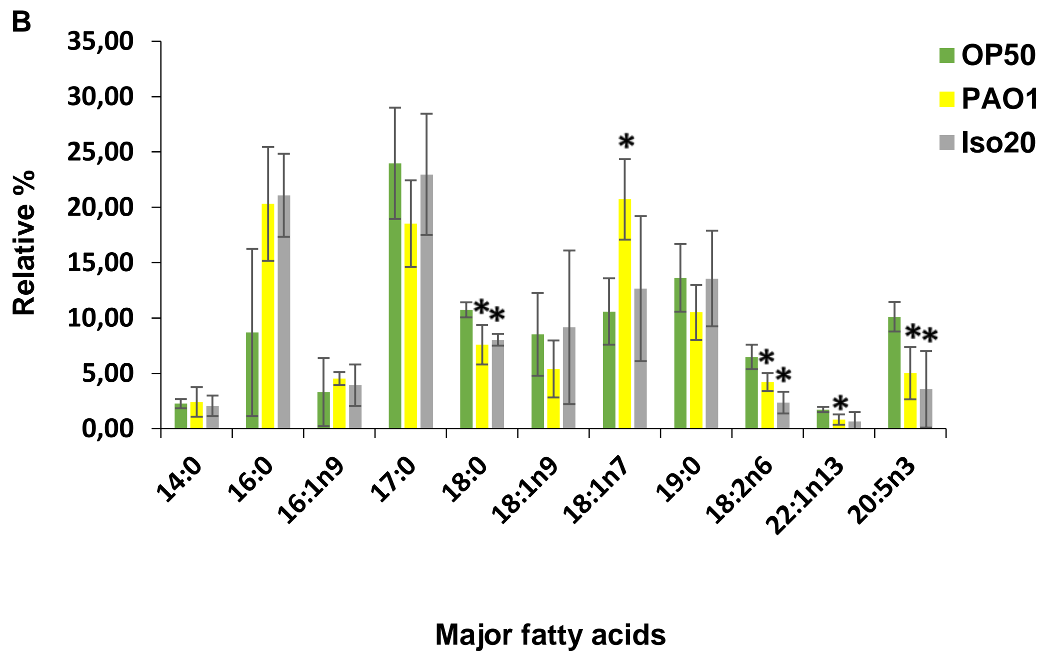


Figure 4. Effect of *C. albicans* (A) and *P. aeruginosa* (B) on relative percentage of major long chain fatty acid as determined by gas chromatography analysis in *C. elegans*. Values represent the mean of three independent experiments and error bars represent the standard deviations and asterisk (*) indicate a significant difference from *E. coli* OP50 with P values <0.05; Student's *t*-test.

Moreover, the unsaturation index of the lipids after co-infection with *C. albicans* and *P. aeruginosa* differed significantly from control nematodes. For the most virulent combination (*C. albicans* SC5314 and *P. aeruginosa* Iso20) the unsaturation index increased, while for the less virulent combination, it decreased (Figure 3). This difference in unsaturation index is largely due to differences in the relative percentages of the saturated fatty acids 17:0, 18:0 and 19:0, which decreased significantly during infection with the more virulent combination and not during the less virulent combination (Figure 5). In addition, the less virulent combination caused a decrease in the relative percentage 20:5n3 in the lipids, contributing to the observed increase in unsaturation index. Interestingly both co-infections again caused a significant increase in the relative percentage 18:1n7.

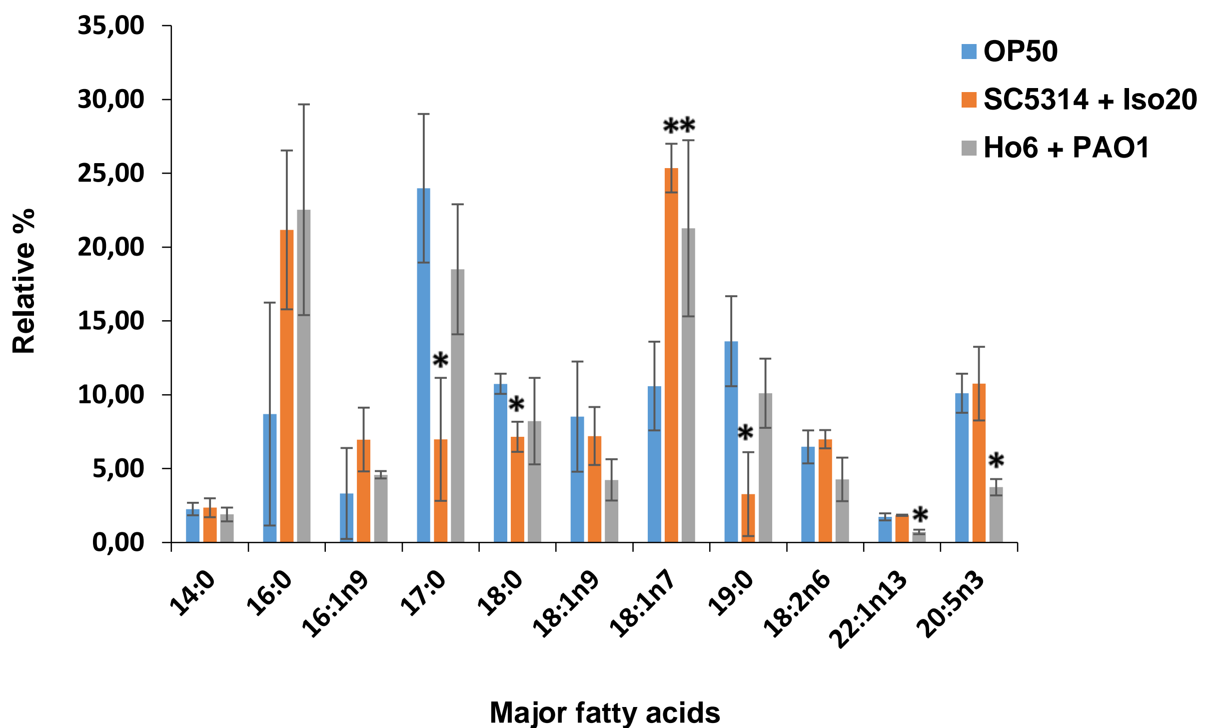


Figure 5. Effect of *C. albicans* and *P. aeruginosa* co-infection on relative percentage of selected major fatty acids as determined by GC analysis in *C. elegans*. Values represent the mean of three independent experiments and error bars represent the standard deviation and asterisk (*) indicate a significant difference from *E. coli* OP50 with P values <0.05.

In order to determine if any of the changes in fatty acid composition associated with infection, are due to differences in the nutritional fatty acid available from the ingested pathogens, the fatty acid compositions of the pathogens were determined. It can be observed that the total lipids of the *Candida* strains had a higher unsaturation index compared to any of the bacterial strains (Figure 6). The higher unsaturation index of the *C. albicans* lipids (Figure 6), can be ascribed to higher levels of 16:1, 18:1 and 18:2 than *E. coli* OP50 (Figure 7). However, these differences were not translated to the fatty acid composition of the nematodes infected with *C. albicans* strains alone (Figure 4A).

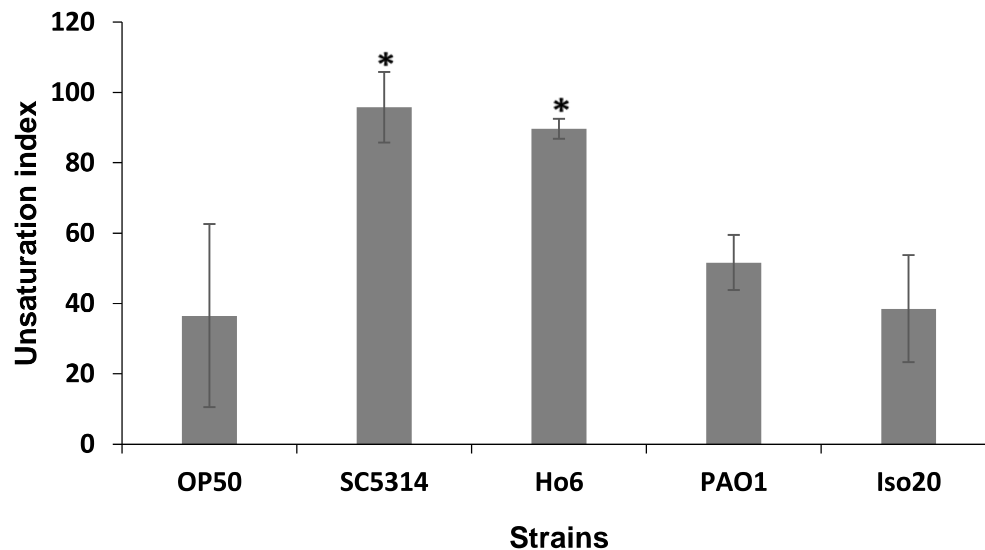


Figure 6. Unsaturation index of major unsubstituted LCFAs in *C. albicans* and *P. aeruginosa* compared to *E. coli*. Values represent the mean of three independent experiments and error bars represent the standard deviations and asterisk (*) indicate a significant difference from *E. coli* OP50 with P values <0.05; Student's *t*-test.

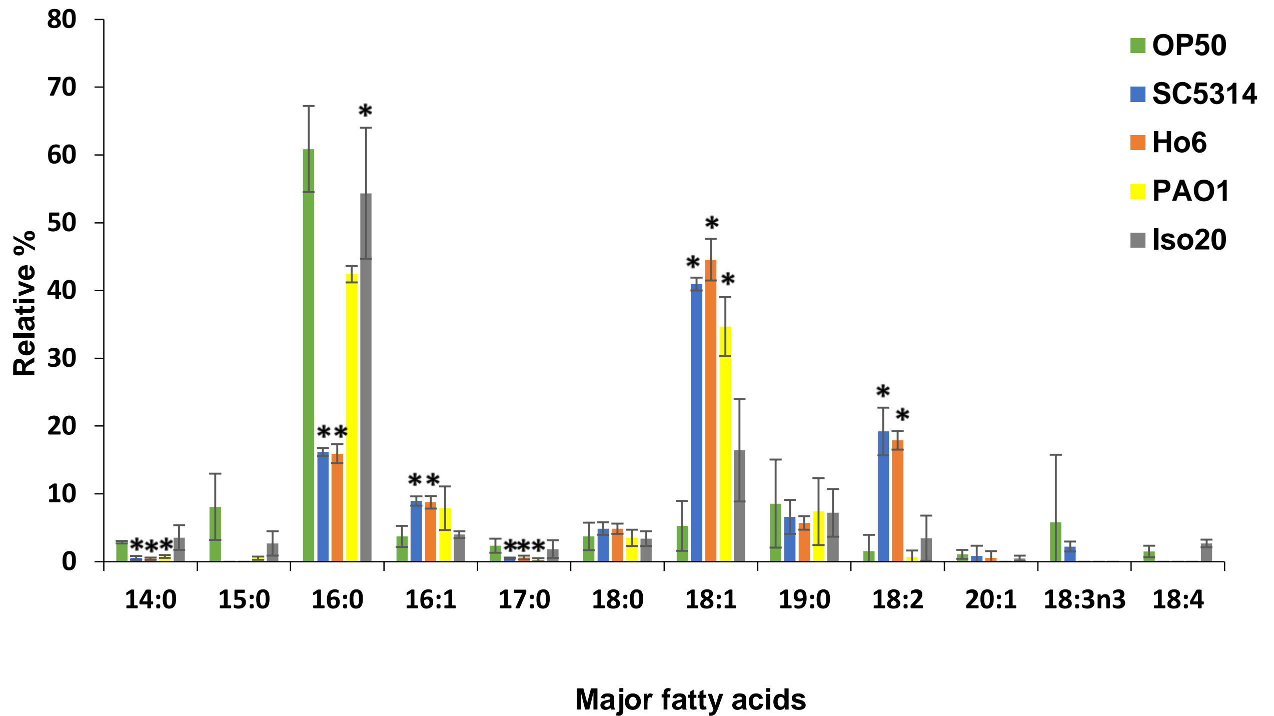


Figure 7. Relative percentage of major long chain fatty acids in *C. albicans* and *P. aeruginosa*, as determined by gas chromatography analysis. Values represent the mean of three independent experiments and error bars represent the standard deviation and asterisk (*) indicate a significant difference from *E. coli* OP50 with P values <0.05.

4.4.2 Prostaglandin E_2

Prostaglandins are an important class of eicosanoids, which are lipid signals derived from polyunsaturated fatty acids (PUFAs) (Funk, 2001). In mammalian cells, prostaglandin E_2 (PGE_2) is derived from metabolism of 20:4n6 by the enzymatic activity of cyclooxygenase (COX-1 or COX-2), which catalyse the rate-limiting step in mammalian prostaglandin synthesis, to firstly produce prostaglandin H_2 (PGH_2), followed by PGE synthase enzymatic activity to produce PGE_2 (Murakami *et al.*, 2003). It is difficult to study these PGE_2 signalling molecules due to their low abundance and reactive nature. Numerous research have assessed the role of several invading pathogenic microorganism specifically on the production of PGE_2 in order to fully understand immunological traits of infection. As we know that *C. albicans* and *P. aeruginosa* are frequently found together at infection sites, having both the ability to form biofilms, resistant to therapeutic intervention. During infection, *C. albicans* and

P. aeruginosa triggers the host cells to release 20:4n6 (Castro *et al*., 1994; Saliba *et al*., 2005). Particularly, during *C. albicans* infection, the release of 20:4n6 is significantly induced by cell wall components as well as phospholipases (such as yeast-derived phospholipase A_2) produced by *C. albicans*, this leads to the increased eicosanoids production, with the most important one being PGE_2, by the host (Castro *et al*., 1994; Filler *et al*., 1994; Brash, 2001; Niewerth and Korting, 2001; Theiss *et al*., 2006; Parti *et al*., 2010). On the other hand, pulmonary infection of *P. aeruginosa* is correlated with PGE_2 overproduction and concurrent decrease in phagocytosis by alveolar macrophages (Ballinger *et al*., 2006; Agard *et al*., 2013).This increase in PGE_2 is due to the large amount of 20:4n6 released during *P. aeruginosa* infection, mediated by ExoU, an intracellular phospholipase (König *et al*., 1996; Saliba *et al*., 2005; Sadikot *et al*., 2007; Agard *et al*., 2013). As such it plays a crucial role in initial infection and infiltration by *P. aeruginosa.* Interestingly, according to the *in vitro* results by Fourie and co-workers (2016), *C. albicans* and *P. aeruginosa* can produce15-hydroxyeicosatetraenoic acid (15-HETE), PGE_2 and $PGF_{2\alpha}$ from exogenous 20:4n6. In their study, a significant increase in eicosanoid production was observed by polymicrobial biofilms compared to monomicrobial biofilms (Fourie *et al*., 2016). At present we have very limited insight concerning the effect of PGE_2 produced by microbes, particularly on the polymicrobial species and also on host. Thus *C. elegans* will enable us to effectively investigate the influence of infection on PGE_2.

In our present study no authentic PGE_2 was detected in all lysates of nematodes infected with monomicrobial *C. albicans* or *P. aeruginosa*. Interestingly, low levels of PGE_2 was detected in the nematode lysates of polymicrobial infections of *C. albicans* SC5314 and *P. aeruginosa* Iso20 (Figure 8), however not in the less virulent combination of *C. albicans* Ho6 and *P. aeruginosa* PAO1.

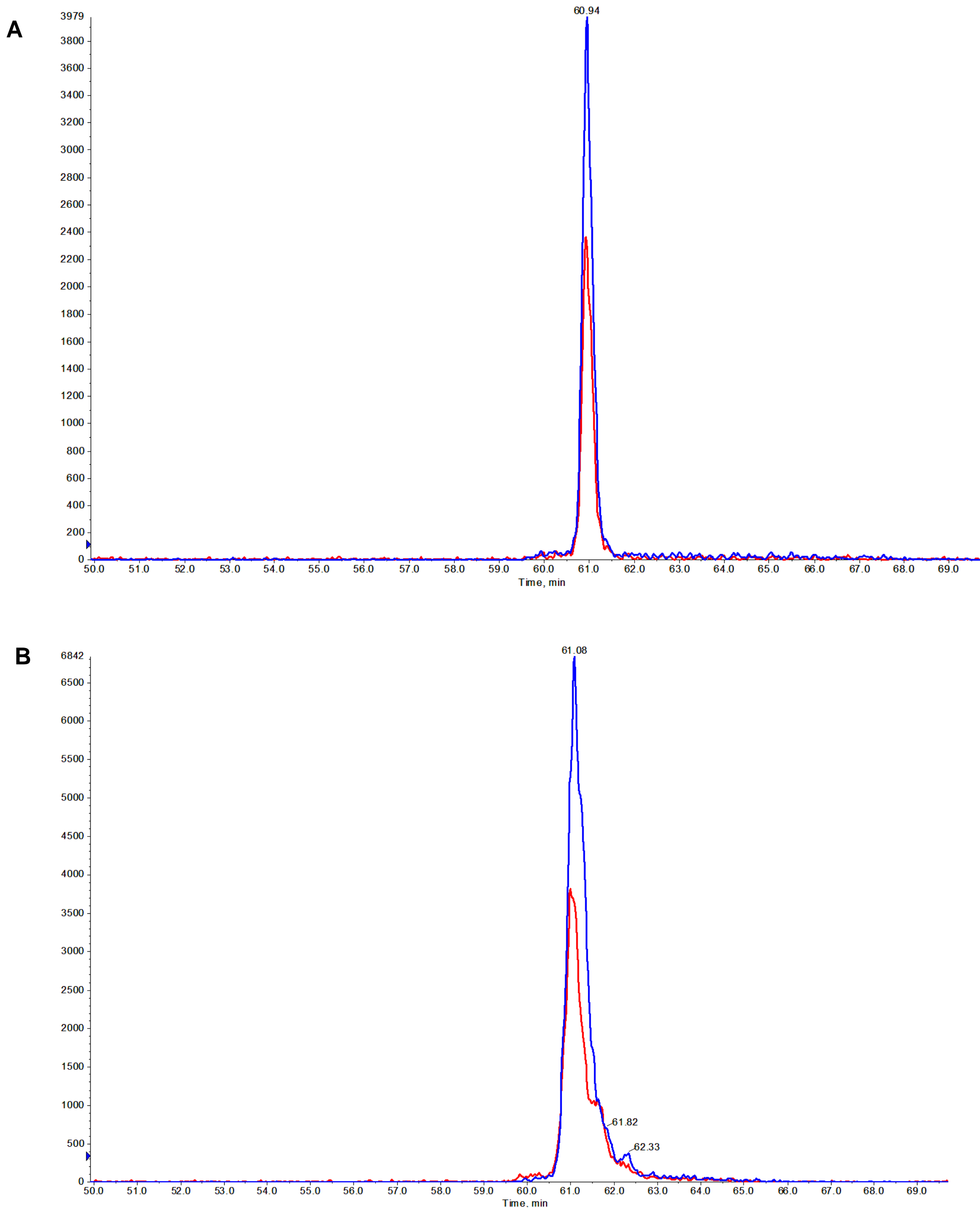


Figure 8. LC-MS/MS spectra of prostaglandin E_2 (PGE_2). (A) PGE_2 standard. (B) PGE_2 from lysate of nematodes infected with both *C. albicans* SC5314 and *P. aeruginosa* Iso20. Although all five diagnostic transitions were present, only two are shown: blue = 351.17 > 271.2; red = 351.17 > 189.0 corresponding to PGE_2 for each sample indicated by arrows.

This may appear to contradict the results of *in vitro* studies by Fourie *et al.* (2016), where authentic PGE_2 was detected in intracellular extracts of *C. albicans* monomicrobial biofilms and also polymicrobial biofilms of both *C. albicans* and *P. aeruginosa*. However, it must be noted that these biofilms were grown in the presence of free 20:4n6.

Literature states that PGE_2 production in response to *P. aeruginosa* infection depends on the release of $cPLA_{2\alpha}$-mediated 20:4n6 (Hurley *et al.*, 2011), moreover, ExoU-expression in *P. aeruginosa* is associated with further PGE_2 production increase in numerous cell lines (Saliba *et al.*, 2005; Agard *et al.*, 2013). Furthermore, the absence of ExoU in *P. aeruginosa* is linked to diminished PGE_2 production and severity of infection (Agard *et al.*, 2013). In our *in vitro* studies from chapter 3, we investigated production of extracellular hydrolytic enzymes, specifically phospholipases, of both *P. aeruginosa* PAO1 and Iso20, however, no secretion of extracellular phospholipases was observed in both strains. This is no surprise since literature clearly states that *P. aeruginosa* PAO1 strain does not naturally express ExoU. This was supported by studies reported by Pazos and co-workers (2017), stating that *P. aeruginosa* cytotoxin ExoU, exhibits phospholipase A_2 (PLA_2) activity in eukaryotic hosts, an enzyme important for production of certain eicosanoids. Using *in vitro* and *in vivo* models of neutrophil transepithelial migration, they investigated the role of ExoU expression on eicosanoid production and function. Infection with PAO1 + ExoU and also PA14 was associated with a significant increase in PGE_2 production compared to infection with their ExoU-negative controls. These results from Pazos and co-workers (2017) support existing literature that states that ExoU functions as a PLA_2 enzyme in mammalian cells with the ability to directly participate in the production of eicosanoids. Thus for future research it will be important to closely investigate secretion of intracellular hydrolytic enzymes particularly phospholipase, ExoU, in *P. aeruginosa* strains since it functions in production levels of PGE_2.

Despite the fact that we did not observe production of PGE_2 in monomicrobial infections by *C. albicans*, previous studies have shown that *C. albicans* can metabolize 20:4n6, liberated from yeast-derived phospholipase A_2 as well as host cells (Castro *et al.*, 1994; Filler *et al.*, 1994; Brash, 2001; Niewerth and Korting, 2001; Theiss *et al.*, 2006; Parti *et al.*, 2010). Additionally, this fungus can induce mammalian cells to produce significant amounts of PGE_2 (Filler *et al.*, 1994; Deva *et al.*, 2001). This apparent discrepancy in results can be explained by the low microbial burden observed for the *C. albicans* monomicrobial infection as well as for the co-infection with the least virulent combination, in contrast to the most virulent combination, observed in chapter 3. Thus indicating that the lack of detection of PGE_2 in these combinations is a factor of microbial burden in the nematodes resulting in low numbers of PGE_2 producing pathogens in the nematodes.

4.5 Conclusions

Discoveries made using model organisms have had significant impact on understanding human pathogenesis. For instance, research on *C. elegans* model has been important for the elucidation of infection process underlying the death of the nematode that occur during infection by various pathogens. This work extends on the studies done on influence of infection process on lipid metabolism, specifically fatty acid production and PGE_2. We observed changes in fatty acid profile being strain dependent, however there is no clear correlation between fatty acid production and virulence. According to literature, C20 PUFAs can be converted into eicosanoids, namely prostaglandins, leukotrienes and thromboxanes, which mediate fever, inflammations, vasodilation, blood pressure, clotting, pain, neurotransmission and modulation of cholesterol metabolism (Funk, 2001). Importantly, the type of eicosanoids produced depends on multiple factors including the kind of cell stimulated and the exact levels of PUFAs substrate in the cell membrane (Funk, 2001). It is believed that prostaglandin synthesis by *C. elegans* does not involve conventional cyclooxygenase pathways, yet $PGF_{1\alpha}$ and $PGF_{2\alpha}$ stereoisomers are still generated with no PGE_2 (Kubagawa *et al.*, 2006; Edmonds *et al.*, 2010; Hoang *et al.*, 2013). Thus the *C. elegans* model could provide unanticipated insight into the mechanisms and actions of prostaglandins, which are among the most widespread and important signalling molecules in mammals. In our present study we observed low levels of PGE_2 production only in the most virulent polymicrobial interaction. However for future research an increase in number of nematodes is required in order to extract more PGE_2 from infected nematodes. It will also be important to closely investigate secretion of intracellular hydrolytic enzymes particularly phospholipase, ExoU, in *P. aeruginosa* strains since it plays crucial role in production levels of PGE_2. Also future studies are required to evaluate if the activity of PUFA is subject to being metabolized into eicosanoids or other signalling molecules, as well as the identification of specific signal transduction pathways that are affected by PUFA composition. This present study also warrants future study into the regulatory mechanisms connecting fatty acid flux and virulence.

4.6 References

Agard, M., Asakrah, S. & Morici, L. A. (2013). PGE_2 suppression of innate immunity during mucosal bacterial infection. *Front Cell Infect Microbiol* **3**, doi: 10.3389/fcimb.2013.00045.

Ashrafi, K., Chang, F. Y., Watts, J. L., Fraser, A. G., Kamath, R. S. *et al.* (2003). Genomewide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421**, 268–272.

Ballinger, M. N., Aronoff, D. M., McMillan, T. R., Cooke, K. R., Olkiewicz, K., Toews, G. B., Peters-Golden, M. & Moore, B. B. (2006). Critical role of prostaglandin E_2 overproduction in impaired pulmonary host response following bone marrow transplantation. *J Immunol* **177**, 5499–5508.

Bragonzi, A., Paroni, M., Nonis, A., Cramer, N., Montanari, S., Rejman, J., Di Serio, C., Döring, G. & Tümmler, B. (2009). *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am J Respir Crit Care Med* **180**, 138-145.

Brash, A. R. (2001). Arachidonic acid as a bioactive molecule. *J Clin Investig* **107**, 1339–1345.

Breger, J., Fuchs, B. B., Aperis, G., Moy, T. I., Ausubel, F. M. & Mylonakis. E. (2007). Antifungal chemical compounds identified using a *Caenorhabditis elegans* pathogenicity assay. *PLoS Pathog* **3,** e18.

Brooks, K. K., Liang, B. & Watts, J .L. (2009). The influence of bacterial diet on fat storage in *C. elegans*. *PLoS One* **4**, 19.

Brose, S. A., Thuen, B. T. & Golovko, M. Y. (2011). LC/MS/MS method for analysis of E_2 series prostaglandins and isoprostanes. *J Lipid Res* **52**, doi: 10.1194/jlr.DOI3441.

Castro, M., Ralston, N. V. C., Morgenthaler, T. I., Rohrbach, M. S. & Limper, A. H. (1994). *Candida albicans* stimulates arachidonic acid liberation from alveolar macrophages through alpha-mannan and beta-glucan cell wall components. *Infect Immun* **62**, 3138–3145.

Corsi, A. K., Wightman, B. & Chalfie, M. (2015). A transparent window into biology: A primer on *Caenorhabditis elegans*. *Genetics* **200**, 387–407.

Dennis, E. A. & Norris, P. C. (2015). Eicosanoid storm in infection and inflammation. *Nat Rev Immunol* **15**, 511-523.

Deva, R., Ciccoli, R., Kock, L. & Nigam, S. (2001). Involvement of aspirin-sensitive oxylipins in vulvovaginal candidiasis. *FEMS Microbiol Lett* **198**, 37–43.

Deva, R., Ciccoli, R., Schewe, T., Kock, J. L. F. & Nigam, S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochim Biophys Acta* **1486**, 299–311.

Diaz, M. T., Alvarez, I., De La Fuente, J., Sanudo, C., Campo, M. M., Oliver, M. A., Fonti Furnols, M., Montossi, F., San Julian, R., Nute, G. R. & Caneque, V. (2005). Fatty acid composition of meat from typical lamb production systems of Spain, United Kingdom, Germany and Uruguay. *Meat Science* **71**, 256–263.

Diaz, P. I., Strausbaugh, L. D. & Dongari-Bagtzoglou, A. (2014). Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. *Front Cell Infect Microbiol* **4**, doi: 10.3389/fcimb.2014.00101.

Edmonds, J. W., Prasain, J. K., Dorand, D., Yang, Y., Hoang, H. D., Vibbert, J., Kubagawa, H. M. & Miller, M. A. (2010). Insulin/FOXO signaling regulates ovarian prostaglandins critical for reproduction. *Dev Cell* **19**, 858–871.

Ells, R., Kemp, G., Albertyn, J., Kock, J. L. F. & Pohl, C. H. (2013). Phenothiazine is a potent inhibitor of prostaglandin E_2 production by *Candida albicans* biofilms. *FEMS Yeast Res* **13**, 849-855.

Erb-Downward J. R. & Huffnagle G. B. (2006). Role of oxylipins and other lipid mediators in fungal pathogenesis. *Future Microbiol* **1**, 219–227.

Erb-Downward, J. R. & Noverr, M. C. (2007). Characterization of prostaglandin E_2 production by *Candida albicans*. *Infect Immun* **75**, 3498–505.

Filler, S. G., Ibe, B. O., Ibrahim, A. S., Ghannoum, M. A., Rai, J. U. & Edwards, J. E. (1994). Mechanisms by which *Candida albicans* induces endothelial cell prostaglandin synthesis. *Infect Immun* **62**, 1064–1069.

Folch, J., Lees, M. & Stanley, G. H. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol Chem* **226**, 497-509.

Fourie, R., Ells, R., Swart, C. W., Sebolai, O. M., Albertyn, J. & Pohl, C. H. (2016). *Candida albicans* and *Pseudomonas aeruginosa* interaction, with focus on the role of eicosanoids. *Front Physiol* **7**, 64.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoids biology. *Science* **294**, 1871-1875.

Henry, P., Owopetu, O., Adisa, D., Nguyen, T., Anthony, K., Ijoni-Animadu, D. *et al.* (2016). Fatty acids composition of *Caenorhabditis elegans* using accurate mass GCMS-QTOF. *J Environ Sci Health B* **51**, 546–552.

Hoang, H. D., Prasain, J. K., Dorand, D. & Miller, M. A. (2013). A heterogeneous mixture of F-series prostaglandins promotes sperm guidance in the *Caenorhabditis elegans* reproductive tract. *PLoS Genet* **9**, e1003271.

Hur, S. J., Ye, B. W., Lee, J. L., Ha., Y. L., Park, G. B. & Joo, S. T. (2004). Effects of conjugated linoleic acid on color and lipid oxidation of beef patties during cold storage. *Meat Science* **66**, 771-775.

Hurley, B. P., Pirzai, W., Mumy, K. L., Gronert, K., McCormick, B. A. (2011). Selective eicosanoid-generating capacity of cytoplasmic phospholipase A_2 in *Pseudomonas aeruginosa*-infected epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **300**, 286–294.

Hutzell, P. A. & Krusberg, L. R. (1982). Fatty Acid Composition of *Caenorhabditis elegans* and *C. briggsae. Comp Biochem Physiol* **73**B, 517-520.

Jenkins, W. R. (1964). A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Dis Rep* **48**, 692.

Kniazeva, M., Crawford, Q, T., Seiber, M., Wang, C. Y., Han, M. (2004). Monomethyl branched-chain fatty acids play an essential role in *Caenorhabditis elegans* development. *PLoS Biol* **2**, e257.

König, B., Jaeger, K. E., Sage, A. E., Vasil, M. L. & König, W. (1996). Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect Immun* **64**, 3252-3258.

Kubagawa, H. M., Watts, J. L., Corrigan, C., Edmonds, J. W., Sztul, E. *et al.* (2006). Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment *in vivo*. *Nat Cell Biol* **8**, 1143–48.

Lamacka, M. & Sajbidor, J. (1995). The occurrence of prostaglandins and related compounds in lower organisms. *Prostaglandins Leukot Essent Fatty Acids* **52**, 357–364.

Lindsay, A. K. & Hogan, D. A. (2014). *Candida albicans*: molecular interactions with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Fungal Biol Rev* **28**, 85-96.

Murakami, M., Nakashima, K., Kamei, D., Masuda, S., Ishikawa, Y., Ishii, T., Ohmiya, Y., Watanabe, K. & Kudo, I. (2003). Cellular prostaglandin E_2 production by membrane-bound prostaglandin E synthase-2 via both cyclooxygenases-1 and -2. *J Biol Chem* **278**, 37937–37947.

Nandakumar, M. & Tan, M. W (2008). Gamma-linolenic and stearidonic acids are required for basal immunity in *Caenorhabditis elegans* through their effects on p38 MAP kinase activity. *PLoS Genet* **4** (11), e1000273.

Niewerth, M. & Korting, H. C. (2001). Phospholipases of *Candida albicans. Mycoses* **44**, 361-367.

Noverr, M. C., Erb-Downward, J. R. & Huffnagle. G. B. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev* **16**, 517–533.

Parti, R. P., Loper, R., Brown, G. D., Gordon, S., Taylor, P. R., Bonventre, J. V., Murphy, R. C., Williams, D. L. & Leslie, C. C. (2010). Cytosolic phospholipase A_2 activation by *Candida albicans* in alveolar macrophages: Role of dectin-1. *Am J Respir Cell Mol Biol* **42**, 415–423.

Pazos, M. A., Lanter, B. B., Yonker, L. M., Eaton, A. D., Pirzai, W., Gronert, K. *et al.* (2017). *Pseudomonas aeruginosa* ExoU augments neutrophil transepithelial migration. *PLoS Pathog* **13** (8), e1006548.

Rhome, R. & Del Poeta, M. (2009). Lipid signaling in pathogenic fungi. *Annu Rev Microbiol* **63**, 119–131.

Sadikot, R. T., Zeng, H., Azim, A. C., Joo, M., Dey, S. K., Breyer, R. M., Peebles, R. S., Blackwell, T. S. & Christman, J. W. (2007). Bacterial clearance of *Pseudomonas aeruginosa* is enhanced by the inhibition of COX-2. *Eur J Immunol* **37**, 1001–1009.

Saliba, A. M., Nascimento, D. O., Silva, M. C. A., Assis, M. C., Gayer, C. R. M., Raymond, B., Coelho, M. G. P., Marques, E. A., Touqui, L. *et al.* (2005). Eicosanoid-mediated proinflammatory activity of *Pseudomonas aeruginosa* ExoU. *Cell Microbiol* **7**, 1811–1822.

Shea, J. M., Kechichian, T. B., Luberto, C. & Del Poeta, M. (2006). The cryptococcal enzyme inositol phosphosphingolipid-phospholipase C confers resistance to the antifungal effects of macrophages and promotes fungal dissemination to the central nervous system. *Infect Immun* **74**, 5977–5988.

Slover, H. T. & Ianza, E. (1979). Quantitative analysis of food fatty acid by capillary gas chromatography. *J Am Oil Chem Soc* **112**, 1032-1040.

Theiss, S., Ishdorj, G., Brenot, A., Kretschmar, M., Lan, C. -Y., Nichterlein, T., Hacker, J., Nigam, S., Agabian, N. & Kohler, G. A. (2006). Inactivation of the phospholipase B gene PLB5 in wild-type *Candida albicans* reduces cell-associated phospholipase A_2 activity and attenuates virulence. *Int J Med Microbiol* **296**, 405–420.

Van Gilst M. R., Hadjivassiliou, H., Jolly, A. & Yamamoto, K. R. (2005). Nuclear hormone receptor NHR-49 controls fat consumption and fatty acid composition in *C. elegans*. *PLoS Biol* **3**, e53.

Vance, R. E., Hong, S., Gronert, K., Serhan, C. N. & Mekalanos, J. J. (2004). The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. *Proc Natl Acad Sci U S A* **101**, 2135–2139.

Wallis, J. G., Watts, J. L. & Browse, J. (2002). Polyunsaturated fatty acid synthesis: what will they think of next? *Trends Biochem Sci* **27**, 467.

Watts, J. L. (2008). Fat synthesis and adiposity regulation in *Caenorhabditis elegans*. *Trends Endocrinol Metab* **20**, 58–65.

Watts, J. L. (2016). Using *Caenorhabditis elegans* to uncover conserved functions of omega-3 and omega-6 Fatty Acids. *J Clin Med* **5**, e19.

Watts, J. L. & Browse, J. (2002). Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **99**, 5854–5859.

Zhu, H. & Han. M. (2014). Exploring developmental and physiological functions of fatty acid and lipid variants through worm and fly genetics. *Annu Rev Genet* **48**, 119–148.

General discussions and conclusions

5.1 *C. elegans* as infection model for *C. albicans* and *P. aeruginosa*

Non-mammalian infection models such as the nematode *Caenorhabditis elegans* have been remarkably useful in the study of host-pathogen interactions (Ewbank, 2002; Mylonakis and Aballay, 2005). Experimental advantages of the *C. elegans* model include simple growth conditions, rapid reproductive cycle, relative low cost, transparent body, and no ethical considerations (Breger *et al*., 2007; Everman *et al*., 2015). Moreover, depending on the temperature conditions, the nematode has been shown to develop at different rates, for instance it shows rapid life cycle at higher temperature than at lower temperatures (Brenner, 1974; Porta-de-la-Riva *et al*., 2012). For instance, the life cycle takes about 90 hours at 15 °C, while about 45 hours when grown at 25 °C (Porta-de-la-Riva *et al*., 2012). Therefore in our present study we firstly determined the duration of the different stages at 15 °C in order to conduct further experiments. Our results confirmed that the life cycle of *C. elegans* takes about 96 hours at 15 °C.

Thereafter we developed *C. elegans* infection model to study pathogenicity of the fungus *Candida albicans* and Gram-negative bacterium *Pseudomonas aeruginosa*. Both *C. albicans* and *P. aeruginosa* are found as commensals in most of the same niches of the human body. However, both are opportunistic pathogens and have frequently been found to be constituents of polymicrobial infections (Richard *et al*., 1999; Rosenthal *et al*., 2006). Despite all these shared features, there is relatively little insight based on how these pathogens affect each other in a biologically significant manner. Thus the main objective was to use *C. elegans* model to study the polymicrobial interaction of *C. albicans* and *P. aeruginosa*. Firstly we developed a monomicrobial infection model using reference and clinical strains of either *C. albicans* or *P. aeruginosa*. Our monomicrobial infection results demonstrated that with either pathogens, the reference strains were more virulent than the clinical strains. In contrast, when we established the *C. elegans* polymicrobial infection model with both pathogens we discovered that combination of reference and clinical strains of *C. albicans* and *P. aeruginosa* had a positive impact on each other’s virulence leading to extremely high mortality of *C. elegans*. Thus this study highlights potentially that polymicrobial interactions show more pathogenicity to *C. elegans* than monomicrobial species. We also evaluated the consequences of the observed *C. albicans*-*P. aeruginosa* interaction on the pathogenicity of *C. albicans* toward *C. elegans*. As it is considered that *C. albicans* hyphal morphogenesis has been shown to be a major virulence determinant mechanism to kill the nematode (Breger *et al*., 2007; Pukkila-Worley *et al*., 2009). However, in our present work, we showed that hyphal formation of *C. albicans* is not required virulence determinant to efficiently kill the nematode, since none of our *C. albicans* strains showed any hyphal formation.

This results showing a synergistic relationship between the two pathogens was unexpected since previous research described mostly antagonistic relationship between *C. albicans* and other pathogenic bacteria in *C. elegans* model (Peleg *et al.*, 2008; Boon *et al.*, 2008; Tampakakis *et al.*, 2009; Cruz *et al.*, 2013). For instance, Peleg *et al.* (2008) described an antagonistic relationship between *Acinetobacter baumannii* and *C. albicans*, where *A. baumannii* inhibits some of the virulence factors of *C. albicans*, including hyphal formation and biofilm formation. This *C. albicans-A. baumannii* interactions resulted in reduced virulence of *C. albicans*, as well as reduced nematode lethality. Moreover, the *C. elegans* was used to investigate the virulence of *Salmonella Typhimurium* and *C. albicans* interaction, and it was observed that this pathogens cause a persistent and lethal gut infection in *C. elegans* (Tampakakis *et al.*, 2009). Likewise with other Gram-negative pathogens, *S. Typhimurium* antagonized *C. albicans* by inhibiting its growth as well as the hyphal formation and biofilm formation (Tampakakis *et al.*, 2009). Furthermore, Boon and colleagues (2008) identified another antagonistic relationship in a cross-kingdom interaction between *C. albicans* and *Burkholderia cenocepacia*, mediated by a secreted diffusible signal factor molecule found in *B. cenocepacia*. Additionally, this *B. cenocepacia* signalling molecule share similar structures with a quorum-sensing molecule, thus able to inhibit formation germ tube and hyphae in *C. albicans* (Boon *et al.*, 2008). Antagonistic relationship was further confirmed by Cruz *et al.* (2013), where *C. albicans* and *Enterococcus faecalis* co-infection drastically reduced killing of *C. elegans*. Moreover, co-infection of *E. faecalis* and *C. albicans* caused lesser tissue damage to nematodes compared to single species infections, furthermore, it also prevented *C. albicans* hyphal formation as well as *E. faecalis* cell death (Cruz *et al.*, 2013).

However research showed that when using other infection models, such as rats and mice, a synergistic relationship was observed between *C. albicans* and other pathogenic bacterium (Carlson, 1983b; Roux *et al.*, 2009; Tan *et al.*, 2016). Roux and co-workers (2009) performed their infection studies using immunocompetent rats to investigate the relationship between the colonization of *C. albicans* and occurrence of *P. aeruginosa* pneumonia. Unlikely, the *in vitro* observations by Hogan and Kolter (2002) that showed that after dense biofilm formation of *P. aeruginosa*, this bacterium can freely attach to *C. albicans* hyphae and kill this fungus. The *in vivo* results by Roux and colleagues (2009) clearly indicated that *C. albicans* might have favoured growth of *P. aeruginosa*, since the existence of *C. albicans* significantly increases the occurrence of *P. aeruginosa* pneumonia. Moreover they rarely observed any *C. albicans* hyphal formation in rat lungs (Roux *et al.*, 2009). Tan and co-workers (2016), further extended the findings by Roux and co-workers (2009) in rat model, by evaluating *C. albicans-A. baumannii* co-infection in rats. Their results showed that colonization of *C. albicans* in the respiratory tract of rats assisted in the subsequent development of severe *A. baumannii*

infection (Tan *et al.*, 2016). In addition, more studies were described in mice models, evaluating the virulence of co-infection of *Staphylococcus aureus* and *C. albicans* (Carlson, 1983b). In the monomicrobial infection of *S. aureus* alone, after intraperitoneally inoculation, no cells could be discovered in peripheral sites of either the pancreas, spleen, or blood, and the mice was able to survive (Carlson, 1983b). However, administration of a similar dose of *S. aureus* coupled with a sublethal dose of *C. albicans* or with heat-inactivated *C. albicans*, caused *S. aureus* to be recovered in all samples for abdominal organs and blood of the mice, and majority of these mice died (Carlson, 1983b). Similar results were observed for Gram-positive bacterium, *Enterococcus faecalis* and for the other Gram-negative bacterium *Serratia marcescens* (Carlson, 1983a). Recently, Bergeron and co-workers (2017), discovered that the co-infection of *C. albicans* and *P. aeruginosa* in the mucosa results to synergistic virulence and enhanced mortality when using Zebrafish swim bladder model. However, it was observed that monomicrobial infection of each species causes little mortality, compared to *C. albicans* and *P. aeruginosa* polymicrobial infection. Moreover, the polymicrobial infection mortality seemed to be more correlated to *C. albicans* invasive pathogenesis and higher microbial burden, which serve as strong virulence determinants for mortality. Moreover, during the polymicrobial infection there are several important aspects of the proinflammatory immune response that are also enhanced. All these data indicated that *P. aeruginosa* augments *C. albicans* pathogenesis and host inflammation, which may contribute to a synergistic virulence associated with mucosal *C. albicans* and *P. aeruginosa* co-infection (Bergeron *et al.*, 2017).

The synergistic virulence and enhanced hyphal invasion of *C. albicans* in polymicrobial infections were unexpected based on known antagonistic interactions of the two microbial species *in vitro* (Hogan and Kolter, 2002; Rinzan, 2009; Shirtliff *et al.*, 2009; Méar *et al.*, 2013; Lindsay and Hogan, 2014; Xu *et al.*, 2014; Fourie *et al.*, 2016). However, we further observe that the enhanced virulence is consistent with most mouse *C. albicans*-bacterial polymicrobial infection models, including organotypic models, which result in enhanced virulence, cytokine production, and/or fungal invasion (Villar *et al.*, 2005; Xu *et al.*, 2014; Nash *et al.*, 2014, 2015; Diaz *et al.*, 2014; Schlecht *et al.*, 2015). The fact that the same strains of *C. albicans* and *P. aeruginosa* were used in these *in vivo* experiments as have been used *in vitro* suggests that the switch from antagonism to synergy is due to the host environment. Many environmental factors are both different and dynamic between *in vitro* and *in vivo* conditions, including microbe-substrate interactions, nutrient levels, immune pressure, secreted products from the host, cell-cell interactions, and biophysical effects of the three dimensional environment (Peleg *et al.*, 2008).

Together, these findings expand the use of *C. elegans* in the study of polymicrobial pathogenesis and provides further evidence of the likely importance of fungi-bacteria

interactions. The evolution of symbiotic, synergistic or antagonistic interactions between diverse organisms in nature or the clinical environment, especially those between prokaryotes and eukaryotes, is likely important for their pathogenesis toward a range of hosts, including humans. The exploitation of the likely evolutionary defence mechanisms used by competing microbes may provide critical insights into novel therapeutic targets, which are desperately needed for pathogens, such as, *C. albicans* and *P. aeruginosa*. Thus, we sought to expand the use of *C. elegans* infection models in order to identify chemical compounds with antifungal activity against the most common human pathogenic fungus, *C. albicans*. Also the search for novel antibacterial therapeutics against bacteria, such as *P. aeruginosa*, greatly depends on representative infection models that mimic pathogenesis in humans and that can be used in a high-throughput fashion.

5.2 Investigating the possible cause for observed differences in virulence

In addition to the ample evidence supporting the polymicrobial interaction of *C. albicans* and *P. aeruginosa*, not only with their host, but also with each other, it is evident that the interaction is multifaceted. In fungi, several virulence factors including secreted factors (with lipid mediators included) and morphogenesis, can affect and cause damage to hosts, in order to establish aggressive and rapid colonization of fungi, leading to infection. While with bacteria, it is important to elucidate the virulence mechanisms that defeat host defences and establish infection. Majority of the pathogenic bacteria are thought to have experienced stepwise additions of virulence factors, as they evolved to survive differently. Furthermore, the survival of bacteria in the host during the early stages of infection is highly associated with secretion of toxins and virulence factors, such as pyoverdine, pyocyanin, proteases (elastase and alkaline protease), and also phospholipases (exotoxin A and phospholipase C) (Sadikot *et al.*, 2005; Rada and Leto, 2013; van 't Wout *et al.*, 2015; Dzvova *et al.*, 2017). For instance, pyocyanin is one of the important virulence factor characterized by a blue phenazine, which is a redox active toxic to several bacteria and fungi, moreover it can also damage mammalian cells (Hassett *et al.*, 1992). Pyocyanin is able to interfere with critical host defence mechanisms and contributes to the symptoms associated with *Pseudomonas*-associated lung diseases (Denning *et al.*, 1998; Sadikot *et al.*, 2005; van 't Wout *et al.*, 2015). Therefore the aim is to test particular bacterial or fungal factors in pathogenesis *in vitro*, by investigating the most virulence strain in comparison to the least virulent strain of the pathogens obtained from the results of the established *C. elegans* infection model.

In our study, production of pyoverdine from *P. aeruginosa* PAO1 strain was evident on king A and King B agar medium, while *P. aeruginosa* Iso20 strain produced none of this toxins. Furthermore we performed pyocyanin assay and obtained very low pyocyanin production from both strains of *P. aeruginosa*. It appears *P. aeruginosa* Iso20 might be genetically tuned to produce more of pyocyanin than PAO1, yet is least virulent. This could be due to lack of other compounds such as pyoverdine, which might further assist in virulence. According to research, pyocyanin is considered as the most important virulence factor of *P. aeruginosa* (Lau *et al.*, 2004), but our data suggest otherwise. Perhaps a combination of virulence factors then is required for increased virulence, and perhaps this is also circumstantial or host specific. In support of this, PAO1 produces multiple compounds, among which pyoverdine might assist in iron uptake (Cornelis and Dingemans, 2013), while pyocyanin might assist in virulence; thus a combination of these might make it more virulent.

Studies show that *P. aeruginosa* can produce two major siderophores, pyochelin and pyoverdine, as part of the virulence factors. Therefore, this biosynthesis of siderophore and iron acquisition are shown to be the most important requirements for virulence of *P. aeruginosa* in both mammalian and plant infections (Meyer *et al.*, 1996; Takase *et al.*, 2000; Nadal Jimenez *et al.*, 2010). For instance, pyoverdine is important to *P. aeruginosa*, since iron acquisition is necessary for both normal development and pathogenesis (Meyer *et al.*, 1996; Takase *et al.*, 2000). Moreover, the iron acquisition by pyochelin or pyoverdine also facilitated in 'red death' phenomenon discovered in *C. elegans* infected with *P. aeruginosa* PAO1 strain (Zaborin *et al.*, 2009). Moreover, Kirienko and co-workers (2013) showed that pyoverdine is important for *P. aeruginosa*-mediated killing of *C. elegans* since it induces hypoxic (suffocates) response and death as it depend on iron-responsive transcription factor hypoxia-inducible factor 1 (HIF-1). Therefore, this might further explain why PAO1 was more virulent (as it produces pyoverdin), and not Iso20. Moreover, PAO1, might be more virulent due to iron assimilating ability in cases where the host iron is limited (nutritional immunity) (Cassat *et al.*, 2013). Therefore, we will investigate this in our future research in order to determine growth of these isolates on iron supplemented medium, one that is limited in this nutrient, to determine nutrient acquisition contributing to virulence. Since iron is very important in virulence of many species including *P. aeruginosa* (Vasil and Ochsner, 1999; Cassat *et al.*, 2013; Kirienko *et al.*, 2013; Nguyen *et al.*, 2014, 2015).

Another virulence factor investigated is swarming motility, which is known as a rapid and coordinated migration of a bacterial population across a semisolid surface (Henrichsen, 1972). We observed swarming motility by *P. aeruginosa* PAO1 while no swarming motility was observed with Iso20. Taken together, all these observations suggested that there are multiple virulence factors of *P. aeruginosa* that influence virulence, including pyoverdin, pyocyanin and

swarming motility. This may be due to the fact that swarming motility is often co-regulated with toxin secretion (Allison *et al*., 1992; Givskov *et al*., 1995). Since PAO1 seems to secrete pyoverdin toxin this might be the reason why it is able to swarm. Some of the virulence factors that contribute to this processes are the extracellular hydrolytic enzymes (Schaller *et al*., 2005). These secreted extracellular hydrolytic enzymes are implicated to efficiently enhance the extracellular nutrient acquisition (Naglik *et al*., 2003). Next, to more broadly explore the involvement of other potential virulence factors, we performed *in vitro* enzymatic tests for the expression of lipase, phospholipase and protease activities by *P. aeruginosa* PAO1 and Iso20 strains. Both strains were found to be positive for the production of protease and lipase, while no production of phospholipase was observed. According to literature different strains of *P. aeruginosa* are known to secrete other several extracellular proteolytic enzymes, including alkaline protease, protease IV, and also LasA and LasB elastases, which are considered as virulence factors (Caballero *et al*., 2001). These proteases have been discovered to promote replication of *P. aeruginosa* within an infected host by causing interference in the host immune system (Miyoshi *et al*., 2002; Hung *et al*., 2005). For future research, these can be other possible virulence factors of *P. aeruginosa* to consider. When we determined overall virulence of selected strains in the *C. elegans* model, some differences in virulence were observed; two strains (both obtained from sputum) that caused high mortality soon after infection were the most virulent. However, there was no indication that the differences in *in vitro* virulence factors were correlated with virulence. It is possible that virulence factors not considered in our study may contribute to the observed virulence in this infection model and that the assays performed are not adequate to predict virulence in the host (Mayer *et al*., 2013; Ells *et al*., 2014).

5.3 Influence of fatty acids on infection

Although only a limited number of investigations on comprehensive lipid analysis in *C. elegans* are published till recent, much has been achieved. The nematode *C. elegans* as model system allows easy setup of experiments related to fat metabolism and storage, and ample knowledge has been collected over the last years. Lipids do not only play a central role in the biology of nematodes, but also in other model organisms, and are often associated with the separation between intracellular and extracellular environments, energy storage, reproduction and lifespan, and may also serve as signalling molecules. Fatty acids specifically fulfil diverse roles in the cell as they are significant constituents of lipids, they play a central role in energy storage, they can regulate the integrity and dynamics of cell membranes, they can form side chains in co-enzymes and metabolites, and they can also form covalent attachments to

proteins (Schweizer and Hofmann, 2004). *Caenorhabditis elegans* is a biologically established model organism of important need in the study of lipid metabolism (Hanse *et al.*, 2013). In contrast to mammals, *C. elegans* contain Δ-12 and ω-3 desaturase enzymes that facilitate polyunsaturated fatty acids (PUFAs) biosynthesis endogenously (Tanaka *et al.*, 1996). The *C. elegans* unsaturated fatty acid synthesis pathway begins with palmitic acid (16:0), which is synthesized either *de novo* or obtained from the *Escherichia coli* diet, which is then converted by FAT-5 to palmitoleic acid (16:1). This 16:1 fatty acid is then elongated to cis-vaccenic acid (18:1n11), which is the most abundant fatty acid in triglycerides and phospholipids (Tanaka *et al.*, 1996). Palmitic acid (16:0) can also be elongated to stearic acid (18:0), the substrate for FAT-6 and FAT-7 desaturation to oleic acid (18:1n9), which is further desaturated and elongated to form all of the PUFAs, including arachidonic acid (20:4n6) and eicosapentaenoic acid (20:5n3) (Watts and Browse 2002).

Here, the objective was to investigate the influence of infection on fatty acid composition. Thus, we used *C. elegans* infected with either *Candida albicans* or *Pseudomonas aeruginosa* and also with both pathogens, as an experimental system to investigate the interplay between major unsubstituted long chain fatty acids and virulence, in context of whole organism. When the nematode was fed on *E. coli* OP50, twenty-three different fatty acids ranging between 12 to 22 carbons in length, of which 35 % were saturated, while 65 % were unsaturated. We then only focused on major unsubstituted long chain fatty acids (LCFAs) and margaric acid (17:0) was the most dominant saturated fatty acid, with an average of 24 % of total major fatty acids. However the *C. elegans* infected with *C. albicans* and *P. aeruginosa* showed no significant change in this 17:0 fatty acid.

According to literature, fatty acids also function in adherence and virulence, since changes in the biosynthesis of fatty acid decrease the adhesion of *C. albicans* to host epithelial cells (Hoberg *et al.*, 1986). Moreover, the fatty acids composition varies greatly depending on *C. albicans* morphogenesis, with the hyphal forms comprising of more PUFA compared to the yeast form (Ghannoum *et al.*, 1986). However in our studies we clearly proved that *C. albicans* does not transition from yeast to hyphal form thus we cannot compare the fatty acid composition between the *C. albicans* morphologies. Another important study highlighted in literature is the importance of lipids in the studies of pathogenesis, revealing that lipases, which are involved in acquisition of lipids from the environment, are important for the virulence of *C. albicans* (Gacser *et al.*, 2007). However in our *in vitro* studies we did not observe any extracellular lipase secretion from *C. albicans.* Thus we can reason that the virulence of *C. albicans* observed may be due to acquisition of lipids from *C. elegans.*

Using the *C. elegans* infection model, we observed changes in fatty acid profiles of the major unsubstituted long chain fatty acids. However, no correlation was found between the virulence of infection and the change in fatty acids of the nematodes. Given that majority of the pathogens secrete hydrolytic enzymes and toxins that can be harmful to host cells, it is important to also note that membrane permeability can have an effect on the outcome of an infection. It is known that fatty acids fulfil diverse roles in the cell, such as regulation of the dynamics and integrity of cell membranes (Schweizer and Hofmann, 2004). This selective permeability of cell membrane in return, functions in retaining cell integrity and also preventing entry of toxins.

5.4 Influence of prostaglandins on infection

Candida albicans and *Pseudomonas aeruginosa* are two most common human opportunistic pathogens, which frequently co-infect hosts and this co-infection may increase morbidity and mortality in infections compared to monomicrobial infections (Azoulay *et al*., 2006; Roux *et al*., 2009; Morales and Hogan, 2010; Hamet *et al*., 2012; Xu *et al*., 2014). In addition, both these microorganisms elicit the release of a large amount of AA from host cells and the metabolism of AA forms PGE_2 (Castro *et al*., 1994; Filler *et al*., 1994; König *et al*., 1996; Brash, 2001; Saliba *et al*., 2005 Sadikot *et al*., 2007; Agard *et al*., 2013). Despite this, less is relatively known with regards to the dynamics within these microbial communities, even on how these interactions affect human disease in the context of host immunity. Although there has been numerous research in *vitro* further research is needed, especially taking the influence of the host environment into consideration. Therefore, the study aimed to investigate the influence of PGE_2 production on *C. albicans* and *P. aeruginosa* polymicrobial infection compared to the monomicrobial infection *in vivo* using *C. elegans* model. No authentic PGE_2 was detected in all intracellular extracts of nematodes infected with monomicrobial *C. albicans* or *P. aeruginosa*. Interestingly, low levels of PGE_2 was detected after intracellular extraction of polymicrobial of most virulent combinations, SC5314 + Iso20, however no PGE_2 was detected in least virulent combination Ho6 + PAO1.

Our results supported the *in vitro* studies by Fourie and co-workers (2016), which highlighted that the possibility of an increased virulence from *C. albicans* and *P. aeruginosa* polymicrobial interaction resulted in significantly higher production of immunomodulatory eicosanoids. Demonstrating that polymicrobial eicosanoid production may generate drastically different eicosanoid profiles compared to monomicrobial counterparts. Additionally, Peters and Noverr (2013) performed studies on the function of eicosanoid production during *Staphylococcus aureus* and *C. albicans* polymicrobial

infection using a murine model. From their studies, during murine peritoneal infection, it was evident that *S. aureus* and *C. albicans* polymicrobial infection caused a significant increase in not only in morbidity but also in mortality, which was an effect not observed in monomicrobial infection. Moreover, polymicrobial infection led to a significantly higher microbial burden and disease progression in the infected mice, compared to monomicrobial infections (Peters and Noverr, 2013). Interestingly, they demonstrated that the immunomodulatory eicosanoid, PGE_2 is synergistically increased during coinfection compared to monomicrobial infection. Importantly, PGE_2 administration during coinfection also significantly increased both the *C. albicans* and *S. aureus* microbial burden in the kidney and spleen. Moreover, *in vitro* studies by Krause and colleagues (2015) showed that *S. aureus* biofilm growth can be significantly enhanced by *C. albicans* PGE_2 production.

In addition, literature states that *P. aeruginosa* triggers the release of large amounts of AA through an intracellular phospholipase, exotoxin U (ExoU) and the subsequent production of PGE_2 by the host (König *et al*., 1996; Saliba *et al*., 2005; Ballinger *et al*., 2006; Agard *et al*., 2013). It is known that the production of PGE_2 in response to *P. aeruginosa* infection depends on the liberation of $cPLA_{2\alpha}$-mediated AA (Hurley *et al*., 2011), and that ExoU-expression is associated with further increased PGE_2 production in numerous cell lines (Saliba *et al*., 2005). In a case whereby ExoU was absent in *P. aeruginosa*, it led to diminished PGE_2 production and, also the infection became more severe (Agard *et al*., 2013). In our *in vitro* studies from chapter 3, we investigated production of extracellular hydrolytic enzymes, specifically phospholipases, of both *P. aeruginosa* PAO1 and Iso20, however, no secretion of extracellular phospholipases was observed. This is no surprise since literature clearly states that *P. aeruginosa* PAO1 strain does not naturally express ExoU. This was supported by studies reported by Pazos and co-workers (2017), stating that *P. aeruginosa* cytotoxin ExoU, exhibits phospholipase A_2 (PLA_2) activity in eukaryotic hosts, an enzyme important for production of certain eicosanoids. Using *in vitro* and *in vivo* models of neutrophil transepithelial migration, they investigated the role of ExoU expression on eicosanoid production and function. Infection with PAO1 + ExoU and also PA14 was associated with a significant increase in PGE_2 production compared to infection with their ExoU-negative controls. These results from Pazos and co-workers (2017) support existing literature that states that ExoU functions as a PLA_2 enzyme in mammalian cells with the ability to directly participate in the production of eicosanoids. Thus for future research it will be important to closely investigate secretion of intracellular hydrolytic enzymes particularly phospholipase, ExoU, in *P. aeruginosa* strains since it functions in production levels of PGE_2.

In summary, this *C. elegans* infection model offers high-resolution longitudinal analysis as a powerful new tool to disentangle the contributions of the co-infecting *C. albicans* and *P. aeruginosa*. The power of this technique is illustrated by the ability to demonstrate that *P. aeruginosa* induces changes that lead to enhanced invasion by *C. albicans* and death of individual nematodes. The unexpected finding that *P. aeruginosa* has a positive influence on *C. albicans* virulence *in vivo* raises questions about what aspects of infection change this bacterial-fungal cross talk from something antagonistic *in vitro* into a positive interaction *in vivo*. With new clinical studies indicating that fungi aggravate bacterial lung infection, which can be treated with antifungal treatment, the importance of understanding bacterium-fungus host interactions *in vivo* is critical (Kim *et al*., 2015; Shwartz *et al*., 2018; Pendleton *et al*., 2017). Hopefully, new *in vivo* tools and models such as the one described in this study will result in a more complete picture of these bacterium-fungus-host interactions in disease.

5.5 References

Agard, M., Asakrah, S. & Morici, L. A. (2013). PGE_2 suppression of innate immunity during mucosal bacterial infection. *Front Cell Infect Microbiol* **3**, doi: 10.3389/fcimb.2013.00045.

Allison. C., Lai, H. C. & Hughes, C. (1992). Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis. Mol Microbiol* **6**, 1583–1591.

Azoulay, E., Timsit, J. F., Tafflet, M., de Lassence, A., Darmon, M., Zahar, J. R., Adrie, C., Garrouste-Orgeas, M., Cohen, Y. *et al.* (2006). *Candida* colonization of the respiratory tract and subsequent *Pseudomonas* ventilator-associated pneumonia. *Chest* **129**, 110–117.

Ballinger, M. N., Aronoff, D. M., McMillan, T. R., Cooke, K. R., Olkiewicz, K., Toews, G. B., Peters-Golden, M. & Moore, B. B. (2006). Critical role of prostaglandin E_2 overproduction in impaired pulmonary host response following bone marrow transplantation. *J Immunol* **177**, 5499–5508.

Bergeron, A. C., Seman, B. G., Hammond, J. H., Archambault, L. S., Hogan, D. A. & Wheeler, R. T. (2017). *Candida albicans* and *Pseudomonas aeruginosa* interact to enhance virulence of mucosal infection in transparent zebrafish. *Infect Immun* **85**, e00475-17.

Boon, C. Deng, Y., Wang, L. H., He, Y., Xu, J. L., Fan, Y., Pan, S. Q. & Zhang, L. H. (2008). A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *ISME J* **2**, 27–36.

Brash, A. R. (2001). Arachidonic acid as a bioactive molecule. *J Clin Investig* **107**, 1339–1345.

Breger, J., Fuchs, B. B., Aperis, G., Moy, T. I., Ausubel, F. M. & Mylonakis, E. (2007). Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS Pathog* **3**, e18.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Caballero, A. R., Moreau, J. M., Engel, L. S., Marquart, M. E., Hill, J. M. & O'Callaghan, R. J. (2001). *Pseudomonas aeruginosa* protease IV enzyme assays and comparison to other *Pseudomonas proteases*. *Anal Biochem* **290** 330–337.

Carlson, E. (1983a). Enhancement by *Candida albicans* of *Staphylococcus aureus*, *Serratia marcescens*, and *Streptococcus faecalis* in the establishment of infection in mice. *Infect Immun* **39**, 193–197.

Carlson, E. (1983b). Effect of strain of *Staphylococcus aureus* on synergism with *Candida albicans* resulting in mouse mortality and morbidity. *Infect Immun* **42**, 285–292.

Cassat, J. E., Hammer, N. D., Campbell, J. P., Benson, M. A., Perrien, D. S., Mrak, L. N. *et al.* (2013). A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis. *Cell Host Microbe* **13** (6), 759–772.

Castro, M., Ralston, N. V. C., Morgenthaler, T. I., Rohrbach, M. S. & Limper, A. H. (1994). *Candida albicans* stimulates arachidonic acid liberation from alveolar macrophages through alpha-mannan and beta-glucan cell wall components. *Infect Immun* **62**, 3138–3145.

Cornelis P. & Dingemans J. (2013). *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. *Front Cell Infect Microbiol* **3**, 75 10.3389/fcimb.2013.00075.

Cruz, M. R., Graham, C. E., Gagliano, B. C., Lorenz, M. C. & Garsin, D. A. (2013). *Enterococcus faecalis* inhibits hyphal morphogenesis and virulence of *Candida albicans*. *Infect Immun* **81**, 189–200.

Denning. G. M., Railsback, M. A., Rasmussen, G. T., Cox, C. D. & Britigan, B. E. (1998). *Pseudomonas* pyocyanine alters calcium signalling in human airway epithelial cells. *Am J Physiol* **274**, 893-900.

Diaz, P. I., Strausbaugh, L. D. & Dongari-Bagtzoglou, A. (2014). Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. *Front Cell Infect Microbiol* **4**, doi: 10.3389/fcimb.2014.00101.

Dzvova, N., Colmer-Hamood, J. A., Griswold, J. A. & Hamood A. N. (2017). Isolation and characterization of HepP: a virulence-related *Pseudomonas aeruginosa* heparinise. *BMC Microbiol* **17**, 233.

Ells, E., Kilian, W., Hugo, A., Albertyn, J., Kock, J. L. F. & Pohl C. H. (2014). Virulence of South African *Candida albicans* strains isolated from different clinical samples. *Med Mycol* **52**, 246-253.

Everman, J. L., Ziaie, N. R., Bechler, J. & Bermudez, L. E. (2015). Establishing *Caenorhabditis elegans* as a model for *Mycobacterium avium* subspecies *hominissuis* infection and intestinal colonization. *Biol Open* **4** (10), 1330-1335.

Ewbank, J. J (2002). Tackling both sides of the host-pathogen equation with *Caenorhabditis elegans*. *Microbes Infect* **4**, 247–256.

Fourie, R., Ells, R., Swart, C. W., Sebolai, O. M., Albertyn, J. & Pohl, C. H. (2016). *Candida albicans* and *Pseudomonas aeruginosa* interaction, with focus on the role of eicosanoids. *Front Physiol* **7**, 64.

Givskov, M., Eberl, L., Christiansen, G., Benedik, M. J. & Molin, S. (1995). Induction of phospholipase- and flagellar synthesis in *Serratia liquefaciens* is controlled by expression of the flagellar master operon *flhD*. *Mol Microbiol* **15**, 445-454.

Filler, S. G., Ibe, B. O., Ibrahim, A. S., Ghannoum, M. A., Rai, J. U. & Edwards, J. E. (1994). Mechanisms by which *Candida albicans* induces endothelial cell prostaglandin synthesis. *Infect Immun* **62**, 1064–1069.

Hamet, M., Pavon, A., Dalle, F., Pechinot, A., Prin, S., Quenot, J. P. & Charles, P. E. (2012). *Candida* spp. airway colonization could promote antibiotic-resistant bacteria selection in patients with suspected ventilator-associated pneumonia. *Intensive Care Med* **38**, 1272–1279.

Hansen, M., Flatt, T. & Aguilaniu, H. (2013). Reproduction, fat metabolism, and lifespan – what is the connection? *Cell Metab* **17** (1), 10–19.

Hassett, D. J., Charniga, L., Bean, K., Ohman, D. E. & Cohen, M. S. (1992). Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defences, and demonstration of a manganese cofactored superoxide dismutase. *Infect Immun* **60**, 328–336.

Henrichsen, J. (1972). Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36**, 478-503.

Hoberg, K. A., Cihlar, R. L. & Calderone, R. A. (1986). Characterization of cerulenin-resistant mutants of *Candida albicans*. *Infect Immm* **51**, 102-109.

Hogan, D. A. & Kolter, R. (2002). *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* **296**, 2229-2232.

Hung, C. Y., Seshan, K. R., Yu, J. J., Schaller, R., Xue, J., Basrur, V., Gardner, M. J. & Cole, G. T. (2005). A metalloproteinase of *Coccidioides posadasii* contributes to evasion of host detection. *Infect Immun* **73**, 6689–6703.

Hurley, B. P., Pirzai, W., Mumy, K. L., Gronert, K., McCormick, B. A. (2011). Selective eicosanoid-generating capacity of cytoplasmic phospholipase A_2 in *Pseudomonas aeruginosa*-infected epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **300**, 286–294.

Gacser, A., Stehr, F., Kroger, C., Kredics, L., Schafer, W. & Nosanchuk, J. D. (2007). Lipase 8 affects the pathogenesis of *Candida albicans*. *Infect Immun* **75** (10), 4710-4718.

Ghannoum, M. A., Khamis, J. L. & Radwan, S. S. (1986). Dimorphism-associated variations in the lipid composition of *Candida albicans*. *J Gen Microbiol* **132**, 2367–2375.

Kim, S. H., Clark, S. T., Surendra, A., Copeland, J. K., Wang, P. W, Ammar, R., Collins, C., Tullis, D. E, Nislow, C., Hwang, D. M. & Guttman, D. S. L. E. (2015). Global analysis of the fungal microbiome in cystic fibrosis patients reveals loss of function of the transcriptional repressor Nrg1 as a mechanism of pathogen adaptation. *PLoS Pathog* **11**, e1005308.

Kirienko, N. V. Kirienko, D. R., Larkins-Ford, J., Wählby, C., Ruvkun, G. & Ausubel, F. M. (2013). *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans* iron homeostasis, causing a hypoxic response and death. *Cell Host Microbe* **13**, 406–416.

König, B., Jaeger, K. E., Sage, A. E., Vasil, M. L. & König, W. (1996). Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect Immun* **64**, 3252-3258.

Krause, J., Geginat, G. & Tammer, I. (2015). Prostaglandin E_2 from *Candida albicans* stimulates the growth of *Staphylococcus aureus* in mixed biofilms. *PLoS One* **10**, doi: 10.1371/journal.pone.0135404.

Lau, G. W., Hassett, D. J., Ran, H. & Kong, F. (2004). The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* **10**, 599–606.

Lindsay, A. K. & Hogan, D. A. (2014). *Candida albicans*: molecular interactions with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Fungal Biol Rev* **28**, 85-96.

Mayer, F. L., Wilson, D. & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* **4**,119–128.

Méar, J. B., Kipnis, E., Faure, E., Dessein, R., Schurtz, G., Faure, K. *et al.* (2013). *Candida albicans* and *Pseudomonas aeruginosa* interactions: more than an opportunistic criminal association? *Médecine Mal Infect* **43**,146-151.

Meyer, J. M., Neely, A., Stintzi, A., Georges, C. & Holder, I. A. (1996). Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun* **64**, 518–523.

Miyoshi, S., Sonoda, Y., Wakiyama, H., Rahman, M. M., Tomochika, K., Shinoda, S., Yamamoto, S. & Tobe, K. (2002). An exocellular thermolysin-like metalloprotease produced by *Vibrio fluvialis*: purification, characterization, and gene cloning. *Microb Pathog* **33**, 127–134.

Morales, D. K. & Hogan, D. A. (2010). *Candida albicans* interactions with bacteria in the context of human health and disease. *PLoS Pathog* **6**, doi: 10.1371/journal.ppat.1000886.

Mylonakis, E. & Aballay, A. (2005). Worms and flies as genetically tractable animal models to study host-pathogen interactions. *Infect Immun* **73,** 3833–3841.

Nadal Jimenez, P., Koch, G., Papaioannou, E., Wahjudi, M., Krzeslak, J., Coenye, T., Cool, R. H. & Quax, W, J. (2010). Role of PvdQ in *Pseudomonas aeruginosa* virulence under iron–limiting conditions. *Microbiology* **156**, 49–59.

Naglik, J. R., Challacombe, S. J. & Hube, B. (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* **67**, 400-428.

Nash, E. E., Peters, B. M., Fidel, P. L. & Noverr, M. C. (2015). Morphology-independent virulence of candida species during polymicrobial intra-abdominal infections with *Staphylococcus aureus*. *Infect Immun* **84**, 90 –98.

Nash, E. E., Peters, B. M., Palmer, G. E., Fidel, P. L. & Noverr, M. C. (2014). Morphogenesis is not required for *Candida albicans*-*Staphylococcus aureus* intraabdominal infection-mediated dissemination and lethal sepsis. *Infect Immun* **82**, 3426–3435.

Nguyen, A. T., Jones, J. W., Ruge, M. A., Kane, M. A. & Oglesby-Sherrouse, A. G. (2015). Iron depletion enhances production of antimicrobials by *Pseudomonas aeruginosa*. *J Bacteriol* **197**, 2265–2275.

Nguyen, A. T., O'Neill, M. J., Watts, A. M., Robson, C. L., Lamont, I. L., Wilks, A. *et al.* (2014). Adaptation of iron homeostasis pathways by a *Pseudomonas aeruginosa* pyoverdine mutant in the cystic fibrosis lung. *J Bacteriol* **196**, 2265–2276.

Pazos, M. A., Lanter, B. B., Yonker, L. M., Eaton, A. D., Pirzai, W., Gronert, K. *et al.* (2017). *Pseudomonas aeruginosa* ExoU augments neutrophil transepithelial migration. *PLoS Pathog* **13** (8), e1006548.

Peleg, A. Y., Tampakakis, E., Fuchs, B. B., Eliopoulos, G. M., Moellering, R. C. *et al.* (2008). Prokaryote-eukaryote interactions identified by using *Caenorhabditis elegans. Proc Natl Acad Sci U S A* **105**, 14585-14590.

Pendleton, K. M., Huffnagle, G. B. & Dickson, R. P. (2017). The significance of *Candida* in the human respiratory tract: our evolving understanding. *Pathog Dis* **75**, ftx029.

Peters, B. M. & Noverr, M. C. (2013). *Candida albicans-Staphylococcus aureus* polymicrobial peritonitis modulates host innate immunity. *Infect Immun* **81**, 2178–2189.

Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A. & Cerón, J. (2012). Basic *Caenorhabditis elegans* Methods: Synchronization and Observation. *J Vis Exp* **64**, e4019 10.3791/4019, doi: 10.3791-4019.

Pukkila-Worley, R., Peleg, A. Y., Tampakakis, E. & Mylonakis, E. (2009). *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. *Eukaryot Cell* **11**, 1750-1758.

Rada, B. & Leto, T. L. (2013). Pyocyanin effects on respiratory epithelium: relevance in *Pseudomonas aeruginosa* airway infections. *Trends Microbiol* **21** (2), 73–81.

Richards, M. J., Edwards, J. R., Culver, D. H. & Gaynes, R. P. (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med* **27**, 887–892.

Rinzan, F. F. (2009). *Pseudomonas aeruginosa-Candida albicans* Interactions from Ecological and Molecular Perspectives. Department of Biology, Georgia State University, Atlanta, USA.

Rosenthal, V. D., Maki, D. G., Salomao, R., Moreno, C. Á., Mehta, Y., Higuera, F. *et al.* (2006). Device-associated nosocomial infections in 55 intensive care units of 8 developing countries. *Ann Intern Med* **145**, 582–591.

Roux, D., Gaudry, S., Dreyfuss, D., El-Benna, J., de Prost, N., Denamur, E., Saumon, G. & Ricard, J. (2009). *Candida albicans* impairs macrophage function and facilitates *Pseudomonas aeruginosa* pneumonia in rat. *Crit Care Med* **37**, 1062–1067.

Sadikot, R. T., Blackwell, T. S., Christman, J. W. & Prince, A. S. (2005). Pathogen–host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* **171** (11), 1209–1223.

Sadikot, R. T., Zeng, H., Azim, A. C., Joo, M., Dey, S. K., Breyer, R. M., Peebles, R. S., Blackwell, T. S. & Christman, J. W. (2007). Bacterial clearance of *Pseudomonas aeruginosa* is enhanced by the inhibition of COX-2. *Eur J Immunol* **37**, 1001–1009.

Saliba, A. M., Nascimento, D. O., Silva, M. C. A., Assis, M. C., Gayer, C. R. M., Raymond, B., Coelho, M. G. P., Marques, E. A., Touqui, L. *et al.* (2005). Eicosanoid-mediated proinflammatory activity of *Pseudomonas aeruginosa* ExoU. *Cell Microbiol* **7**, 1811–1822.

Schaller, M., Borelli, C., Korting, H. C. & Hube, B. (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**, 365-77.

Schlecht, L. M., Peters, B. M., Krom, B. P., Freiberg, J. A., Hänsch, G. M., Filler, S. G., Jabra-Rizk, M. A. & Shirtliff, M. E. (2015). Systemic *Staphylococcus aureus* infection mediated by *Candida albicans* hyphal invasion of mucosal tissue. *Microbiology* **161**, 168–181.

Schwarz, C., Hartl, D., Eickmeier, O., Hector, A., Benden, C., Durieu, I., Sole, A., Gartner, S., Milla, C. E. & Barry, P. J. (2018). Progress in definition, prevention and treatment of fungal infections in cystic fibrosis. *Mycopathologia* **183** (1), 21-32.

Schweizer, E. & Hofmann, J. (2004). Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. *Microbiol Mol Biol Rev* **68** (3), 501-517.

Shirtliff, M. E., Peters, B. M. & Jabra-Rizk, M. A. (2009). Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett* **299**, doi: 10.1111/j.1574-6968.2009.01668.x.

Tampakakis, E., Peleg, A. Y. & Mylonakis, E. (2009). The interaction of *Candida albicans* with an intestinal pathogen; *Salmonella enterica* serovar *Typhimurium*. *Eukaryot. Cell* **8**, 732–737.

Tan, X., Chen, R., Zhu, S., Wang, H., Yan, D., Zhang, X. & Mylonakis, E. (2016). *Candida albicans* airway colonization facilitates subsequent *Acinetobacter baumannii* pneumonia in a rat model. *Antimicrob Agents Chemother* **60** (6), 3348–3354.

Tanaka, T., Ikita, K., Ashida, T., Motoyama, Y., Yamaguchi, Y. & Satouchi, K. (1996). Effects of growth temperature on the fatty acid composition of the free-living nematode *Caenorhabditis elegans*. *Lipids* **31**, 1173–78.

Takase, H., Nitanai, H., Hoshino, K. & Otani, T. (2000). Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. *Infect Immun* **68**, 1834–1839.

van 't Wout, E. F. A., van Schadewijk, A., van Boxtel, R., Dalton, L. E., Clarke, H. J., Tommassen, J. *et al.* (2015). Virulence factors of *Pseudomonas aeruginosa* induce both the unfolded protein and integrated stress responses in airway epithelial cells. *PLoS Pathog* **11** (6), e1004946.

Vasil, M. L. & Ochsner, U. A (1999). The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* **34**, 399–413.

Villar, C. C., Kashleva, H., Mitchell, A. P. & Dongari-Bagtzoglou, A. (2005). Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. *Infect Immun* **73**, 4588–4595.

Watts, J. L. & Browse, J. (2002). Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **99**, 5854–5859.

Xu, H., Sobue, T., Thompson, A., Xie, Z., Poon, K., Ricker, A., Cervantes, J., Diaz, P. I. & Dongari-Bagtzoglou, A. (2014). Streptococcal co-infection augments *Candida* pathogenicity by amplifying the mucosal inflammatory response. *Cell Microbiol* **16,** 214–231.

Zaborin, A., Romanowski, K., Gerdes, S., Holbrook, C., Lepine, F., Long, J., Poroyko, V., Diggle, S. P., Wilke, A. & Righetti, K. *et al.* (2009). Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proc Natl Acad Sci U S A* **106**, 6327–6332.