Investigating molecular pathways and identifying genes and microbes that promote heat stress resistance and healthy ageing

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Investigating molecular pathways and identifying genes and microbes that promote heat stress resistance and healthy ageing

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Abstract

Several studies have linked stress resistance to lifespan extension, and ageing has been associated with dysbiosis. With *C. elegans* longevity correlating with severe heat resistance and the recent recognition of a key role for the gut microbiota in defining worm healthspan, I looked at how *C. elegans* gut microbes found in its natural environment may interact with the worm physiology to mediate stress resistance and healthy ageing.

I exposed young adult *C. elegans* to their natural gut microbiota and assayed for heat (42°C) and oxidative stress (7% tertbutyl hydroperoxide) resistance using LFASS (Label-Free Automated Survival Scoring) assays. To determine if microbial effects on stress resistance involve known gut-brain axis pathways, I conducted LFASS assays on Insulin/IGF-1 Signalling (IIS) and Kynurenine Pathway (KP) *C. elegans* mutants. My results showed that both the KP and IIS pathways are involved in severe stress resistance, with bacterial strains modulating the KP differentially and the gut microbiota influencing stress responses through both IIS-dependent and independent mechanisms.

I then conducted health-span (movement, brood size) experiments and lifespan assays to determine whether enhanced stress resistance translates into healthier ageing and longevity. MYb71 (*Ochrobactrum vermis)*, MYb330 (*Pseudomonas)*, BIGb170 (*Sphingobacterium multivorum)*, MYb396(a) (*Comamonas)*, and MYb21 (*Comamonas)*, were found to be beneficial, as they improved stress resistance without negatively impacting reproduction. Enhanced stress resistance did not lead to increased lifespan, suggesting that stress resistance and longevity are physiologically distinct. To identify new host genetic pathways potentially mediating gut microbiotas effects on severe stress resistance and healthy ageing, I conducted a genome-wide RNAi screen on young adult *C. elegans* exposed to severe heat stress. I found known thermoprotective genes, F44E5.5, *sip-1*, and dnj-5, and thermosensitising gene *skn-1.* I also identified some new thermoprotective candidate genes, such as *clec-20, F20G2.5, pals-30* (immunity) and *R08F11.1, fat-7* (fat metabolism*).* The genes from the RNAi screen will be used in conjunction with the beneficial microbial isolates to determine whether they produce a synergistic response.

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Declaration:

I declare that this is my own work and has not been submitted in the same form elsewhere. Some of the methods were published in my paper, which I have cited.

Experimental contributions:

Dr Alejandra Zárate-Potes contributed to RNAi screen preparation and data annotations (section 3.2 and 3.3) and CeMbio+ method optimisation (2.3, 2.4.1).
Dr Jack Martin contributed to CeMbio+ bacterial preparation and method optimisation (section 2.3, 2.4.1, 2.5).
Tory Higgins provided BIGb170 and CEent1 brood size data (section 3.1.10).

1. Context of the study

1.1 Importance of studying ageing

1.1.1 What is ageing?

Ageing can be challenging to define. However, in a broader sense, it is defined as the cumulative impact of deleterious biological processes that gradually increase an organism's vulnerability to mortality over time (Mc Auley, M.T., 2025).

Ageing can be categorised either as a chronological or biological process. Chronological ageing refers to the passage of time from birth onwards, meaning all individuals age chronologically at the same rate. This way age is easy to measure and helps to determine the needs of older people in society by providing cutoff points (Balcombe, N.R. and Sinclair, A., 2001).

On the other hand, the concept of biological ageing which was first introduced in 1988 by Baker and Sprott (Elliott et al., 2023), states that on a biological level, ageing is a complex process characterised by a progressive decline in bodily functions (physical and mental capacity) possibly due to the gradual accumulation of damaged organelles (Carmona, J.J. and Michan, S., 2016).

In humans, the molecular and cellular damage observed with age includes mitochondrial dysfunction, impaired intercellular communication, abnormal cell senescence and reduced regenerative capacity ***(figure.1)*** (López-Otín et al., 2023). This results in late-life frailty (weight loss, slow responses, and reduced physical activity) and declined functional capacities such as impaired hearing, sight and poor memory and ultimately death (Marcucci et al., 2017). Therefore, López-Otín et al. proposed 12 hallmarks of ageing – genomic instability (increased DNA mutations), telomere attrition (telomere shortening), epigenetic alterations (heritable changes in gene expression), loss of proteostasis (protein homeostasis), disabled macroautophagy (removal of damaged organelles), dysregulated nutrient sensing, mitochondrial dysfunction (increased mtDNA mutations and ROS production), cellular senescence (permanent arrest of proliferation), stem cell exhaustion (decline in stem cell regenerative potential), altered intercellular communication, chronic inflammation and dysbiosis (loss of beneficial bacteria, overgrowth of opportunistic bacteria) (López-Otín et al., 2023).

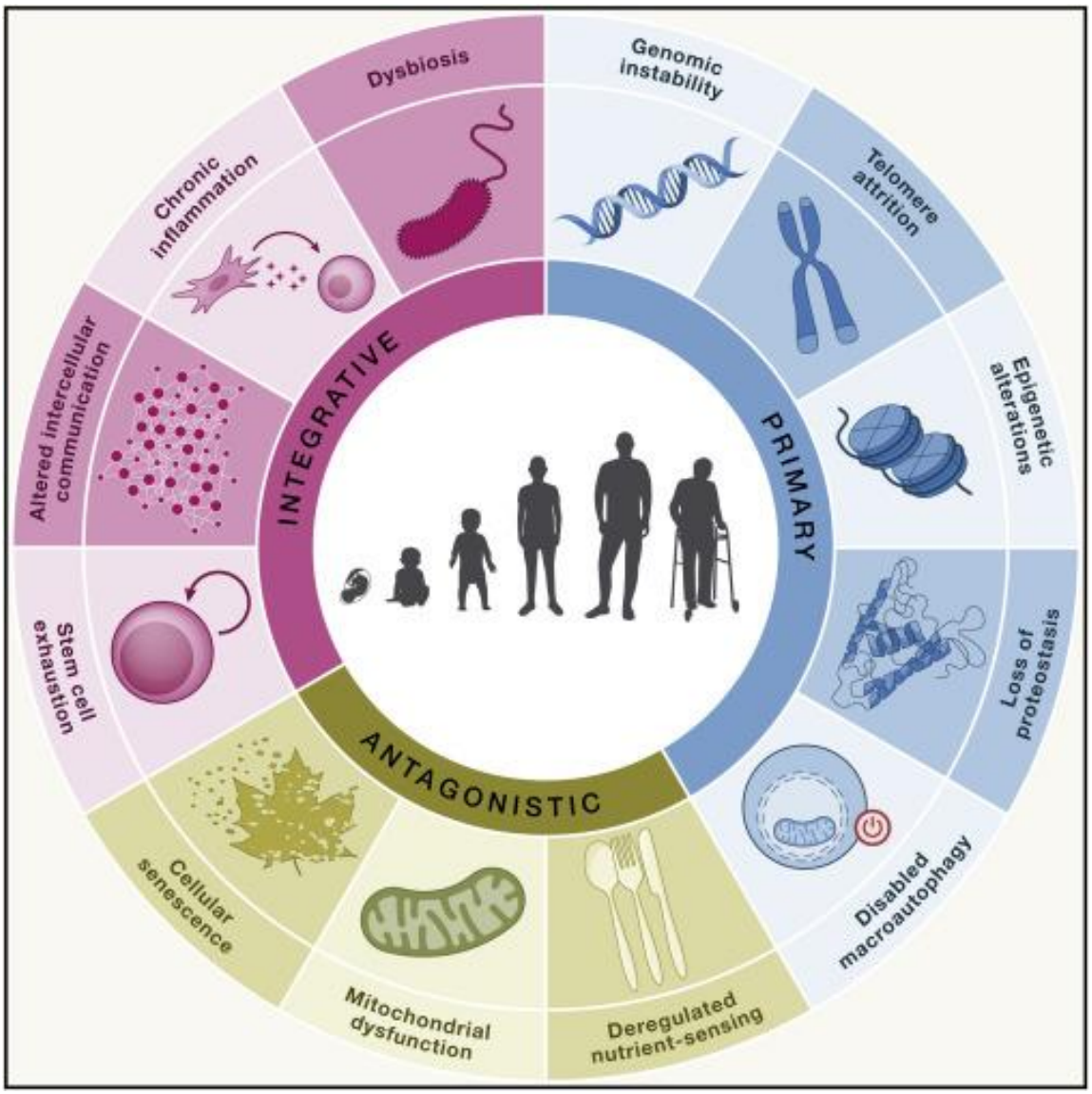


Figure 1. The hallmarks of ageing. The potential molecular and genetic damage with age includes, genomic instability (increased DNA mutations), telomere attrition (telomere shortening), epigenetic alterations (heritable changes in gene expression), loss of proteostasis (protein homeostasis), disabled macroautophagy (removal of damaged organelles), dysregulated nutrient sensing, mitochondrial dysfunction (increased mtDNA mutations and ROS production), cellular senescence (permanent arrest of proliferation), stem cell exhaustion (decline in stem cell regenerative potential), altered intercellular communication, chronic inflammation and dysbiosis (loss of beneficial bacteria, overgrowth of opportunistic bacteria) (López-Otín et al., 2023).

1.1.2 Can biological ageing be quantified?

People of the same chronological age may not be of the same biological age ***(figure.2).*** Biological ageing occurs at varying rates among different species and can also differ among individuals within the same species. In humans, for instance, some adults may experience age-related deterioration at a faster pace than others. While some individuals can maintain independent living well into their eighties, others may suffer from organ failure, dementia, and mortality before reaching their sixties. Therefore, biological age serves as a more accurate marker of health status than chronological age (Elliott et al., 2021).

To measure human biological age, we need quantitative markers that change predictably with biological age, referred to as biomarkers of ageing, which could provide a more accurate picture of health status. This would be beneficial for predicting age-related conditions and evaluating the effectiveness of treatments (Bortz et al., 2023). Additionally, this may be beneficial in providing early treatment before disease onset (Belsky et al., 2015).

However, this can be challenging due to the variety of ageing biomarkers and the difficulty in selecting which ones to use. Multibiomarker algorithms may be more effective than single biomarker ageing indicators (Belsky et al., 2015).

Levine and colleagues looked at the validity and usefulness of biological age algorithms in predicting mortality outcomes (Levine M.E et al., 2013). The criteria were that the algorithm needs to be a better predictor of multiple age-associated biological and functional outcomes than the chronological age, it should be able to predict longevity and disease-specific mortality in a population where 90% of the individuals are still alive and finally, that the method should not decrease life expectancy or impact future age-related measurements. The study included participants aged 30–75 years from National Health and Nutrition Examination Survey (NHANES), between 1988 and 1994. They found that the Klemera and Doubal mathematical model was the most reliable predictor of mortality compared to other algorithms (Levine M.E., 2013).

As most ageing research examines older chronic patients, there is a lack of knowledge about ageing in younger individuals. Therefore, Blesky et al examined changes in 18 biomarkers, such as metabolic, cardiovascular, kidneys, lungs etc across chronological ages 26, 32, and 38 in 954 young individuals over the course of 12 years (Belsky et al., 2015). They used the Klemera and Doubal algorithm on individuals that were all 38 years old to see if the individuals of the same chronological age differ in biological age. The results showed that some individuals

were biologically older than others. Next, by assessing an individual at different points in their life they were able to calculate their personal pace of ageing and again the results showed that some individuals were ageing faster than others. Interestingly, observational experiments also showed that biologically older individuals felt older and looked older to independent observers. (Belsky et al., 2015).

Over the years, more ageing biomarker algorithms are being developed, such as the Levine et al PhenoAge in 2018. This model uses a Cox proportional-hazards model with an Elastic-Net penalty on data consisting of 42 blood-based biomarkers and all-cause mortality collected within the NHANES programme to estimate biological age (Bortz, J et al., 2023). And recently, Bortz, J et al have shown created an Elastic-Net derived Cox model with 25 selected biomarkers to predict mortality risk and concluded that this model outperforms PhenoAge model by 11% (Bortz, J et al., 2023).

Another recent approach involves examining the brain age gap (BAG), which is the difference between a person's estimated brain age and their chronological age, determined through magnetic resonance imaging (MRI). These regression-based machine learning models for brain age prediction use MRI scans from participants with healthy cognitive function, combined with learning algorithms. The trained model can then be applied to new participants to estimate their brain age. This approach enables early detection of brain-based disorders and supports differential diagnosis, prognosis, and treatment decisions, leading to more timely and targeted interventions for age-related conditions (Yi et al., 2025).

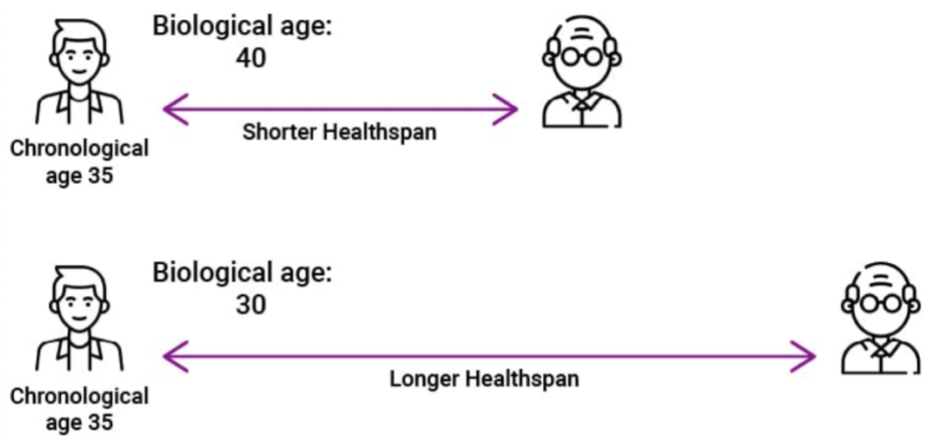


Figure 2. Biological age versus chronological age. People of the same chronological age may not be of the same biological age. Biological age diagnostics to transform how we see ageing - Longevity Technology Reports (2022).

1.1.3 A societal issue with a huge impact

The living standards including education, sanitation (clean water, food and hygiene), advances in medical research and access to better healthcare (immunisation against infectious diseases, antibiotics) have improved over the past 200 years doubling the average human life expectancy in most developed countries (Partridge, L., Deelen, J. and Slagboom, P.E., 2018). For example, the life expectancy in wealthier countries, such as Switzerland, the UK, and Australia is above 80 years. In 2019, it was the highest in Japan with close to 85 and lowest in the Central African Republic with 53 years (Roser, M., Ortiz-Ospina, E. and Ritchie, H., 2021). Overall, the average life expectancy in the world has risen by 26 years, from 47 in the 1950s to 73 in 2020 (Garmany et al., 2021).

However, the increase in healthspan has not increased as much as lifespan, for example, a global increase of five years in total life expectancy between 2000 and 2015 was accompanied by only 4.6 years of healthy life expectancy (Partridge, L., Deelen, J. and Slagboom, P.E., 2018). Healthspan can be defined as the functional ability of the organism to deal with a molecular and cellular decline for the longest length of lifespan (Ferrucci et al., 2020). In other words, lifespan is the total number of years lived, whereas healthspan is years lived disease-free (Garmany et al., 2021).

In humans, ageing is accompanied by the onset of various pathologies such as diabetes, chronic inflammation, cardiovascular disease, abnormal protein aggregation and neurodegeneration (Rea et al., 2018). Therefore, older people are more likely to develop chronic conditions with several morbidities, which leads to a greater risk of physical and cognitive disabilities, reducing the quality of life and requiring complex and expensive drug treatments (Ferrucci et al., 2020). An average of 16–20% of life (longer in women) is now spent in late-life morbidity (Partridge, L., Deelen, J. and Slagboom, P.E., 2018).

Currently, there are 15.5 million people (23% of the population) aged 60 or over in the UK, 40% of which have a long-term illness or disability. In total, two-thirds of patients receiving care are older people (Mha.org.uk. 2021). Moreover, in the year 2017/18 public spending on adult social care was £21.7 billion, half of which was accounted for older people >60 (Ageuk.org.uk. 2021).

The global population of older individuals (> 65) is increasing and expected to reach 1.6 billion by 2050 (Ferrucci et al., 2020). As the world population is reaching eight billion, with the older population (>70) outpacing younger individuals (<70), currently, the gap between the lifespan

and healthspan is estimated to be around nine years (***figure.3)*** (Garmany et al., 2021). Therefore, it is essential to develop interventions that prevent the onset of age-related pathologies and promote healthy ageing (Partridge et al., 2020).

Additionally, extreme temperatures due to climate change mean that older people are exposed to more heat waves. Older people have heightened susceptibility to hyperthermia, and health conditions such as cardiovascular disease are worsened by heat exposure. This is because older individuals have a reduced capacity to thermoregulate and may also rely on medications that cause dehydration. Compared to 14% today, around 23% of people aged 69 or above will live in areas where the temperature could exceed the critical threshold of 37.5°C in the coming years, resulting in an additional 177-246 million older individuals being exposed to dangerous acute heat (Falchetta et al., 2024).

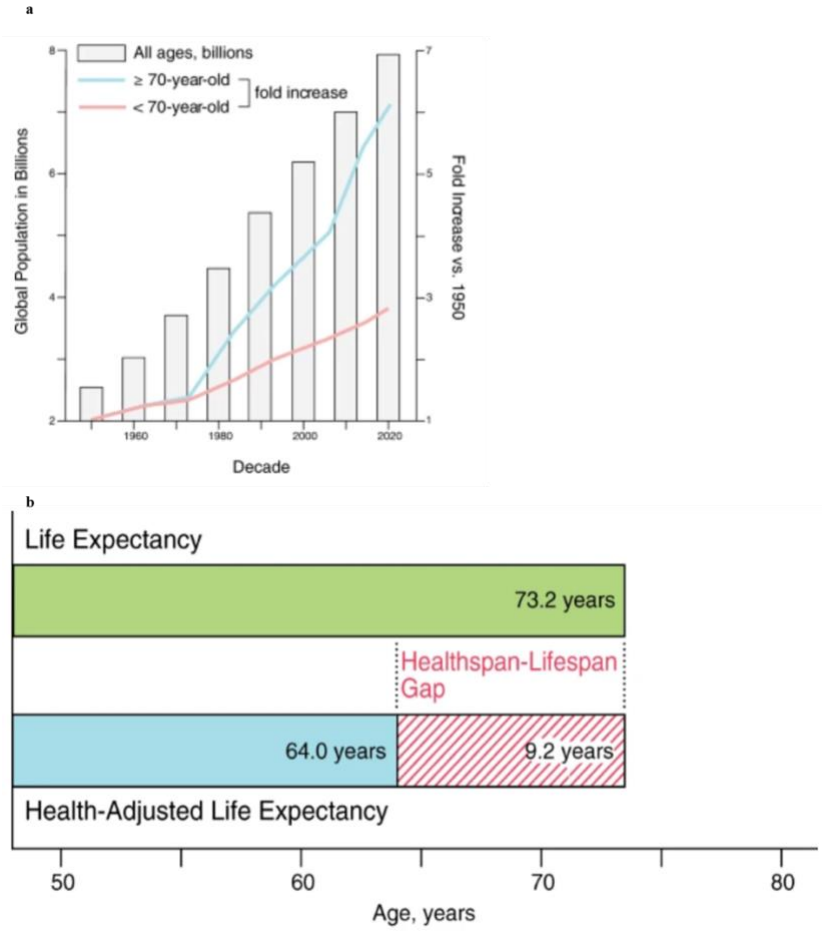


Figure 3. Healthspan-lifespan gap. a) The world population is reaching eight billion with the older population >70 (blue line) outpacing younger individuals <70 (pink line); b) The average life expectancy is around 73 years. However, health-adjusted life expectancy is lower at 64 years, highlighting the nine years gap between lifespan and healthspan (Garmany et al., 2021).

1.1.4 Current efforts to tackle ageing

The Geroprotection approach consists in combining multiple strategies to combat ageing mechanisms and prevent age-related diseases, as lifestyle recommendations such as diet and exercise alone may not be enough to preserve functional capacity with age. The aim is to extend the healthspan, reduce the health care burden and improve the quality of life by maintaining independence (Partridge et al., 2020).

Currently, the project DrugAge database contains more than 400 geroprotector compounds tested on lifespan, and the geroprotectors.org database includes 259 candidates that have been shown to increase lifespan in different model ageing organisms. Ongoing clinical trials for potential geroprotectors include compounds that treat age-related diseases, for example, anti-β-amyloid to stop the accumulation of amyloid aggregates, Urolithin A to improve muscle health and already approved FDA drug Metformin for diabetes as a preventative agent in lowering the risk of developing diseases in people without diabetes (Moskalev A., 2020).

Currently, there are no clinically proven human geroprotectors, as most of them failed in clinical trials (Moskalev A., 2020). For example, Rapamycin is an FDA-approved drug used for kidney transplant patients and some cancer treatments. It has been shown to increase lifespan in mice; however, in humans, prolonged treatment with rapamycin is associated with an increased risk of various adverse metabolic side effects, such as hyperglycaemia, new-onset diabetes, and dyslipidaemia in transplant patients. In healthy individuals, even lower doses of rapamycin have resulted in increased levels of glycated haemoglobin, triglycerides, and very-low-density lipoproteins (VLDL). Therefore, an optimal dose of rapamycin needs to be established to extend healthy lifespan with minimal adverse effects (Elliehausen et al., 2023).

The strategies for intervention in the ageing process are based on healthspan and resilience (the capability of recovering back to baseline functional health status after an acute event). Older individuals have lower resilience compared to younger individuals which result in longer recovery times, higher risk of death and lower independence when faced with the same physiological stressor, for example, surgery (Newman et al., 2016).

Studies which focus on interventions that extend healthspan are longitudinal, double-blinded, and placebo-controlled, to examine if an intervention can prevent or slow adverse health events over a relatively long period. On the other hand, studies of resilience test the ability of intervention in improving health and functional outcomes after acute stress (Newman et al., 2016).

However, there are many challenges with ageing studies. The main challenge is designing a study that caters for the need of all the different individuals in an ageing population. This is because older individuals show disease heterogeneity (co-comorbidities and disabilities), and are genetically, ethnically and physically diverse. This has resulted in the exclusion of many older individuals from clinical studies, but diversity across the older population must be embraced to study the impact of interventions on the ageing process. Other challenges include the complex nature of human physiology in which changes in one system have effects on multiple systems, such as type 2 diabetes could lead to Alzheimer's disease (and *vice versa*). Therefore, the study needs to consider the association between a disease and increased risk of other diseases to assess independent outcomes of ageing (Newman et al., 2016).

1.2 Twelve Hallmarks of ageing

Twelve hallmarks of ageing include genomic instability (increased DNA mutations), telomere attrition (telomere shortening), epigenetic alterations (heritable changes in gene expression), loss of proteostasis (protein homeostasis), disabled macroautophagy (removal of damaged organelles), dysregulated nutrient sensing, mitochondrial dysfunction (increased mtDNA mutations and ROS production), cellular senescence (permanent arrest of proliferation), stem cell exhaustion (decline in stem cell regenerative potential), altered intercellular communication, chronic inflammation and dysbiosis (loss of beneficial bacteria, overgrowth of opportunistic bacteria). To be classified as a hallmark, the following criteria must be met: age-associated manifestations, acceleration of ageing when experimentally accentuated, and the potential to decelerate, stop, or reverse ageing through therapeutic interventions. These hallmarks are interconnected with each other and may contribute to the ageing process across different organisms (López-Otín et al., 2023). On one hand, the hallmarks representation provides an overview of biogerontology and creates a shared perspective that unites the ageing research community. However, on the other hand, some hallmarks may play little role in the ageing of certain model organisms, such as *C.elegans* (Gems, D. and De Magalhães, J.P., 2021).

1.2.1 Genomic instability

Genomic instability refers to the increased tendency for DNA mutations and defects in cell division process leading to genetic alterations ***(figure.4)*** (Schumacher et al., 2021). DNA alterations, including point mutations, insertions, deletions, and chromosomal rearrangements, can irreversibly change the genome's content. The genome is unstable due to the continuous induction of DNA damage. DNA can be damaged by endogenous sources, such as free radicals, and exogenous sources, such as ultraviolet light or ionising radiation. DNA damage refers to physical alterations in the nucleic acid structure, such as breaks, depurination, depyrimidination, crosslinks, and modified bases. Mutations, on the other hand, arise from errors during the repair or replication of damaged DNA and can also occur spontaneously. While DNA damage is typically repaired successfully, DNA mutations are not recognised by repair enzymes and are therefore irreversible (Vijg, J. and Montagna, C., 2017).

In 1952, Medawar proposed that mutations resulting from DNA damage lead to a loss of genetic information, thereby driving ageing. Over the years, double-stranded DNA break, occurring at a rate of 10–50 per cell per day, is the type of DNA damage that is consistently linked to ageing. (Yang et al., 2023). Cagan et al. looked at somatic mutation rates and lifespan across 16 species, such as cats, dogs, mice, naked mole-rats, and humans, by examining their intestinal crypts. They showed that mutation rate is inversely proportional to lifespan (Cagan et al., 2022).

Genomic instability is functionally interconnected with all other hallmarks of ageing because it can involve telomere length shortening, senescence upon DNA damage, and mutations during DNA repair processes. Mutations in mitochondrial genomes lead to mitochondrial dysfunction and disabled macro-autophagy. DNA mutation and damage can trigger inflammation and may induce dysbiosis through the accumulation of genomic mutations in intestinal cells (López-Gil et al., 2023).

Research in some mice has also shown a progressive decline in genomic stability with age. De Majo et al. found that genomic instability does not contribute to the natural ageing of murine hearts, as naturally ageing hearts did not show an accumulation of mutations through genome sequencing; however, the small intestine and liver did. This suggests that different tissues accumulate mutations at divergent rates (De Majo et al., 2021).

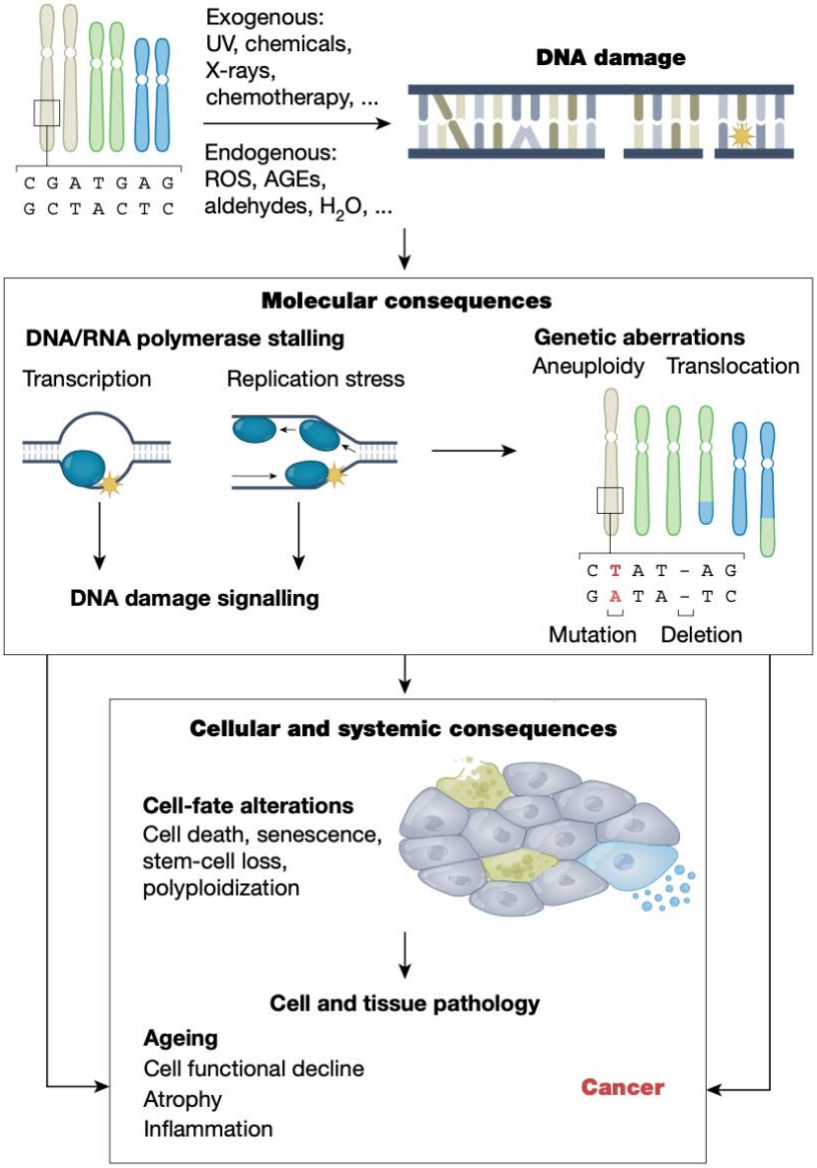


Figure 4. Genomic instability. Exogenous agents such as chemicals, UV radiation, chemotherapy, and X-rays, and endogenous sources, such as ROS, aldehydes, and advanced glycation end products (AGEs), can cause DNA damage. This can lead to molecular consequences, such as, genetic aberrations and DNA/RNA polymerase stalling by DNA lesions, promoting DNA damage signalling. Cellular and systemic consequences of DNA damage include cell fate alterations (cell death, stem cell loss, and senescence) leading to cell and tissue pathologies, such as functional decline, atrophy, and inflammation associated with ageing (Schumacher et al., 2021).

1.2.2 Telomere attrition

Telomere attrition is another hallmark of ageing in humans ***(figure. 5)*** (Vaiserman, A. and Krasnienkov, D., 2021). Telomeres are made up of DNA repeat sequences (TTAGGG) to form the protective ends of eukaryotic chromosomes, protecting chromosomes from improper recombination and degradation. Human telomeres are 5 kilobase (kb) pairs that shorten with age, due to many rounds of DNA replication known as the 'end-replication problem' (Zhu et al., 2019). This is because telomeric DNA can't be completely replicated. In 1985, Greider and Blackburn discovered Telomerase which is a ribonucleoprotein enzyme that elongates chromosomes by adding DNA sequence repeats to their terminal regions, thus maintaining the length of telomeres. However, most somatic cells have very little to no levels of telomerases as the enzyme is mostly expressed in stem cells (Schellnegger et al., 2024).

On the other hand, lifestyle factors such as smoking, lack of exercise and obesity may also increase the rate of telomere shortening by possibly increasing inflammation and oxidative stress (Shammas, M.A., 2011). A meta-analysis conducted by Astuti et al., looked at 84 primary studies on smoking and telomere length and concluded that telomere length was shorter in smokers compared to those that had never smoked. Free radicals generated by smoking can cause oxidative damage, which could be a possible mechanism leading to telomere shortening (Astuti et al., 2017). Biological sex/gender does not seem to impact the rate of telomere loss (Shammas, M.A., 2011).

Whittemore et al examined the rate of telomere shortening in various bird and mammal species, such as gull, mouse and dolphin and observed a correlation between the rate of telomere shortening and lifespan. They concluded that the initial length of telomere does not correlate with longevity, however, the rate of telomere shortening could predict species lifespan. For example, humans have a shorter initial telomere length (5–15 kb) compared to mice (50 kb), yet have a longer lifespan. This could be due to a lower rate of telomere shortening in humans than in mice, as the rate of telomere shortening in humans is approximately 70 base pairs (bp) per year, whereas in mice it is around 7,000 bp per year (Whittemore et al., 2019).

Cawthon *et al.* looked at the association between telomere shortening and survival rate in 143 normal unrelated individuals (>60 age). They showed that individuals with shorter telomeres in blood DNA had higher mortality rates from heart (3.18-fold) and 8.54-fold higher in infectious diseases. This suggested that the telomere shortening may contribute to early onset of mortality in many age-related diseases (Cawthon et al., 2003).

If the shortening of telomeres results in ageing, the leading question is whether restoring telomere length could reverse or slow ageing in humans?

Long telomeres may promote cancer by allowing more cell divisions. Zhu et al. conducted a meta-analysis and found that short telomeres were associated with gastrointestinal and head and neck cancers, while long telomeres were associated with lung adenocarcinoma. This suggests that telomeres play diverse roles in different cancers and therefore the answer is not that simple (Zhu, X et al., 2016).

Delivering modified mRNA encoding telomerase reverse transcriptase (TERT) has been shown to increase telomerase activity in human fibroblasts and myoblasts without causing cell immortalisation and is therefore being explored further as a potential approach to increase telomere length. (Ramunas, J et al., 2015).

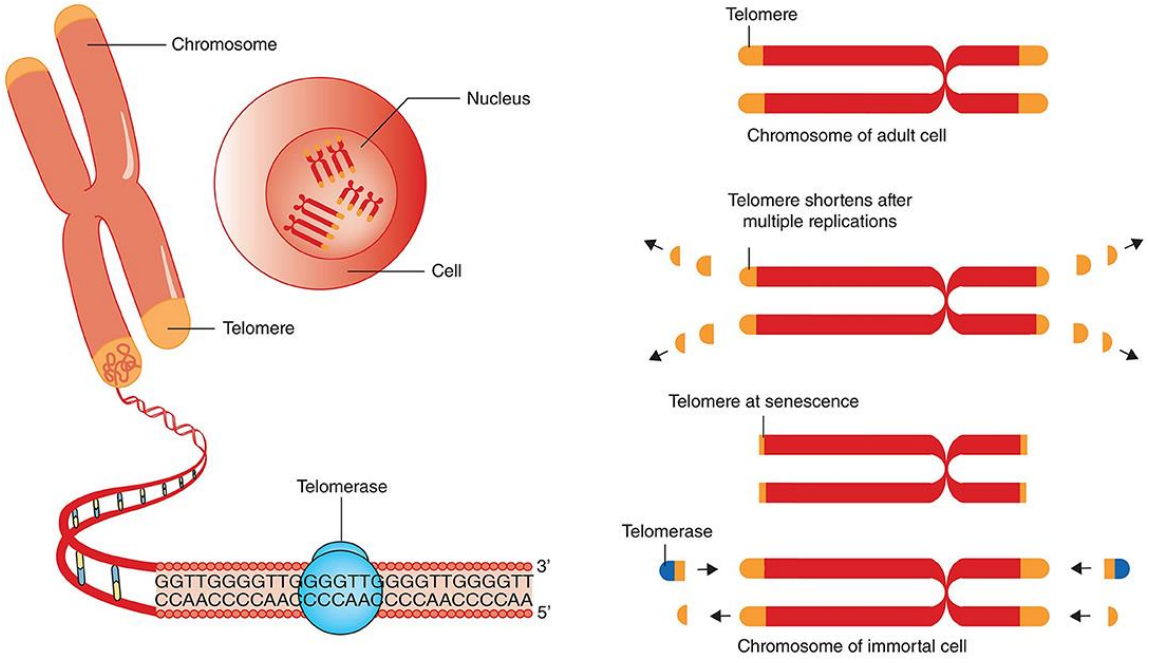


Figure 5. Telomere attrition. Telomeres are the protective ends of the eukaryotic chromosomes consisting of DNA repeat sequences (TTAGGG) which protect chromosomes from improper recombination and degradation. Telomeres shorten after many rounds of DNA replication. Telomerase (ribonucleoprotein enzyme) elongates chromosomes by adding DNA sequence repeats to their terminal regions, therefore maintaining the length of telomeres. However, most somatic cells have very little to no levels of telomerases as the enzyme is mostly expressed in stem cells. This results in telomere shortening with ageing (Vaiserman, A. and Krasnienkov, D., 2021; Schellnegger et al., 2024).

1.2.3 Epigenetic alterations

In 1942, C. Waddington coined the term 'epigenetics' (Galow, A.M. and Peleg, S., 2022). Epigenetics refers to meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Egger et al., 2004). Epigenetic mechanisms control gene expression, ensuring that different cell types selectively express specific genes rather than activating all genes simultaneously (Moore et al., 2013). These modifications include DNA methylation, histone modification, and RNA-mediated gene silencing (***figure.6)*** (Dhar et al., 2022). During development epigenetic changes determine whether genes are turned on or off. Genes required later in development are transiently held in a repressed state during early developmental stages through histone modifications and are activated when their expression is needed. During differentiation, genes crucial for pluripotency are silenced by both histone modifications and DNA methylation (Reik, W., 2007).

DNA methylation is the transfer of a methyl group from *S*-adenyl methionine onto cytosine to form 5-methylcytosin. In mammals, the majority of DNA methylation occurs at CpG sites (cytosines that precede a guanine nucleotide). This process is catalysed by a family of DNA methyltransferases (DNMTs): the de novo methyltransferases DNMT3A and DNMT3B, which establish new methylation patterns on unmodified DNA, and DNMT1, which maintains DNA methylation during replication by copying the methylation pattern from the parental strand onto the newly synthesised daughter strand. Promoters with methylated CpG sites prevent transcription factor binding, directly suppressing gene transcription (Moore et al., 2013).

In eukaryotes, DNA is wrapped around histone proteins forming compact structures called nucleosomes. Post-translational modifications such as methylation, acetylation, ubiquitination, and phosphorylation influence how tightly DNA is wrapped around histones, thereby regulating gene activation or silencing. Histone modifications that tightly package DNA repress gene expression, while those that loosen DNA allow transcription (Moore et al., 2013).

MicroRNAs (miRNA), which were first discovered in *C. elegans*, are a non-coding RNA between 18–25 nucleotides in length that can silence gene expression through post-transcriptional modifications. miRNA binds to mRNA, blocking its translation or leading to mRNA degradation, thereby reducing protein output. One miRNA can target multiple mRNAs, and one mRNA can be targeted by multiple miRNAs. So far, 1,048 miRNA sequences have been identified in humans through cloning, sequencing, and computational analysis (Jin Jung, H. and Suh, Y., 2012).

Exposure to an environmental stimulus can cause an epigenetic change; however, epigenetic changes in an individual can also arise sporadically. If this change is passed on to the next generation, inheritance in the immediate offspring is known as 'intergenerational'. Usually, some epigenetic changes are lost; however, sometimes, an epigenetic signal is passed on to several generations, even in the absence of the initial stimulus or epigenetic trigger, and is therefore termed 'transgenerational' epigenetic inheritance (Fitz-James, M.H. and Cavalli, G., 2022).

Epigenetic 'age estimators' are sets of CpG sites coupled with mathematical algorithms that estimate the age of a DNA source, such as cells, tissues, or organs. Estimating epigenetic age using DNA methylation data provides insight into the effects of endogenous and exogenous stress factors on biological ageing. This is particularly useful because epigenetic changes are reversible, allowing anti-ageing interventions to be identified and validated (Horvath, S. and Raj, K., 2018).

The first DNA methylation-based age estimator was constructed by Bocklandt et al. using DNA extracted from saliva. This was followed by Hannum et al. who developed a highly accurate age estimator based on 71 CpG sites from blood-derived DNA. Since then, attention has shifted towards multi-tissue age estimators, as DNA methylation patterns vary across different cell types and tissues (Horvath, S. and Raj, K., 2018).

The first multi-tissue age estimator, known as Horvath's clock, was developed by Horvath using 8,000 publicly available DNA methylation microarray samples from 51 healthy tissues and cell types. It has been widely used in numerous studies, most of which suggest that tissues and organs from the same individual generally exhibit similar epigenetic ages. However, some studies have revealed intriguing findings, for instance, the cerebellum may be epigenetically younger than other parts of the brain and female breast tissue may be epigenetically older than other tissues (Horvath, S. and Raj, K., 2018).

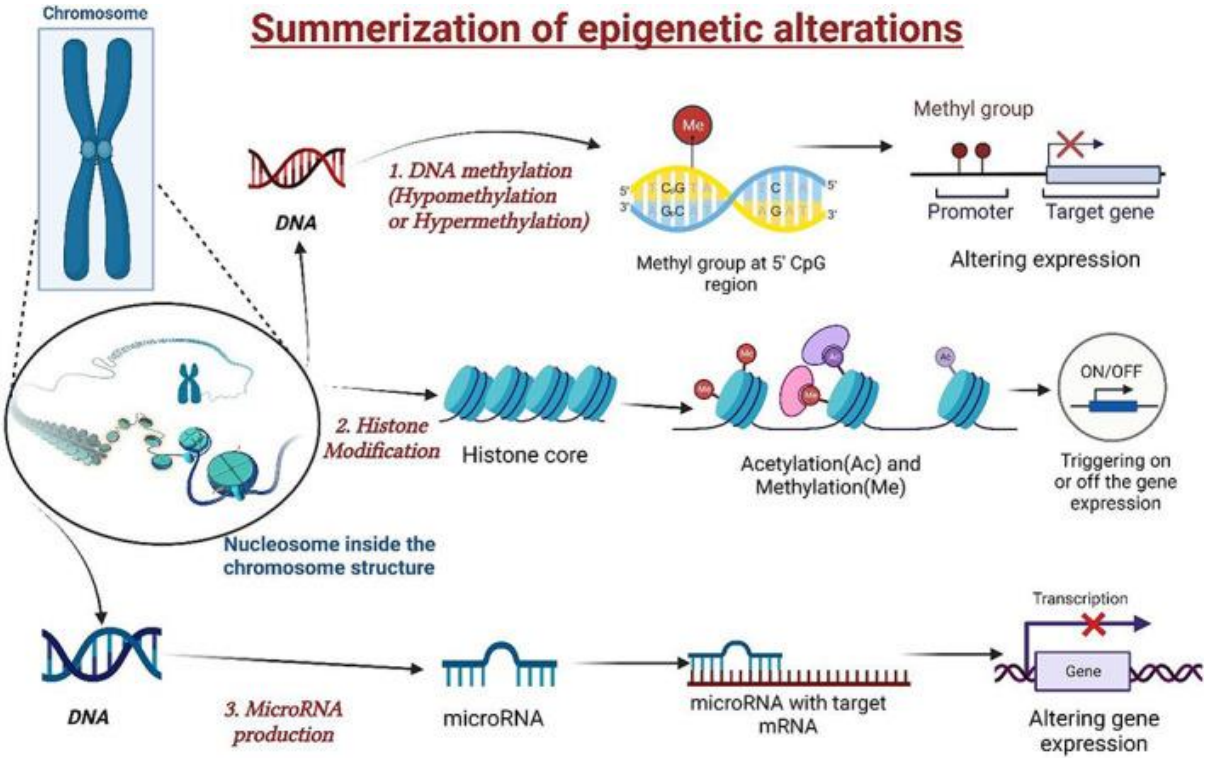


Figure 6. Epigenetic alterations. 1) DNA methylation- a methyl group from S-adenyl methionine is transferred onto cytosine to form 5-methylcytosin. 2) Histone modifications- Post-translational modifications such as methylation, acetylation, ubiquitination, and phosphorylation influence how tightly DNA is wrapped around histones, thereby regulating gene activation or silencing. 3) MicroRNA production- miRNA binds to mRNA blocking gene transcription (Dhar et al., 2022).

1.2.4 Loss of proteostasis

Protein homeostasis is known as proteostasis. Proteins are folded into a 3D structure which is maintained throughout their lifetime. This is essential for their biological function. In addition, the abundance of different proteins in a mammalian cell is also carefully controlled and misfolded proteins are either removed by autophagy or degraded by proteosome to stop them from aggregating. It requires an extensive network of molecular chaperones (ensure protein folding), proteolytic systems and their regulators to maintain this balanced proteome. This makes up to almost 2,000 proteins in human cells. In eukaryotes, chaperones prevent aggregation and promote protein folding through both ATP-dependent and ATP-independent mechanisms. These chaperones are classified into different protein families, including small heat shock proteins (sHSPs), HSP60, HSP70, and HSP90 (Hipp et al., 2019). Protein folding

states are maintained independently within cellular compartments, such as the cytosol, endoplasmic reticulum (ER), and mitochondria, by HSPs.

Different HSPs play significant roles, for example, HSP70 operates primarily in the cytoplasm, while HSP60 is predominantly found in the mitochondria and is upregulated during the mitochondrial unfolded protein response. In the ER, the unfolded protein response is regulated by glucose-regulated proteins 78 (GRP78) and 94 (GRP94) (Salway, K.D et al., 2011).

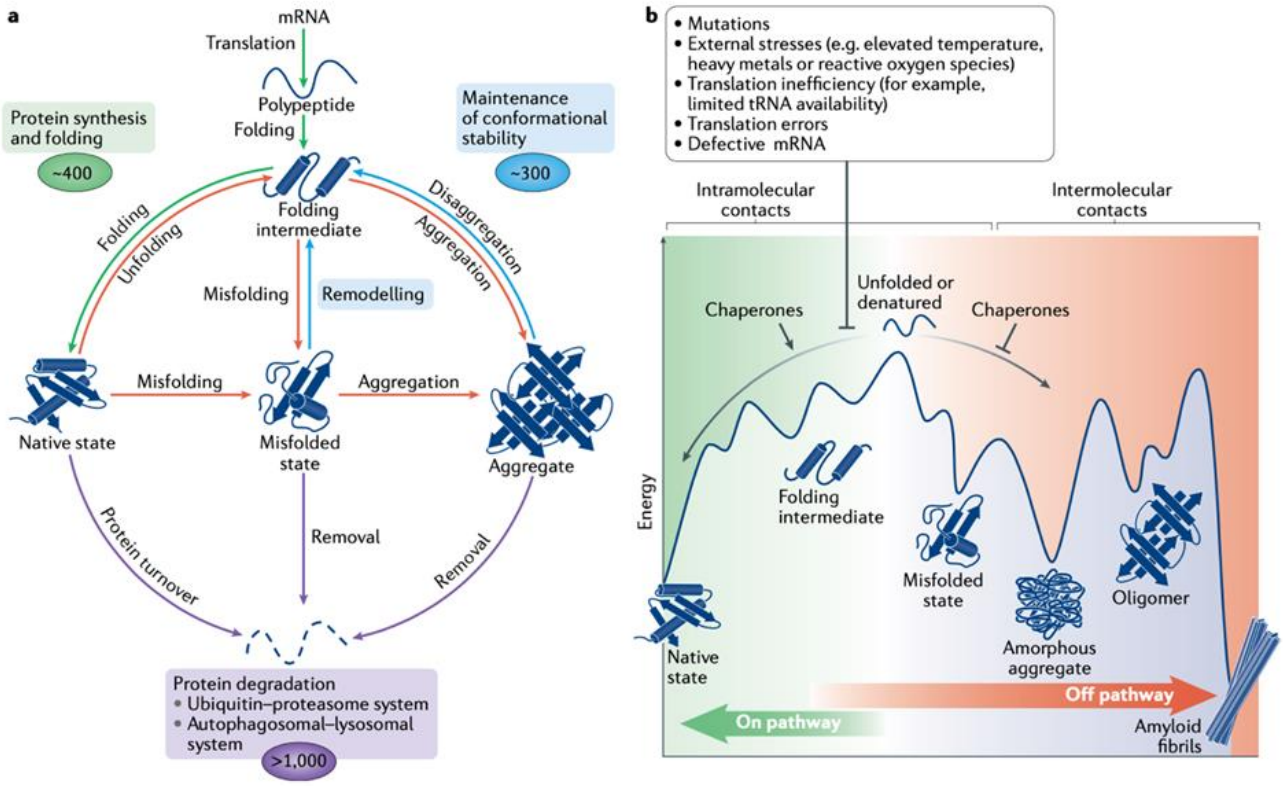
Some studies suggest that longer-lived species exhibit higher constitutive expression of HSPs, indicating improved protein homeostasis in longer-lived species. For example, Salway et al. found a positive correlation between some of the constitutive expression of mitochondrial, cytosolic, and ER HSPs in liver, heart, and brain tissue samples from 13 species of mammals and birds and their lifespan. This included samples from mouse, hamster, squirrel, rabbit, guinea pig, rat, cow, pig, sheep, deer, finch, and gerbil. Specifically, levels of HSP60 and HSP70 in the liver, heart, and brain were positively correlated with lifespan across these species. However, no upregulation of HSP60, HSP70, or GRP78 was observed in the heart and brain tissues of long-lived Snell dwarf mice, suggesting that heat shock proteins are not upregulated in this longevity model (Salway, K.D et al., 2011).

Overexpression of HSP70 in unhealthy mice has been shown to increase longevity; however, in certain disease models, it may lead to growth retardation and early death (Salway, K.D et al; 2011). In one study, intranasal administration of exogenous human HSP70 increased lifespan in both old and middle-aged healthy mice. The survival rate after 22 months was approximately 22% in the untreated middle-aged group, compared to 55.5% in the treated group. In old mice, the survival rate was 35.2% in the untreated group, compared to 54.5% in the treated group (Bobkova et al., 2015).

Mutations, stresses such as high temperatures, heavy metals and ROS, translational errors or defects in mRNA can disrupt the correct folding process which can lead to misfolded proteins and protein aggregates. Loss of proteostasis is observed in various age-related diseases such as neurodegenerative disorders, suggesting that the capacity of the proteostasis network declines with age (***figure. 7)*** (Hipp et al., 2019).

In *C. elegans*, the levels of ribosomal proteins and mitochondrial chaperones decrease with ageing, suggesting that proteostasis is altered during ageing. Mitochondrial enzymes involved in the tricarboxylic acid cycle and electron transport chain are also reduced, which may explain the age-related decline in energy (Liang, V et al., 2014). Liang et al. examined de novo synthesized proteins using an azidohomoalanine (AHA) tag, which showed that, with age, there

is an overall decline in the synthesis of new proteins, including ribosomal proteins. In Day 10 animals, the abundance of small HSPs following heat shock was reduced, suggesting that the heat shock response also diminishes with age (Liang, V et al., 2014). Moreover, In *C. elegans*, several proteins become more insoluble with age and have been shown to form aggregates in vivo, indicating that widespread protein insolubility and aggregation are inherent aspects of ageing (David et al., 2010).



Molecular chaperone family	Characteristics	Function
HSP70	• ~70 kDa • ATP-dependent	Major chaperone family, comprising at least eight homologous chaperone proteins, located in the cytoplasm, mitochondria and the endoplasmic reticulum. These chaperones are required for aggregation prevention, folding of newly synthesized proteins and conformational maintenance. They also cooperate with HSP40 and HSP110 in protein disaggregation
HSP40 (also known as J proteins)	• ~40 kDa	A diverse group of proteins, all containing the HSP70-interacting J domain, with homologues in the cytoplasm, mitochondria and the endoplasmic reticulum. They function as co-chaperones of HSP70 and regulators of the HSP70 ATPase cycle of protein substrate binding and release. They recruit HSP70 to different substrates and cellular locations
HSP110	• ~100 kDa	Serves as a nucleotide exchange factor for HSP70. Cooperates with HSP70 in protein folding and degradation of misfolded proteins, and it is crucial for protein disaggregation in metazoans
HSP90	• ~90 kDa • ATP-dependent	Functions as a homodimer in the folding and conformational regulation of functionally and structurally diverse client proteins that are involved in many different cellular pathways. Major substrate classes are kinases, steroid receptor molecules and other signalling proteins. Cooperates with multiple co-chaperones containing TPR domains
HSP60	• ~60 kDa subunit • ATP-dependent	The chaperonin of mitochondria. Consists of two heptameric rings composed of ~60 kDa subunits, which are stacked back-to-back. Cooperates with a cofactor, HSP10, and is required for the folding of a subset of mitochondrial proteins following their import from the cytosol
TRiC	• ~1 MDa • ATP-dependent	The chaperonin of the eukaryotic cytosol. Consists of two octameric rings composed of ~60 kDa subunits, which are stacked back-to-back. Required for the folding of a subset of cytosolic proteins, including actin and tubulins. TRiC has also been shown to interfere with the aggregation of huntingtin
Hsp100	• ~100 kDa subunit • ATP-dependent	A family of proteins in fungi, bacteria and chloroplasts in plants (comprising Hsp104, Hsp78, ClpA, ClpB, ClpC, ClpX and HslU) that belongs to a large superfamily of AAA^+ ATPases. These chaperones are typically composed of hexameric rings. Hsp104 in yeast and other fungi mediates protein disaggregation in cooperation with Hsp70 and Hsp40
Small heat shock proteins	• ~12–45 kDa subunit	ATP-independent chaperones that form large (~1 MDa) heterogeneous oligomers. Subunits contain a conserved α-crystallin domain, which is packed with β-sheets and involved in oligomerization. Ten different forms are present in humans (HSPB1–HSPB10). Prevent aggregation by binding to non-native states ('holdase' function) but also mediate sequestration of misfolded proteins into less-toxic aggregates

HSP, heat shock protein; TPR, tetratricopeptide repeat.

Figure 7. Proteostasis. The proteostasis network consists of three components, protein synthesis and folding (green), conformational maintenance (blue) and degradation (purple). Together, these mechanisms ensure that proteins are folded correctly and that misfolded proteins are removed to prevent them from forming aggregates. a) Around 300 different chaperones mediate the processes of folding, refolding, and disaggregation by cooperating with the autophagosomal-lysosomal and ubiquitin-proteasome systems in humans. b) As proteins fold, they form intramolecular contacts to move towards their thermodynamically stable, native state. Misfolded proteins and folding intermediates need to overcome energy barriers to form functional forms. However, incorrect intermolecular interactions between misfolded proteins can lead to different types of aggregates. Additionally, mutations, stresses such as high temperatures, heavy metals and ROS, translational errors or defects in mRNA can disrupt the correct folding process which can lead to misfolded proteins and protein aggregates. c) In eukaryotes, chaperones that prevent aggregation and promote protein folding are classified into different protein families, including small heat shock proteins (sHSPs), HSP60, HSP70, and HSP90 (Hipp et al., 2019).

1.2.5 Disabled macroautophagy

Autophagy is an evolutionarily conserved physiological process by which cells remove damaged proteins and dysfunctional organelles to maintain intracellular homeostasis. It can be initiated by stresses such as nutrient deprivation, hypoxia and oxidative stress (Chen et al., 2020).

There are three identified types of autophagy: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy, with macroautophagy being a hallmark of ageing (***figure.8*).** All three different types of autophagy are required for the different physiological processes, such as immunity, recycling and clearance of protein aggregates, starvation, fat store metabolisation and cell sensing (Feng et al., 2014).

In microautophagy, cytoplasmic components are degraded through direct invagination of the lysosomal membrane. In contrast, in chaperone-mediated autophagy (CMA), the chaperone protein Hsc70 recognizes a specific pentapeptide motif, known as KFERQ, on unfolded cytosolic proteins and directs them to the lysosome-associated membrane protein type 2A (LAMP-2A). There, the proteins are unfolded at the membrane and translocated into the lysosome for degradation (Badadani, M., 2012). These two types of autophagy can be either selective (targeting damaged organelles) or non-selective, such as during starvation conditions where bulk cytoplasmic material is degraded (Feng et al., 2014).

This maintains quality control by selectively targeting invading protein aggregates and dysfunctional organelles (Kubli and Gustafsson., 2012).

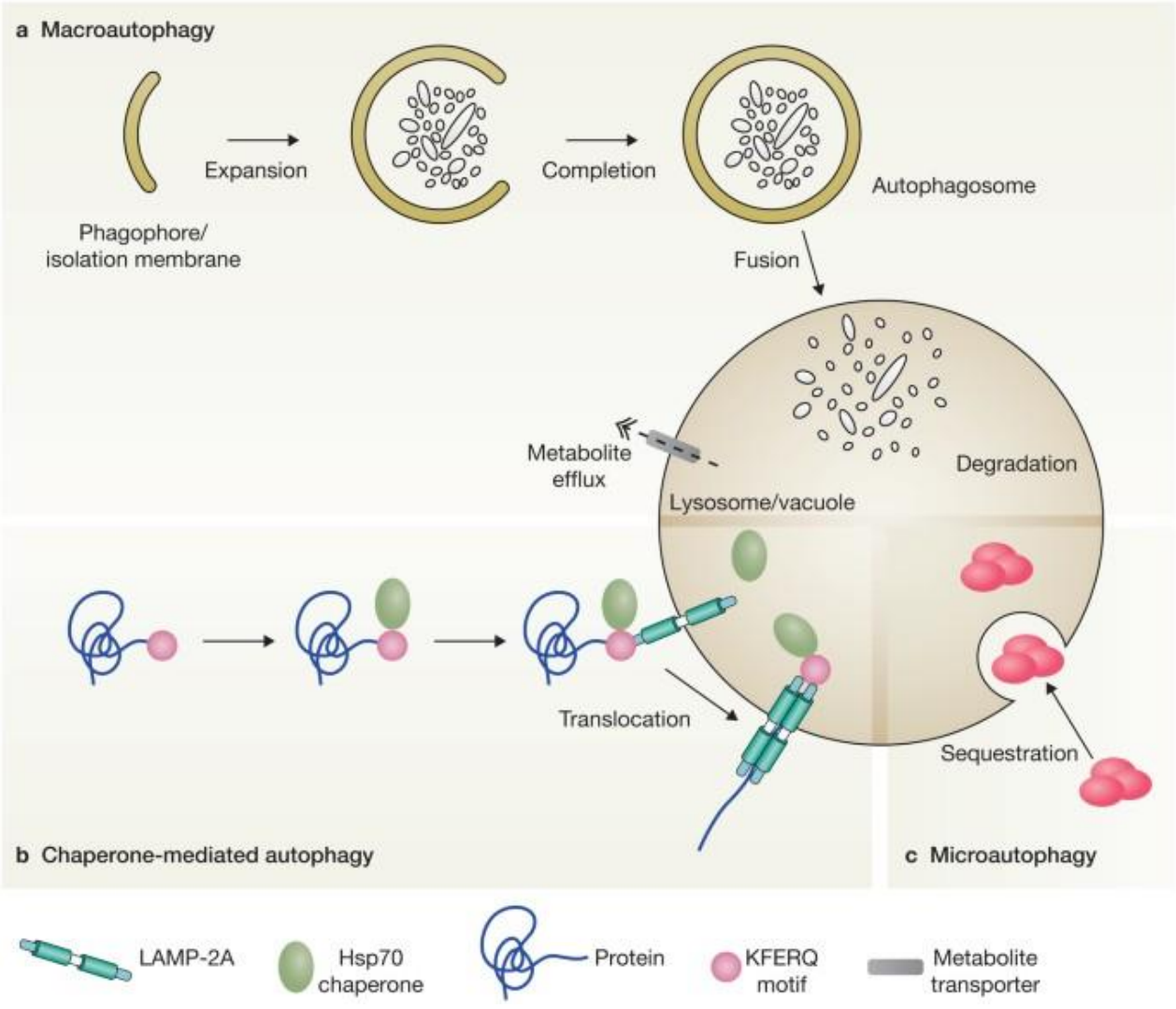
Macroautophagy involves the formation of autophagosomes that are double-membrane structures which sequester cytoplasmic materials and deliver them to lysosomes for degradation. After degradation, resulting macromolecules are released back into the cytosol for re-use. Macro-autophagy is the most studied type because it mediates the large-scale degradation of organelles and large macromolecules. Therefore, has many associated physiological and pathological consequences (Gelino and Hansen., 2012).

In yeast, more than 30 Autophagy-related genes (ATG) required for autophagy have been identified and most of these ATGs are conserved from yeast to mammals. ATGs are organized into five groups to allow initiation, formation, elongation and auto-phagosome fusion with the lysosome (Feng et al., 2014).

Many short-lived model organisms have defects in macroautophagy; for example, *C. elegans* mutants with loss-of-function in *Atg1* (*Unc-51*), *Atg7*, *Atg18*, and *Beclin-1* have a

shortened lifespan (Martinez-Lopez et al., 2015). Atg5 is essential for the early stages of autophagosome formation and plays a critical role in development, cell differentiation, and adaptation to early neonatal starvation. This has been demonstrated in mice, where Atg5-knockout animals die at the neonatal stage, and Atg5-deficient mice develop progressive motor dysfunction due to neurodegeneration. Increasing Atg5 expression has been shown to extend the median lifespan of mice by 17.2% and improve their motor function, possibly due to increasing autophagy (Pyo et al., 2013).

A cell's ability for autophagy declines with age which results in the gradual accumulation of damaged organelles that may disrupt cellular homeostasis and interfere with functioning molecules. As a result, cellular integrity declines leading to age-related disorders, such as cancer, cardiac and neurodegenerative disease (Srivastava, S., 2017).



c)

Group	Components	Function
ULK1/ATG1 kinase complex	ULK1, FIP200, ATG101, ATG13	Autophagy initiation and nucleation
PI(3)P kinase complex	VPS34, Beclin1, ATG14, VPS15	Generation of PI(3)P for autophagosome formation
ATG2/ATG18 complex	ATG2, WIPI2 (ATG18 homolog)	Isolation membrane elongation
ATG9	ATG9	Potential membrane source for isolation membrane nucleation and elongation
Ubiquitin-like conjugation systems	ATG8 family (LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAPL1, GABARAPL2), ATG7 (E1 enzyme), ATG3 (E2 enzyme), ATG10 (E2 enzyme), ATG12, ATG5, ATG16L	Isolation membrane elongation, isolation membrane closure and autophagosome–lysosome fusion

Figure 8. The three different types of autophagy. A) Macroautophagy is the main form of autophagy. It involves the formation of autophagosomes, double-membrane structures containing degradable contents, for sequestration of cytoplasmic materials and their delivery to lysosome for degradation. B) In Chaperone-mediated autophagy chaperone protein Hsc70 recognizes KFERQ, a specific pentapeptide motif on unfolded cytosolic proteins and chaperones them directly to lysosome-associated receptor protein type 2A, where the protein is unfolded at the membrane and translocated to the lysosome. C) Microautophagy refers to the engulfment of cytoplasmic cargo by direct invagination of the lysosomal membrane (Boya et al., 2013). D) Function of evolutionarily conserved ATG proteins in autophagosome formation in mammalian cells. (Zhao, Y.G. and Zhang, H., 2018).

1.2.6 Dysregulated nutrient sensing

In humans, amino acids, glucose, and lipids are important nutrients sensed through different nutrient pathways. This is achieved by molecule binding to its sensor directly or being detected indirectly by a surrogate molecule (Efeyan et al., 2015).

The presence of nutrients promotes growth and reproduction, and excess nutrients are stored. In contrast, when nutrients are limited, catabolic mechanisms save energy and maintain

essential functions. There are multiple nutrient pathways, and they interact with each other ***(figure.9)*** (Yuan et al., 2013)**.** This includes the evolutionarily conserved insulin and insulin-like growth factor 1 (IGF1) pathways, key regulators such as mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), sirtuins (SIRTs), and fibroblast growth factor 21 (FGF21) pathways (Pignatti et al., 2020).

The IIS is a nutrient responsive pathway that promotes morphogenesis, reproductive growth and survival (Badadani, 2012). AMPK serves as an energy sensor and promotes ATP generating pathways during energy stress (increased cellular AMP/ATP ratio) such as glycolysis, while switching off ATP consuming pathways, glycogen and fatty acid synthesis. The AMPK pathway also inhibits mTOR pathway (involved in protein and lipid synthesis) (Yuan et al., 2013). The increased NAD+/NADH ratio during stress activates sirtuins which activate FOXO to increase the expression of antioxidant enzymes and autophagy. On the other hand, FGF21 is synthesised mainly in the liver during starvation and activates fatty acid oxidation, late hepatic gluconeogenesis, lipolysis and inhibits IIS pathway. This is important for starving to refeeding state (Pignatti et al., 2020).

Nutrient responsive pathways have been shown to become impaired with ageing and are implicated in diseases such as diabetes. For example, insulin resistance is commonly observed in older individuals and is associated with metabolic syndromes such as impaired glycogen synthesis, reduced adipose lipogenesis, and unrestrained hepatic gluconeogenesis. With aging, fat distribution also changes (subcutaneous fat decreases while visceral fat in the abdomen increases) leading many older adults to appear abdominally obese. Age-related inflammation in adipose tissue interferes with insulin signalling. Impaired mTOR signalling has been linked with age-related diseases, such as cancer and Alzheimer's (Barzilai et al., 2012).

Lifestyle factors, such as diet, can modify nutrient responsive pathways by influencing metabolic signalling (Yang et al., 2023).

Some studies have suggested a positive role for dietary restriction (DR) in promoting health. This includes intermittent fasting and the reduction of specific nutrient components, such as lipids, carbohydrates, or proteins (López-Lluch, G. and Navas, P., 2016). Calorie restriction (CR), a type of DR, has been studied extensively, with some studies showing beneficial effects, and others reporting no significant impact (Micó et al., 2017). CR refers to a nutritional intervention in which calorie consumption is reduced by lowering total food intake, while avoiding malnutrition (López-Lluch, G. and Navas, P., 2016).

For example, in the Wisconsin National Primate Research Center (WNPRC) study, a 30% CR in adult rhesus monkeys was associated with improved survival and a lower incidence of age-

related diseases. In contrast, the National Institute on Aging (NIA) study on rhesus monkeys found that CR did not extend lifespan, regardless of whether it was initiated in young or old monkeys. These differences in findings may be attributed to variations in experimental design (Micó et al., 2017). Other animal studies have suggested that reducing calorie intake by approximately 20–50% can extend lifespan. In rodents, CR has been shown to improve health, decrease age-related diseases, and increase maximum lifespan by up to 50% (Pifferi et al., 2018). CR may also delay the onset of a wide range of chronic diseases in rodents, including cancer, which is the leading cause of death in this species, accounting for 70–80% of cases (Omodei, D. and Fontana, L., 2011).

CR has been studied extensively in laboratory animals, however, in humans, it is difficult to determine whether CR has beneficial effects on ageing and lifespan. This is due to several reasons, such as the difficulty of conducting randomised, diet-controlled, survival studies in normal-weight individuals for the long-term or difficulty to differentiate if results are due to the better quality of diet or dietary restriction. However, there is some data from epidemiologist studies which supports evidence for the beneficial effects of CR on longevity (Omodei, D. and Fontana, L., 2011). This includes the data of residents of Okinawa (Japan) that had lower mortality rate mortality from coronary heart disease and cancer than the average mainland Japanese and US population. This was because the population of Okinawa consumed fewer calories (1785 kcal/day) than the population on main Japanese island (2068 kcal/day) and US (2980 kcal/day). This may also explain Okinawa having one of the highest numbers of centenarians (50 centenarians per 100,000 residents) in the world (Omodei, D. and Fontana, L., 2011).

The increase in longevity and healthspan observed with CR may be due to its ability to modulate several metabolic pathways, such as mitochondrial function, IIS, autophagy, and mTOR (López-Lluch, G. and Navas, P., 2016).

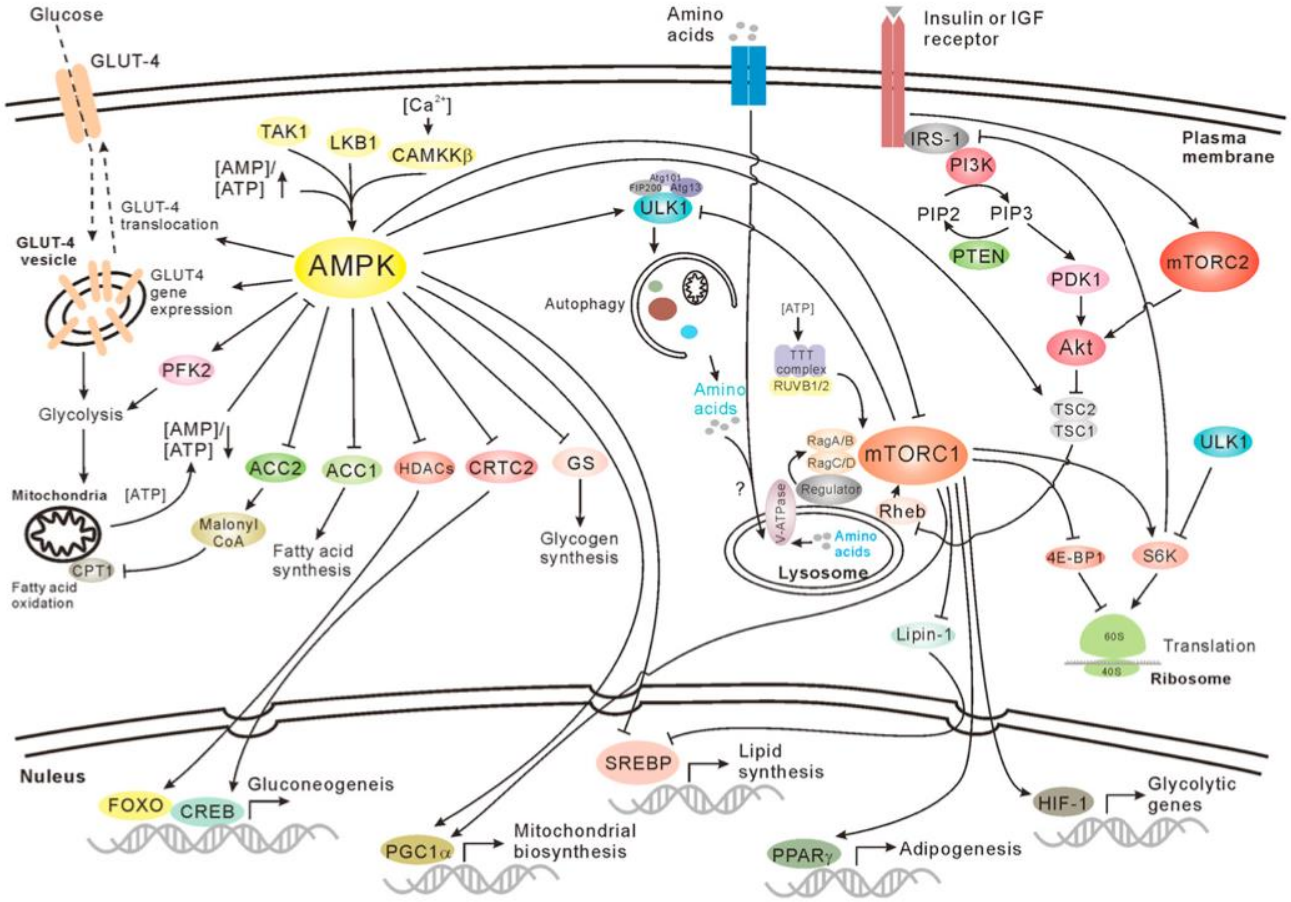


Figure 9. Nutrient sensing pathways. There are multiple nutrient pathways, and they interact with each other. This includes the evolutionarily conserved insulin and insulin-like growth factor 1 (IGF1) pathways, key regulators such as mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). AMPK serves as an energy sensor and promotes ATP generating pathways during energy stress such as glycolysis, while switching off ATP consuming pathways, such as, glycogen and fatty acid synthesis. The AMPK pathway also inhibits mTOR pathway (involved in protein and lipid synthesis) (Yuan et al., 2013).

1.2.7 Mitochondrial dysfunction

Mitochondria generate energy through the production of adenosine triphosphate (ATP). Pyruvate is oxidised in mitochondria by oxygen to carbon dioxide and water, generating 30 ATP molecules per glucose (Alberts et al., 2002). Approximately 50Kg of ATP is produced per day in a healthy adult (Kühlbrandt, W., 2015). Mitochondria are abundant in many cell types and can occupy 10–40% of the total cellular volume. The number varies depending on the cell's energy demand; cells with higher energy requirements contain more mitochondria, for example, they make up 22–37% of the volume in cardiac cells (Hamacher-Brady and Brady., 2016). Moreover, mitochondria regulate calcium signalling, maintain the cellular redox state, and activate the mitochondrial unfolded protein response to degrade protein aggregates during stress (Herst et al., 2017).

Mitochondria are unique as they carry their own genome (mtDNA) which encodes for transfer ribonucleic acid (RNAs) and ribosomal RNAs. mtDNA encodes for multiple subunits of the oxidative phosphorylation complexes (Wang et al., 2016). mtDNA in humans is approximately 16,569 base pairs (bp) in a double-stranded circular molecule containing 37 genes that encode 13 mRNAs, 22 tRNAs, and 2 rRNAs (Srivastava, S., 2017).

Mitochondrial network morphology, changes in number and size is regularly controlled by fusion and fission. Fission splits one mitochondrion into two, whereas fusion occurs when two adjacent mitochondria join. Increased fusion results in extended interconnected mitochondria, whereas fission causes increased mitochondrial fragmentation. Mitochondrial morphology is adjusted by the cell to coordinate between energy demand and availability of resources. Elongated mitochondria are associated with increased ATP production and reduced generation of ROS, whereas fragmented mitochondrial networks are associated with reduced ATP production and mitochondrial uncoupling (Chaudhari and Kipreos., 2017).

Increased mitochondrial fusion or a reduction in mitochondrial fission can protect against mitochondrial autophagy (mitophagy). For example, during starvation, cells with elongated mitochondria are protected from elimination, whereas cells with fragmented mitochondria are selected for degradation (Wang et al., 2016).

With age, spontaneous errors during mtDNA replication can result in point mutations and deletions, which can accumulate and cause mitochondrial dysfunction (Srivastava, S., 2017). One of the most common mtDNA mutation is T414G which has been observed in nearly 50% mtDNA molecules of half of the individuals tested above 65 years. However, it is not clear

whether mitochondrial mutations are a cause of ageing or merely correlated with it (Chocron et al., 2019). Age-related defects in mitophagy, as well as mitochondrial fission and fusion processes, can lead to the accumulation of damaged or dysfunctional mitochondria. This is associated with increased production of ROS, which are by-products of mitochondrial respiration. Elevated ROS levels contribute to cellular damage by affecting macromolecules, ultimately reducing respiratory chain activity and ATP generation, and leading to the deterioration of cellular function and protective responses ***(figure.10)*** (Srivastava, S., 2017).

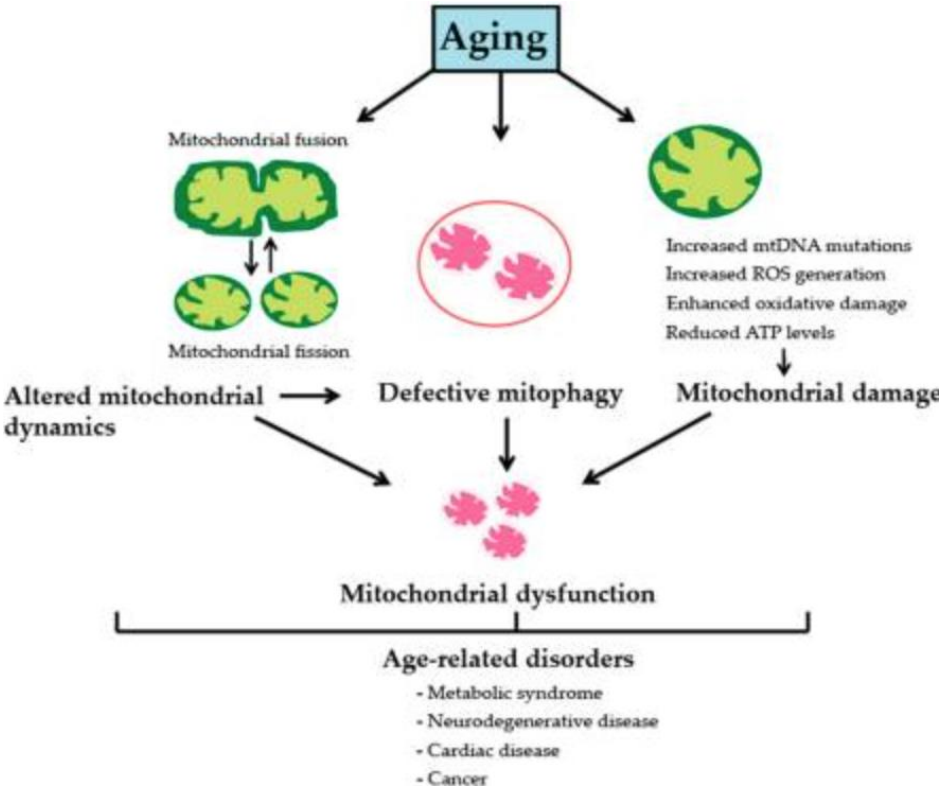


Figure 10 Age related mitochondrial dysfunction. Age-related defects in mitochondrial function include defective mitophagy, altered mitochondrial dynamics (fission and fusion processes) and mitochondrial damage caused by excessive ROS production and increased mtDNA mutations (Srivastava, S., 2017).

1.2.8 Cellular senescence

Cellular senescence was first described in vitro in 1961 and refers to the permanent arrest of proliferation caused by possible triggers, such as DNA damage, telomere dysfunction and oncogenic stressors ***(figure.11).***

Damage to the DNA triggers a DNA damage response, which is mediated by phosphorylated histone H2AX (γH2AX), 53BP1, and MDC1, along with the apical kinases ataxia telangiectasia mutated (ATM) and ATR, and the downstream kinases CHK2 and CHK1. This signalling activates p53, resulting in cell cycle arrest. The increased expression of cell cycle inhibitory proteins, called cyclin-dependent kinase inhibitors such as p16 and p21, maintains proliferative arrest. Furthermore, senescent cells are resistant to apoptotic cell death due to the upregulation of antiapoptotic proteins of the BCL-2 family (Di Micco et al., 2021).

Senescent cells release 'senescence-associated secretory phenotype' (SASP). These are pro-inflammatory cytokines, growth factors and chemokines to activate the innate immune response. Immune cells such as macrophages remove the SASP-producing senescent cells by phagocytosis to prevent tumour development. However, with increasing age, senescent cells are not removed effectively and start to accumulate. The accumulation of senescent cells increases SASP levels to potentially trigger cellular senescence in bystander cells (Haynes, L., 2020). Cells need to continuously proliferate in order to replace damaged cells and therefore senescent cell accumulation may result in tissue ageing (Di Micco et al., 2021). In mice, targeting cellular senescence has been shown to improve age-related bone loss, physical dysfunction, and neurodegenerative diseases (Justice et al., 2019).

Idiopathic pulmonary fibrosis (a chronic lung disease) is one of the age-related diseases associated with cellular senescence. Drugs that selectively induce apoptosis in senescent cells by transiently disabling the senescent cell anti-apoptotic pathways are known as senolytics. The impact of the senolytic combination of Dasatinib and Quercetin (DQ) was tested in fourteen participants. The three-week pilot study showed improvement in physical functions such as the six-minute walk test, five repeated chair stands, and the pulmonary FEV1 test (forced expiratory volume in one second). SASP factors were evaluated using multiplex analysis, microarray, and targeted ELISA with serum or plasma samples before and after treatment. The effects of treatment on SASP factors were inconclusive, but there was a correlation between changes in function and changes in SASP-related microRNAs and pro-inflammatory cytokines, including interleukin-6, the matrix remodelling protein MMP7, and

the metallopeptidase inhibitor TIMP2. Although the study could not confirm whether lung senolytics directly clear senescent lung cells to improve pulmonary function in humans with IPF, it suggested that senolytics may alleviate the functional consequences of age related diseases in humans and supports the potential use of DQ in larger randomised controlled trials for senescence related diseases (Justice et al., 2019).

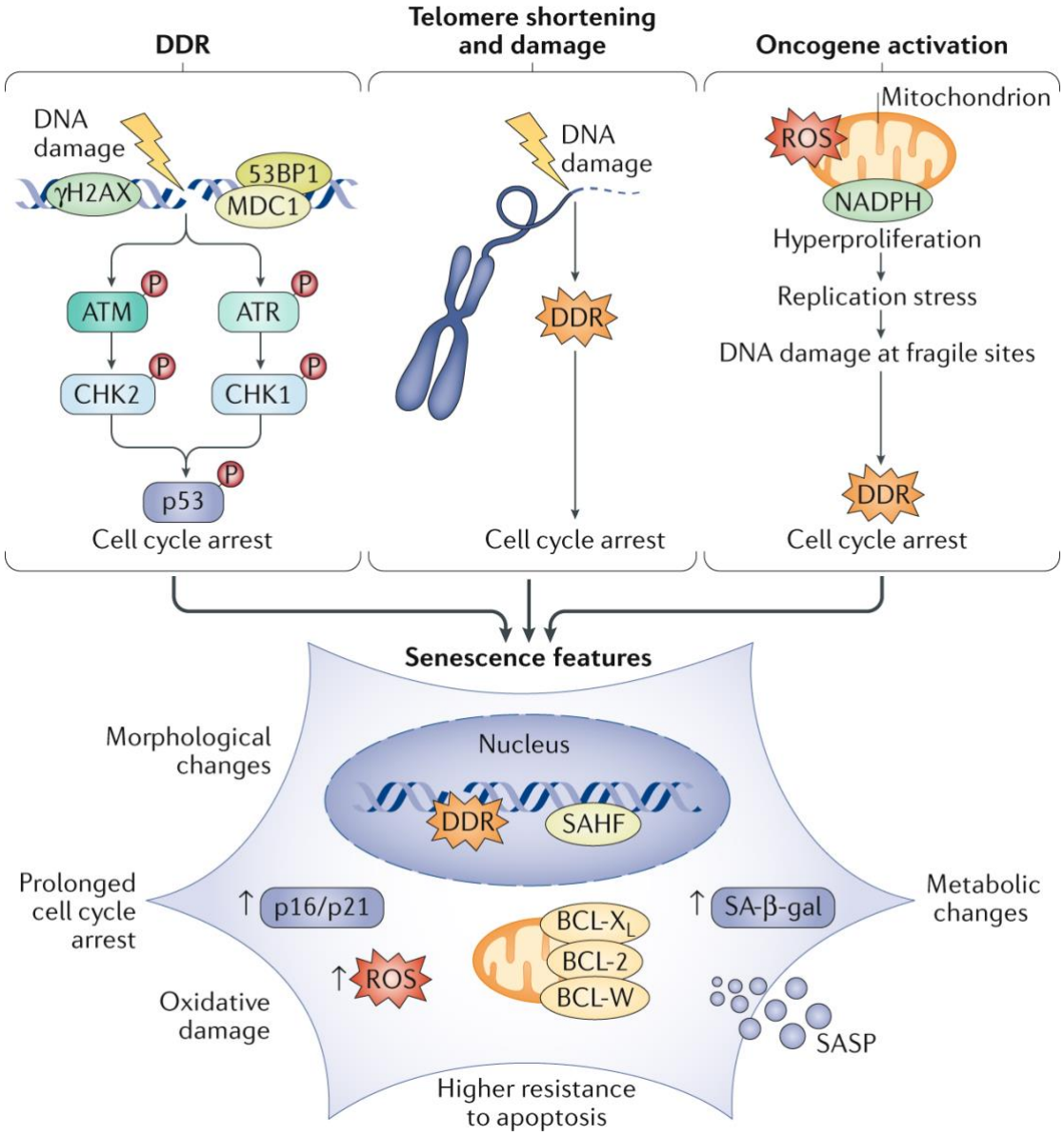


Figure 11. Cellular senescence. DNA damage, telomere shortening and oncogene activation triggers DNA damage response resulting in cell cycle arrest. This is mediated by phosphorylated histone H2AX (γH2AX), 53BP1 and MDC1, the apical kinases ataxia telangiectasia mutated (ATM) and ATR and the downstream kinases CHK2 and CHK1 which signal p53 activation. Senescence features include metabolic changes characterised by increased senescence-associated-β-galactosidase (SA-β-gal)

accumulation, senescence-associated heterochromatin foci (SAHF) and SASP, higher resistance to apoptosis by upregulation of antiapoptotic proteins of the BCL-2 family, oxidative damage detected by increased levels of ROS, and prolonged cell cycle arrest by upregulation of p21 and p16 cell cycle inhibitors (Di Micco et al., 2021).

1.2.9 Stem cell exhaustion

Stem cells are undifferentiated cells with the ability to regenerate into a variety of different cells in various tissues and organs. Stem cells are characterised into pluripotent stems cells and adult stem cells. Pluripotent stem cells include embryonic stem cells derived from the inner cell mass of the embryos and induced pluripotent stem cells which are derived from reprogramming somatic cells. Pluripotent cells can differentiate into cells derived from all three germ layers (endoderm, mesoderm, and ectoderm), whereas adult stem cells originate from their embryonic counterparts and are multipotent or unipotent and therefore can only produce tissue-specific terminally differentiated cells (Tian et al., 2023).

Most adult organs contain adult stem cells in their specific micro-environments (niches) which can be used for regular tissue homeostasis and cell renewal in response to an injury. Cells in different organs have different cell turnover, for example neurons in the brain and cardiomyocytes in the heart last a lifetime, whereas epithelial cells of the skin are continuously lost and replenished quickly. These cells with rapid turnover rates are replaced by adult stem cells. The cells in the blood are regenerated by haematopoietic stem cells located in the bone marrow and epithelial cells that line the gut are regenerated by intestinal stem cells located in crypts of the intestine (Brunet et al., 2023).

However, with ageing there is a decline in stem cell regenerative potential and ability to give rise to differentiated cells in the tissue ***(figure.12).*** Studies on the expansion and in vitro differentiation potential of stem cells from human adipose tissue have shown that advancing age negatively impacts stem cell function (Garay, R.P., 2023). This is associated with a deterioration of tissue integrity and health. In addition, with ageing there is an increase in the number of quiescent adult stem cells that have decreased ability to activate. On the other hand, some adult stem cells can undergo excessive activation or become senescent (Brunet et al., 2023).

Most mammals have non-renewable cells such as neurons. However, some organisms, such as hydra, can be viewed as non-ageing organisms, as they can fully control damage accumulation

through constant cell renewal and are therefore virtually immortal in lab settings. (Gladyshev et al., 2021).

Stem cells from the umbilical cord, adult mesenchymal stem cells from human bone marrow, and those from adipose tissue are being investigated in clinical trials to slow or reverse normal ageing processes. Stem cells from bone marrow are obtained in lower yields compared to those from adipose tissue (Garay, R.P., 2023).

Some of these trials focus on physical frailty, which is defined as age-associated decline in locomotive activity and immune dysfunction. Up to 15% of individuals over 65 years old and 25% of those over 85 years old are considered frail (Garay, R.P., 2023). Currently, there are no effective treatments for frailty. The safety of bone marrow mesenchymal stem cells administered via peripheral intravenous infusion was assessed in a non-randomised, dose-escalation study involving 15 patients over the age of 60. The treatment was well tolerated across different dose groups, and no treatment related serious adverse events were observed during the 12-month follow-up period. Inflammatory biomarkers and the 6-minute walk test were assessed at 3 and 6 months. The 6-minute walk distance increased at both time points, and TNF-α levels decreased at 6 months (Golpanian et al., 2017). The Phase II randomised, double-blinded trial in 30 patients showed similar results, with 100M being a superior dose level compared to 200M (Tompkins et al., 2017).

In mice, transplantation of mesenchymal stem cells from the bone marrow of young mice significantly slowed bone density loss and prolonged the lifespan of old mice, likely by improving the functions of multiple vital organs. In contrast, transplantation of stem cells from old animals had no such effect, reiterating that with advancing age, stem cells lose their ability to adhere, proliferate, and differentiate. Whereas, stem cells from young donors can actively migrate and differentiate into various cell types (Shen et al., 2011).

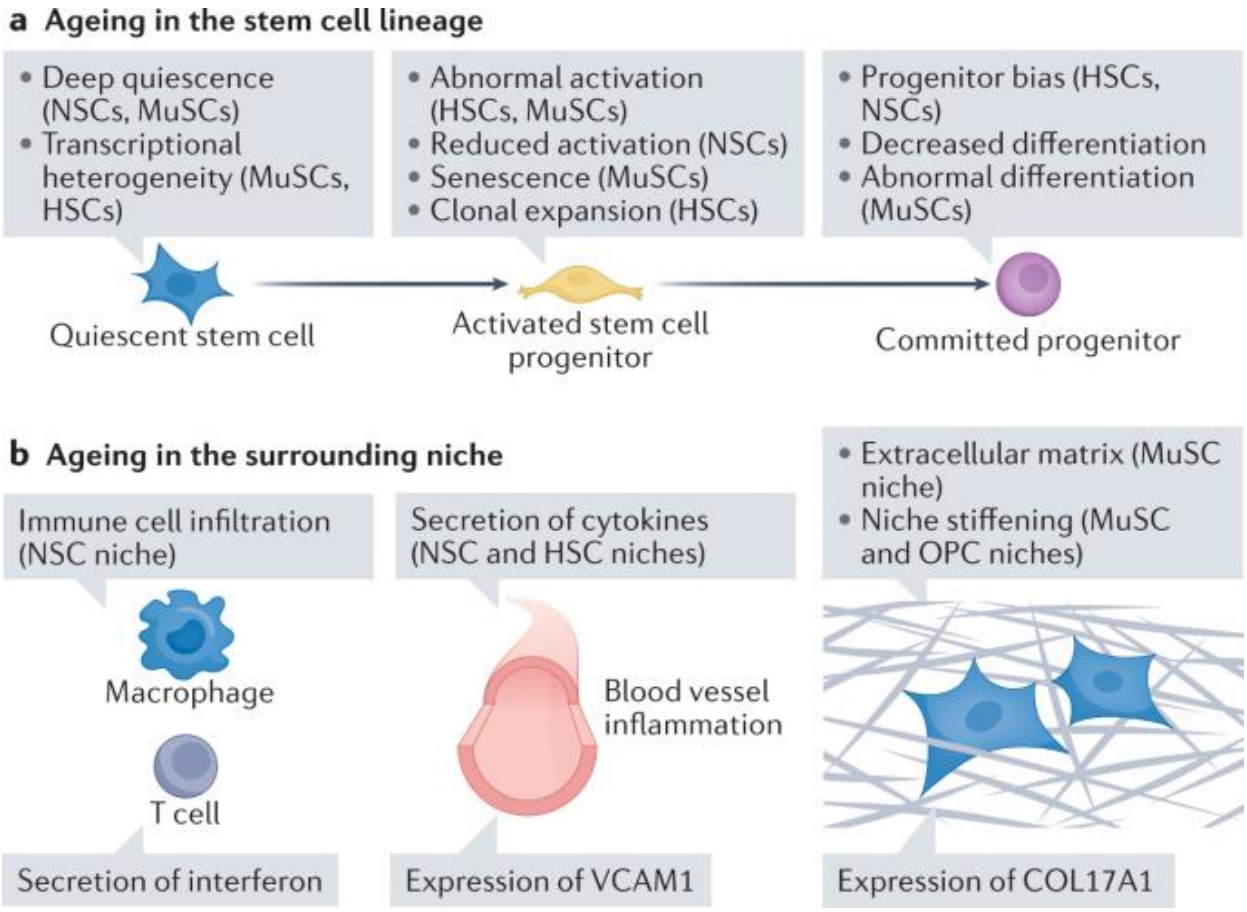


Figure 12. Stem cell exhaustion with ageing. a) With ageing, stem cells start to lose their ability to activate and enter a deep quiescence stage. Some become abnormally activated, while others exhibit reduced activation and clonal expansion. b) With ageing, there are also changes in the stem cell surrounding niches, such as immune cell infiltration, secretion of cytokines, and niche stiffening (Brunet et al., 2023).

1.2.10 Altered intercellular communication

Intercellular communication refers to the way cells communicate with each other, both directly through interactions between neighbouring cells and indirectly via different signalling molecules ***(figure.13)***. The four types of cell-cell interactions are autocrine, paracrine, juxtracrine, and endocrine. Autocrine signalling occurs when cells secrete ligands that bind to their surface receptors to induce a cellular response. Paracrine signalling happens when cells secrete signalling molecules that diffuse from one cell to another without direct contact. Juxtracrine signalling is contact-dependent; cells pass signalling molecules to each other through gap junctions or membrane nanotubes without secreting the molecules into the

extracellular space. Endocrine signalling refers to signalling molecules, such as hormones, being released into extracellular fluids, such as blood plasma (Armingol et al., 2021).

The most studied type of intercellular communication are soluble factors which are released by cells and affect the function of neighbouring cells. This includes proteins, growth factors, chemokines, and cytokines found in blood or extracellular matrix that can easily cross cell membrane (Tan et al., 2021).

Altered intercellular communication includes increased SASP levels, resulting in chronic inflammation and passive senescence of healthy cells, as well as SASP-related extracellular vesicles (EVs), which amplify senescent signals in paracrine, autocrine and endocrine ways. EVs are small lipid bilayer membrane vesicles released by cells found in mostly all bloody fluids (Yin et al., 2021). They contain a wide range of biomolecules, including proteins, lipids, metabolites, and nucleic acids. Evs may play a role in age-related diseases, such as cancer, and are related to other hallmarks of ageing (Romero-García et al., 2023). For example, cancer cells with the *HRAS* oncogene exhibit considerable genetic instability, as indicated by the formation of micronuclei. These cancer cells release EVs that carry genomic DNA, including oncogenic sequences, and transfer this material to endothelial cells. When EVs are taken up by primary endothelial cells, they induce abnormal micronuclei formation, as well as increased cell migration and proliferation (Chennakrishnaiah et al., 2020).

In mice, miR-192 levels in circulating EVs increase with age. Administration of the pro-inflammatory cytokine IL-6 (elevated in the serum of aged mice compared to young mice) led to an increase in miR-192 levels in the serum EVs of young mice. This finding indicates that the age-associated hyperinflammatory state contributes to elevated miR-192 levels. EV-associated miR-192 exhibited anti-inflammatory effects on macrophages, suggesting that circulating EVs can regulate immune responses (Tsukamoto et al., 2020). Treatment of aged mice with small EVs derived from adipose-derived stem cells of young mice resulted in reduced frailty and improvements in motor coordination, fatigue resistance, fur regeneration, grip strength, and renal function (Sanz-Ros., 2022).

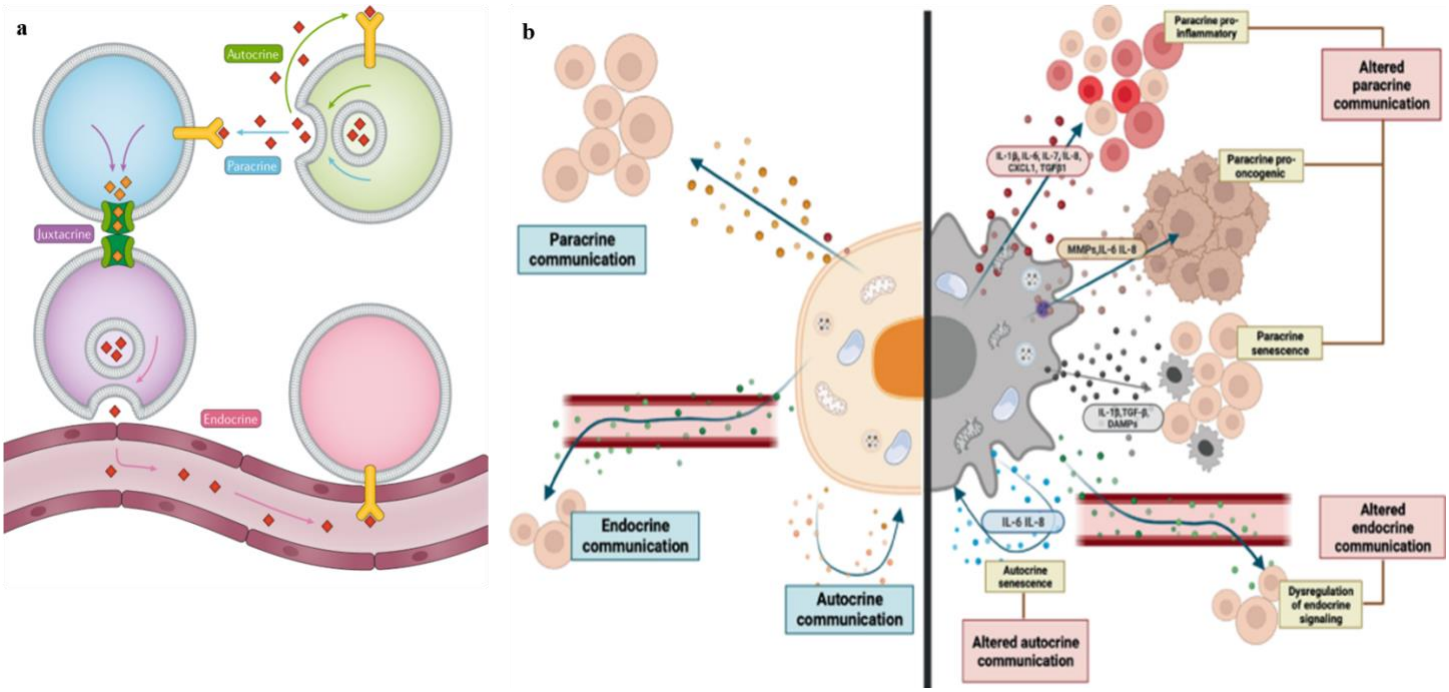


Figure 13. Cell-Cell interactions. a) Autocrine signalling: Cells secrete ligands that bind to their surface receptors to induce a cellular response. Paracrine signalling: Cells secrete signalling molecules that diffuse from one cell to another without direct contact. Juxtracrine signalling: This is contact-dependent; cells pass signalling molecules to each other through gap junctions or membrane nanotubes without secreting the molecules into the extracellular space. Endocrine signalling: Signalling molecules, such as hormones, are released into extracellular fluids, such as blood plasma (Armingol et al., 2021). b) Altered cellular communication results in pro-inflammatory cytokines and SASPs (Mas-Bargues et al., 2014).

1.2.11 Chronic inflammation

Inflammaging is the chronic activation of the immune system which causes an increase in low-grade inflammation. With age, molecular damage such as changes in the composition of the microbiome, mitochondrial dysfunction and defective autophagy could trigger inflammation (Haynes, L., 2020).

Healthy older people have elevated blood pro-inflammatory biomarkers, such as interleukin cytokines, C-reactive protein (CRP), transforming growth factor-β, tumour necrosis factor and serum amyloid A, suggesting that with age there is an increase in inflammation (Ferrucci, L. and Fabbri, E., 2018). This could suggest a trade-off between the early beneficial effects of inflammation and its later negative outcomes (Franceschi et al., 2000).

Inflammaging was first described in 2000 by Claudio Franceschi who argued that antigens were a type of stressors and continuous load of stress provokes ageing. The theory is based on

two-hit hypothesis ***(figure.14).*** The 'first hit' is that over time, inflammatory reactions accumulate and result in increased susceptibility to disease. The 'second hit' is the presence of frail gene variants or the absence of robust gene variants. Individuals with high inflammatory status, combined with the absence of robust gene variants or the presence of frail gene variants, may be more susceptible to certain age-related diseases than others (Franceschi et al., 2000).
Immunosenescence results in a decrease in naive T and B lymphocytes, the accumulation of memory and effector T and B cells, the production of defective antibodies, and chronic low-grade inflammation (Puzianowska-Kuźnicka et al., 2016).
The plasma levels of IL-6 are undetectable in young individuals but start to increase at age 50-60. High levels of IL-6 with age have been detected in both healthy individuals and those with pathologies. Proinflammatory cytokines stimulate the synthesis of CRP in the liver (Puzianowska-Kuźnicka et al., 2016). Therefore, IL-6 and CRP are used as indicators of inflammation. Puzianowska-Kuźnicka et al. measured the levels of IL-6 and CRP in healthy individuals aged 65 years or older to examine their association with physical and cognitive performance and to determine if these levels could be used as predictors of mortality in healthy individuals. They found that higher levels of IL-6 and CRP were associated with poor cognitive performance and increased mortality risk (Puzianowska-Kuźnicka et al., 2016).

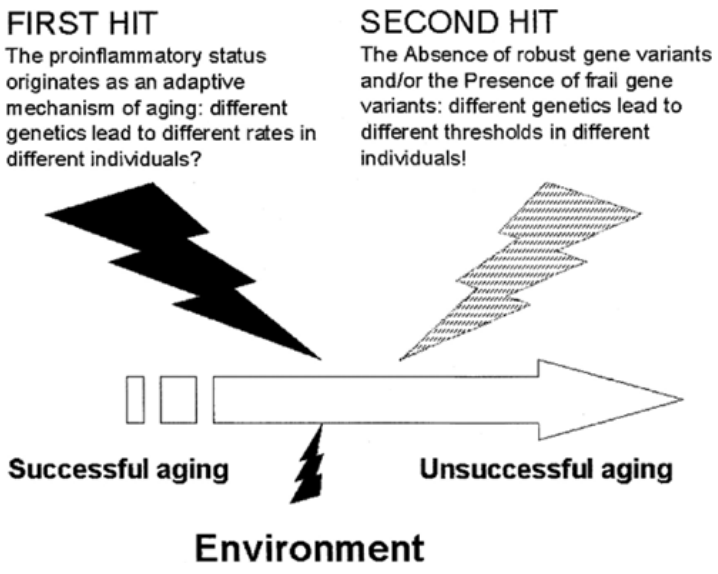


Figure 14. The two-hit theory of inflammaging. The first hit refers to the accumulation of inflammatory reactions over time, resulting in increased susceptibility to disease. The second hit refers to the absence of robust genes and/or the presence of frail gene variants, which leads to age-related diseases such as atherosclerosis and osteoarthritis (Franceschi et al., 2000).

1.2.12 Dysbiosis

The human body contains an estimated 100 trillion microbes (bacteria, fungi, viruses), most of which are found in the gut, skin, and mucosal environments (Krishna et al., 2019). The genome of all the microorganisms inside the human body is known as the microbiome, whereas microbiota refers to the microbes found in a certain environment (Zheng, D., Liwinski, T. and Elinav, E., 2020).

The human microbiome plays a vital role in host immunity and therefore is studied extensively. In 2007, the National Institutes of Health launched the human microbiome project to analyse the role of the human microbiome in health and disease (Turnbaugh et al., 2007). The composition of the microbiome is determined by host genetics, lifestyle and diet which results in individual variability among the population (Vaga et al., 2020).

Gut microbiota comprises thousands of microbial species that provide the host with various advantages, such as protection against pathogens, vitamin production and homeostasis maintenance. *Bacteroidetes, Firmicutes,* and *Actinobacteria* are the main bacterial families that dominate a healthy human gut (Vaga et al., 2020). Fungi, viruses, and archaea are also present in the gut (Amon, P. and Sanderson, I., 2017).

Microbiome density and composition differs at different sites with an organ. The upper region of the respiratory tract is more densely populated compared to the lower region. The gastrointestinal tract, jejunum, caecum, and colon are densely populated whereas the stomach, duodenum, and ileum have low population densities. However, microbiota composition can be alerted by localised and systemic changes which may lead to dysbiosis (loss of beneficial bacteria, overgrowth of opportunistic bacteria) resulting in various pathologies such as inflammatory bowel disease, diabetes and obesity (Vaga et al., 2020).

The gut epithelium acts as a barrier between gut microbes and systemic circulation. However, with age, there is a decline in intestinal integrity, resulting in microbial leakage into the systemic circulation. This could result in increased levels of pro-inflammatory cytokines ***(figure.15)*** (Nagpal et al., 2018).

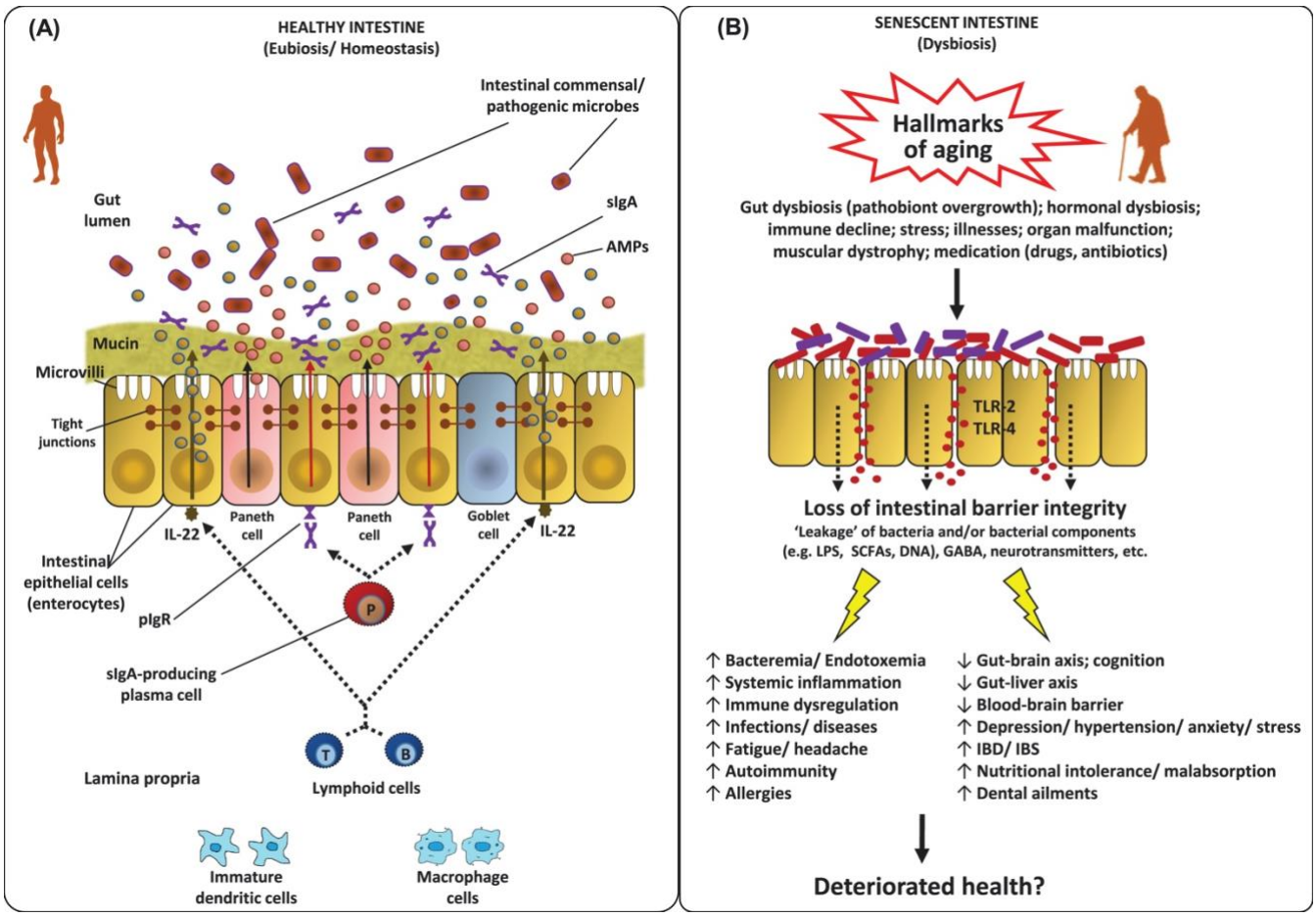


Figure 15. Healthy Intestine vs. Senescent Intestine: With age, there might be a loss of intestinal integrity, resulting in the leakage of bacteria into systemic circulation. This eventually leads to an increased antigenic load and subsequent inflammaging (Nagpal et al., 2018).

1.3 Ageing theories

Over the years many theories of ageing have been proposed ***(figure.16)*** (Libertini, G., 2019). The different theories of ageing can be broadly divided into those based on evolutionary principles and those based on molecular damage. Evolutionary theories, such as Mutation Accumulation (MA) and Antagonistic Pleiotropy (AP), focus on how genes influence ageing through trade-offs that benefit early-life fitness at the expense of late-life health (Johnson et al., 2019).

In contrast, molecular damage theories propose that ageing results from the gradual accumulation of cellular and molecular damage over time, with many of the underlying mechanisms being evolutionarily conserved (Guo et al., 2020). This includes, telomere attrition

theory of ageing, free radical theory of ageing, rate of living theory and the membrane pacemaker theory of ageing. Other theories include Disposable soma theory and hyperfunction theory of ageing.

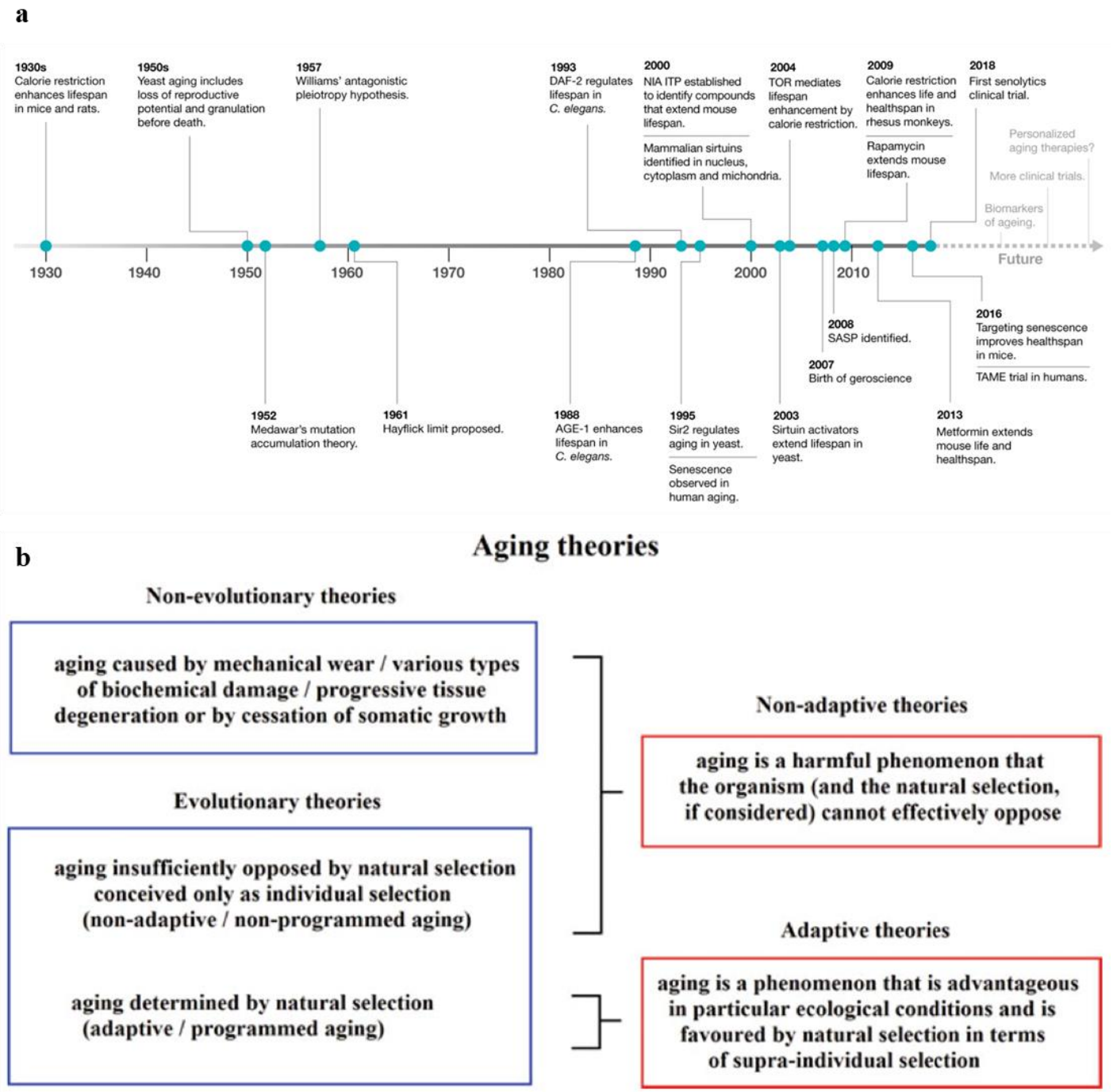


Figure 16. a) Timeline of research in ageing. This includes notable discoveries, such as the role of calorie restriction, DAF-2, AGE-1, sirtuins, rapamycin and metformin, and ageing theories, such as mutation accumulation, antagonistic pleiotropy and telomere attrition (Campisi et al., 2019); b) Evolutionary and non-evolutionary ageing theories. Evolutionary theories suggest that ageing could be the result of accumulation of detrimental mutations over time due to a decline in natural selection potency after reproduction. Non-evolutionary theories suggest that ageing maybe the result of increase in molecular damage with age (Libertini, G., 2019).

1.3.1 Evolutionary theories of ageing

1.3.1.1 Mutation accumulation theory

Mutation Accumulation (MA theory) was proposed by Peter Medawar in 1952 which suggests that ageing is the result of the accumulation of detrimental mutations over time due to a decline in natural selection potency after reproduction (Medawar, P.B., 1952). This was demonstrated through work by Michael Rose in 1984, which showed that culturing *Drosophila* populations at later ages increases female longevity, enhances late fecundity, and reduces early fecundity (Johnson et al., 2019). Various other studies in *Drosophila* have also shown that late-reproducing flies live almost twice as long as early-reproducing flies (Campisi et al., 2019). As most organisms in nature would die from accidents, diseases and predators, etc before reaching old age, there is no need for the organisms to be robust beyond the age of reproduction and survive into old age (Kyriazis, M., 2020). Therefore, there is little to no evolutionary pressure to favour genetic changes that slow ageing and extend lifespan (Johnson et al., 2019). This results in random detrimental mutations, predominantly in the germline, to accumulate and elevate the risk of chronic degeneration. Some mutations would have already been passed onto the offspring, but the effects would become apparent only in later life leading to ageing-related phenotypes (Kyriazis, M., 2020).

However, this theory does not appear to apply to all organisms; for example, naked mole rats maintain high reproductive potential even in old age (Johnson et al., 2019). Reznick et al. examined guppies (*Poecilia reticulata*) from high- and low-mortality environments in Trinidad and found that guppies from high-predation environments exhibited slower rates of ageing and showed no significant differences in reproductive senescence compared to those from low-predation areas (Reznick et al., 2004).

1.3.1.2 Antagonistic pleiotropy theory

The MA theory was developed further by George C. Williams in 1957 into AP, which proposes that certain genes have both, beneficial and detrimental effects on the organism. In early life, when selection pressure is strong, these genes provide health benefits, however later in life when selection pressure is weaker the same genes become detrimental. AP differs from MA, as it implies that the AP genes are involved throughout the ageing process and explains that

genetic trade-offs are the cause of ageing. The priority is to assure a large healthy number of off-springs and therefore, mechanisms have evolved to protect the organism while young, but with time these genes start to lose their protective effect and become detrimental (Johnson et al., 2019).

An example is Huntington's disease (HD), an autosomal dominant neurodegenerative disorder that increases fecundity. HD is caused by an excessive number of CAG trinucleotide repeats within the *Huntingtin* (*HTT*) gene. The symptoms of the disease, including uncontrolled and involuntary movements, typically appear after reproductive age (30-45). Individuals with HD have been shown to have more offspring compared to unaffected individuals. For example, one study comparing Huntington's patients with 170 related unaffected individuals found that the patients had, on average, 39% more offspring than their unaffected siblings and 18% more than an unrelated control group matched for age and sex (Carter, A.J. and Nguyen, A.Q., 2011).

Another example is Apolipoprotein E alleles, which in early life are positively involved in immune responses but later in life are involved in vascular diseases (Johnson et al., 2019).

Van Exel et al. found that the APOE ε4 allele provides an advantage in terms of survival and fertility in high pathogenic conditions within a rural Ghanaian population. They observed a non-significant, but positive, survival benefit and higher fertility and offspring survival in women exposed to high pathogen levels. This may explain why APOE ε4, despite being linked to Alzheimer's and atherosclerosis, is found globally at frequencies from 5% to 45%, with higher prevalence in African populations, indicating benefits in certain environments (van Exel et al., 2017). However, this theory does not account for species with negligible senescence, such as *Hydra*, which exhibit little to no decline in function or reproduction with age (Johnson et al., 2019).

1.3.2 Molecular damage focused theories of ageing

1.3.2.1 Telomere theory of ageing

The Telomere Theory of Ageing was proposed in 1971 by Alexey Olovnikov and was based on the DNA end-replication problem discovered by Leonard Hayflick and Moorhead (Olovnikov, A.M., 1973).

In 1961, Leonard Hayflick and Moorhead demonstrated that normal cell replication is finite, after which cells stop dividing and are removed by programmed cell death. This is known as

the Hayflick limit (Hayflick, L. and Moorhead, P.S., 1961). Cancer cells such as HeLa cells are an exception to this as they are immortal. Hayflick experiments revealed that human fibroblasts cells could only undergo a specific number of population doublings after which they stop dividing and old cells could potentially remember the number of times they had divided even when surrounded by younger cells. This was demonstrated by mixing equal numbers of human male and female fibroblasts. The female fibroblasts added to the mixture were young as they had only divided a few times, but the male fibroblasts added to the mixture were old as they had divided many times. When the control mixture of only male containing fibroblasts had stopped dividing it was compared to the mixed culture of male and female fibroblasts, which showed only the presence of female fibroblasts. Therefore, older male fibroblasts stop dividing before younger female fibroblasts suggesting that cells have a finite capacity to divide, and the presence of younger cells does not affect the division of older cells suggesting that cells remember the number of times they divide. The finite capacity of cells to divide is based on the rounds of DNA replication during which the ends of the linear DNA (Telomeres) are not copied (Shay, J.W. and Wright, W.E., 2000). Olovnikov proposed that telomere shortening, resulting from the inability of DNA polymerase to fully replicate chromosome ends, eventually leads to cellular senescence and therefore artificially lengthening telomeres may delay ageing (Olovnikov, A.M., 1973).

Greider and Blackburn discovered telomerase in 1985. They observed that the ciliate *Tetrahymena thermophila* contained the six-base sequence TTGGGG, repeated 20–70 times (Blackburn, E.H. and Gall, J.G., 1978) and telomeres lengthened steadily by approximately 4–10 base pairs per cell generation over the course of 200–300 generations (Greider, C.W. and Blackburn, E.H., 1985). This suggested that telomere length can increase, and that the replication of telomere ends is not accomplished solely by conventional DNA replication enzymes. Instead, it likely involves a terminal transferase-like activity capable of adding telomeric repeats to chromosome ends. To test this, they added a synthetic single-stranded DNA primer to cell-free extracts of *Tetrahymena* and observed elongation of the unlabelled DNA by the addition of repeated six-base sequences. Up to 30 repeats were added, indicating the presence of an enzyme that could extend telomeres. This enzyme was later named telomerase (Greider, C.W. and Blackburn, E.H., 1989).

This discovery led to a surge in telomerase research and in 1989, Gregg Morin demonstrated telomerase activity in human HeLa cells, showing that the enzyme adds the human-specific telomeric repeat sequence TTAGGG to chromosome ends (Morin, G.B., 1989).

However, there are several criticisms of this theory, such as that not all cells in the body proliferate constantly and therefore would not lose telomere length. On the other hand, cells that do proliferate regularly have active telomerase activity to maintain the length of the telomere. Moreover, cells such as fibroblasts that proliferate occasionally may not reach the Hayflick limit in the lifetime of an animal (Blagosklonny, M.V., 2006).

1.3.2.2 Free radical theory of ageing

One of the most studied ageing theory is probably the free radical theory of ageing (Vina et al., 2013). The 'free radical theory of ageing' was proposed by Denham Harman in 1956, which anticipated an association between oxidative stress and ageing (Harman, D., 1955). This suggested that increased ROS as by-products of mitochondrial respiration lead to cellular damage and consequently deterioration of cellular function and protective responses (key ageing phenotypes). Elderly individuals have shown increased levels of peroxides (oxidative stress marker) and reduced antioxidant power (measured by iron-reducing power) (Gorni, D. and Finco, A., 2020).

However, there is conflicting evidence for this theory which links the reduction in ROS levels to both an increase and a decrease in lifespan. Nonetheless, 'free radical theory of ageing' has provided the basis of vast majority of the ageing research and has led to significant advances in the understanding of ageing (Vina et al., 2013). And, mitochondria remain a prevalent organelle to study ageing, due to their integral role in cell homeostasis of almost every tissue. Mitochondria are related to the main aspects of ageing, such as cellular senescence, decline in stem cell functions, metabolism, and inflammation (Yang and Hekimi., 2010).

1.3.2.3 The rate of living theory of ageing

The rate-of-living theory was proposed by Raymond Pearl in 1928 (Wright, S., 1928).

In 1908, Max Rubner, suggested that a slower metabolism may be associated with longevity. This was based on the observation that larger animals tend to outlive smaller ones (Ferrucci et al., 2012). He examined the energy metabolism and lifespans of five domestic animals (guinea pig, cat, dog, cow, and horse) as well as humans, and found that energy expenditure per gram

was approximately constant across these species. This suggested that total metabolic energy consumption per lifespan is fixed (Escala, A., 2022).

This idea was supported by Pearl's discovery that *Drosophila melanogaster* live much longer at low temperatures than at high temperatures, leading to what became known as the rate-of-living theory (Hulbert, A.J., 2010).

Over the years, this relationship has been partially confirmed in many mammals, birds, ectotherms, and some unicellular organisms. However, larger datasets have shown that total metabolic energy expended per lifespan per unit of body mass is not constant across different animal classes. (Escala, A., 2022).

Basal metabolic rate (BMR) refers to the daily energy required to maintain structural and functional homeostasis and accounts for up to 60%–70% of total energy expenditure. The relationship between BMR and mortality was examined in healthy participants of the Baltimore Longitudinal Study of Ageing (BLSA). The study included 1,227 individuals (972 men and 255 women) enrolled between 1958 and 1982, with over 40 years of follow-up for mortality. An age-related decline in BMR was observed, which was associated with an increased risk of mortality. The rate of BMR decline accelerated at older ages. However, Participants who died had a higher BMR compared to those who survived, independent of age (Ruggiero et al., 2008). This could be due to that people with chronic conditions may require additional energy for recovery, repair, and homeostatic restoration. For example, in chronic respiratory disease, the energy cost of ventilation may be significantly higher, suggesting that people in poorer health require more energy than healthy individuals (Ferrucci et al., 2012).

1.3.2.4 The membrane pacemaker theory

The membrane pacemaker theory suggests that variation in the fatty acid composition of cell membranes influences the rate of aging through its effect on lipid peroxidation (Hulbert et al., 2005).

Lipids are categorized into three main groups: fatty acids, phospholipids (including glycerophospholipids and sphingolipids), and neutral lipids (such as triglycerides and cholesteryl esters). Fatty acids can be saturated, monounsaturated, or polyunsaturated. In eukaryotic cells, the ratio of saturated to unsaturated fatty acids is approximately 40:60, which plays a key role in determining membrane fluidity and flexibility. This composition also contributes to selective permeability and affects the membrane's susceptibility to peroxidation.

During ageing, the accumulation of ROS can lead to lipid peroxidation, resulting in membrane damage (Mutlu et al., 2021).

Fatty acids vary in their susceptibility to peroxidation, with polyunsaturated fatty acids being significantly more prone to peroxidation than saturated and monounsaturated fatty acids. Docosahexaenoic acid (DHA), found in mammalian cell membranes, is the most polyunsaturated fatty acid and, therefore, highly prone to peroxidation. For example, DHA is approximately 320 times more susceptible to peroxidation than monounsaturated oleic acid. Naked mole-rats (NMRs) exhibit a lifespan approximately nine times longer than that of mice. Although mice and NMRs have similar total amounts of unsaturated fatty acids, NMRs possess about one-ninth the amount of DHA compared to mice. This lower DHA content may lead to reduced membrane peroxidation in NMRs, potentially contributing to their extended lifespan (Mitchell et al., 2007).

With age, lipid remodelling has been shown to occur at the plasma membrane in humans. For example, Cabré et al. found an increase in saturated fatty acid content and a decrease in polyunsaturated fatty acid content beginning at around 50 years of age, followed by an anti-inflammatory response (Cabré et al., 2018).

1.3.3 Other ageing theories

1.3.3.1 Disposable soma theory (DST)

Disposable Soma Theory (DST) was proposed by Thomas Kirkwood in 1977 and suggests that there is a finite amount of energy available which means there is a trade-off in the body between mechanisms involved in repair and reproduction (Kirkwood, T.B., 1997) . Continuation of life is a priority and because organisms could be killed by external causes, energy is prioritised for reproduction during early life. This compromises the energy available for bodily repairs, leading to inadequate repair of functions over time which results in the deterioration of the body. One of the main criticism of DST is based on its hypothesis that if less energy (food) is available organism lifespan would be reduced as less energy is available for somatic repairs. However, CR has provided evidence that lowering calorie intake increases lifespan (Johnson et al., 2019).

Moreover, using *C. elegans*, Ermolaeva et al. showed that when certain stresses, such as heat and oxidative stresses, are applied to the germ line, they can protect the soma through an innate

immune response. This suggests that there is not a unidirectional path from germline to soma, but rather trans-cellular signalling (Ermolaeva et al., 2013).

When DNA is damaged, the germ line mitogen-activating protein kinase (MPK) triggers a protective cascade that ultimately results in the secretion of an extracellular signal from the germ line to the soma. This activates the ubiquitin-proteasome system (UPS) in somatic tissues. This leads to the soma increasing protein turnover through the UPS in response to the stress signal (Ermolaeva et al., 2013).

In humans, it has not been shown that there is a trade-off between fertility and lifespan. Kuningas et al. looked at menarche and menopause, along with mortality, in 3,575 married female participants and found no association between fertility and age at menarche and menopause. Moreover, women who had two or three children lived longer than those who did not have any or had many children. This could be because repeated pregnancies have detrimental effects on lipid and glucose metabolism, which may increase the risk of stroke, coronary artery disease, and diabetes. Mechanisms that lead to infertility may have detrimental effects on other processes, resulting in higher mortality (Kuningas et al., 2011). Corbo et al. looked at the impact of longevity genes, including ACE, PON1, PPAR-γ, and APOE, on fertility in 151 participants and found that the APOE*2 allele results in a trade-off with fertility (Corbo et al., 2008).

1.3.3.2 Hyperfunction theory of ageing

A newer theory 'hyperfunction' developed by Blagosklonny suggests that rather than the accumulation of molecular damage, ageing could be the result of hyperfunctions driven by inappropriate activation of signalling pathways such as mTOR (Blagosklonny, M.V., 2006). Blagosklonny defines hyperfunction as an unchanged or a decrease of function which is still higher than the optimal level required for longevity (Blagosklonny, M.V., 2021).

The theory suggests that molecular damage accumulates with age, and both molecular damage and cellular hyperfunctions occur in parallel. However, hyperfunction may cause death long before the damage accumulates to a sufficient level to cause lethal pathologies. This is based on studies that have shown that Rapamycin increases lifespan in short-lived mice by inhibiting the mTOR signalling pathway and therefore, hyperfunction could be life-limiting. In comparison, molecular damage occurs in parallel to hyperfunction but progresses slowly to reach the death threshold. Some studies have shown that a decrease in molecular damage does

not prolong lifespan and in some cases, mild levels of molecular damage increase lifespan possibly by inhibiting mTOR (Blagosklonny, M.V., 2021).

Most age-related pathologies observed are caused by hyperfunction. This includes the late-life pathologies associated with hypertrophy, such as cancer due to hyperplasia and heart diseases due to hypertension (Gems, D. and de la Guardia, Y., 2013). Therefore, ageing could be defined as the sum of age-related diseases caused by cellular hyperfunctions (Blagosklonny, M.V., 2021).

Evolutionary ageing theories suggest that ageing is not genetically programmed, however, identification of genes that can increase lifespan demonstrates that ageing could be programmed. Hyperfunction theory suggests that as ageing can be altered by growth and development control pathways, it is quasi-programmed. This means that the ageing is the potential unpremeditated continuation of the growth and development program (Gems, D. and de la Guardia, Y., 2013).

1.4 Major molecular pathways underlying ageing

Over the years, the research on ageing has uncovered multiple molecular pathways, some of them interlinked, that are involved in the modulation of ageing and longevity.

In 1939, it was discovered that restriction of caloric intake increases lifespan. In 1988, it was discovered that mutations in *age-1*, determined the lifespan of *C. elegans.* The *age-1* mutant worms had an average increase in lifespan by 40–60%. Further work discovered the importance of IIS and mTOR signalling, in lifespan extension. IIS and mTOR signalling pathways are the major pathways involved in growth, metabolism and nutrient sensing (Campisi et al., 2019).

IIS and mTOR longevity pathways are interlinked with mTOR, FOXO transcription factors and sirtuins (NAD+-dependent deacetylases) being the key regulators of these signalling pathways which control gene transcription and post-translational protein activity. CR lowers IGF-1 secretion resulting in downstream inhibition of mTOR and activation of FOXO. Inhibition of mTOR complex 1 (mTORC1) enhances catabolic processes, such as apoptosis and autophagy, while FOXO proteins regulate the transcription of autophagic genes and genes that promote resistance to oxidative stress. Similarly, the AMPK pathway is activated during nutrient-limited conditions and works by inhibiting mTOR and stimulating FOXO (Zhang et al., 2020).

1.4.1 Insulin/Insulin-like signalling (IIS) pathway

The IIS is a nutrient responsive pathway that promotes morphogenesis, reproductive growth and survival (Badadani, 2012). IIS is evolutionarily conserved from worms to mammals and is also involved in longevity ***(figure.17)*** (Altintas et al., 2016).

Insulin-like growth factor-1 (IGF-1) was first identified in the late 1950s during studies investigating the mechanisms of growth hormone action (Salmon Jr, W.D., 1957).

The study demonstrated that sulfate incorporation into cartilage was not a direct action of growth hormone, but rather an indirect process mediated by a 'sulfation factor'. This led to the conclusion that a hormonally regulated serum factor stimulated sulfate incorporation into cartilage in *vitro*, indicating its role as a mediator of growth hormone effects (Werner, H., 2023). Further research led to the identification, in 1976, of two peptides isolated from human serum that exhibited insulin-like activities. These peptides were subsequently renamed from sulfation factors to insulin-like growth factors I and II (IGF-I and IGF-II) (Rinderknecht, E. and Humbel, R.E., 1976).

Both IGF-1 and IGF-2 bind to the transmembrane insulin-like growth factor 1 receptor (IGF1R). Ligand binding activates the receptor's intrinsic tyrosine kinase activity, leading to phosphorylation of insulin receptor substrate 1 (IRS1). This, in turn, activates two major downstream signalling pathways: the RAS–MAPK (ERK) pathway, which promotes cell proliferation, and the PI3K–PDK1–Akt/PKB pathway, which is primarily involved in cell survival and metabolic regulation (Werner, H., 2023).

The role of IIS in mediating longevity was first discovered in *C.elegans* where inhibiting IIS in worms doubled their lifespan (Klass, M.R., 1983). In *C. elegans*, insulin-like peptides (ILP) activate insulin receptor DAF-2 which phosphorylates the FOXO transcription factor, DAF-16. Phosphorylated DAF-16 is retained in the cytoplasm, but under stress conditions such as starvation, heat or oxidative stress, is unphosphorylated and translocated to the nucleus where it activates the transcription of genes involved in development and longevity (Princz et al., 2020).

In the presence of nutrients, DAF-2 inhibits DAF-16 promoting growth and reproduction, whereas nutrient limitation downregulates signalling through DAF-2 allowing activation of DAF-16. In the absence or limitation of food, *C. elegans* larvae can undergo development arrest and enter dauer state or L1 arrest. DAF-16 activity promotes dauer formation in the early larval stage and in adults causes fat accumulation, reduces the reproductive rate and increases

lifespan. *daf-2* mutants partially express L2 arrest phenotype even in the presence of food which means they are better at persisting in harsh conditions compared to the wild-type. Additionally, *daf-2* mutants live twice as long as the wild-type. Therefore, stress resistance is linked with an increase in lifespan (Altintas et al., 2016).

Daf-2 and *age-1* are the two key components of the IIS pathway that are involved in ageing. *Daf-2* encodes the insulin receptor and *age-1* encodes the phosphatidylinositol-3-OH kinase (PI3K). ILP binds to its receptor, DAF-2 which in turn recruits an insulin receptor substrate IST-1 leading to the activation of AGE-1. Activated AGE-1 increases the level of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) which provides signals to activate the downstream kinase cascade, composed of 3-phosphoinositide-dependent protein kinase 1 (PDK-1) protein kinase B (AKT-1/-2) and serum and glucocorticoid-inducible kinase-1 (SGK-1). This, in turn, phosphorylates and inactivates DAF-16 by promoting its nucleus to cytosol translocation. Conversely, in unfavourable conditions, IIS is downregulated and DAF-16 is activated and translocated from the cytoplasm to the nucleus, where it switches on the expression of genes that promote longevity (Altintas et al., 2016).

Moreover, *daf-2* mutants have elevated autophagy activity that can be observed through electron microscopy showing increased autophagy vesicles. Beclin 1 (autophagy protein) RNAi knockdown shortens *daf-2* lifespan, demonstrating that autophagy activity is essential for *daf-2* longevity. Mutations in this pathway in human centenarian suggest that longevity pathway is conserved in humans (Nakamura and Yoshimori, 2018).

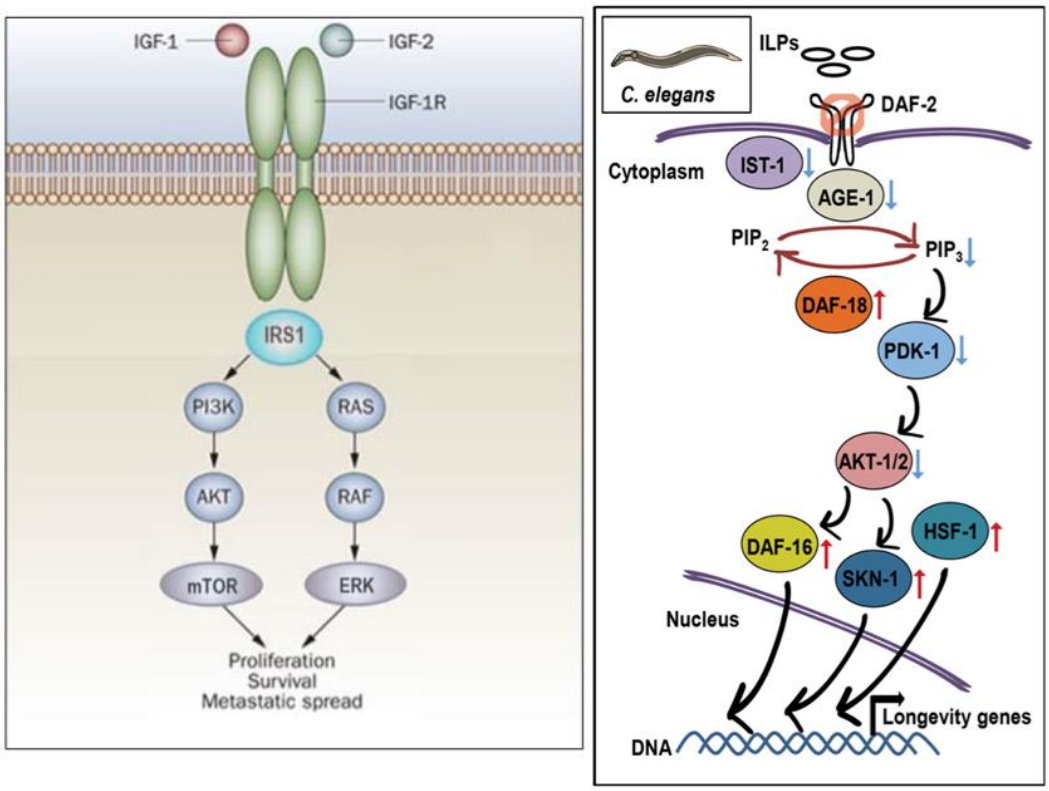


Figure 17. a) Insulin-like signalling pathway in humans. IGF-1 binding activates the IGF-1R receptor's intrinsic tyrosine kinase activity, leading to phosphorylation of insulin receptor substrate 1 (IRS1). This, in turn, activates two major downstream signalling pathways: the RAS–MAPK (ERK) pathway, which promotes cell proliferation, and the PI3K–PDK1–Akt/PKB pathway, which is primarily involved in cell survival and metabolic regulation (Werner, H., 2023). b) Insulin-like signalling pathway in C. elegans. Downregulation of Insulin signalling in unfavourable conditions can promote longevity through the activation of DAF-16. In favourable conditions, insulin-like peptide binds to its receptor, DAF-2 activating downstream kinase cascade which inactivates DAF-16 to promote growth and reproduction (Altintas et al., 2016).

1.4.2 Mechanistic target of Rapamycin (mTOR) pathway

The mTOR signalling pathway is an evolutionary conserved serine-threonine kinase that directs cellular responses by responding to nutrients and growth factors (Weichhart, T., 2018). mTOR is involved in various cellular processes, such as growth, proliferation and autophagy (Srivastava., et al., 2019).

Rapamycin (isolated from soil bacterium in the 1970s) can directly form a complex with FK506-binding protein 12 (FKBP12) to inhibit the activity of mTOR which give it its name-

mechanistic Target of Rapamycin. mTOR is composed of two functionally different proteins complexes - complex 1 (mTORC1) and mTORC2 (Weichhart, T., 2018). mTORC1 is sensitive to nutrient availability including amino acids, cholesterol, and glucose, whereas mTORC2 is primarily sensitive to signalling from hormones, such as insulin and leptin (Dumas, S.N. and Lamming, D.W., 2020).

At the cell membrane, growth factor receptors such as insulin/IGF-1 receptor or the epidermal growth factor (EGF) receptor activate tyrosine kinase adaptor molecules ***(figure.18).*** This recruits PI3K which phosphorylates phosphatidylinositol 4,5-bisphosphate [PI(3,4,5)P2] to generate phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. PI(3,4,5)P3 recruits and activates the serine-threonine kinase Akt which targets tuberous sclerosis 2 (TSC2). TSC2 then forms a complex with TSC1 and inhibits mTORC1 activity (Weichhart, T., 2018).

The correlation between longevity and mTOR inhibition was first demonstrated in *C. elegans* and *Drosophila melanogaster*, followed by experiments in yeast strain *S. cerevisiae* in which the inhibition of mTOR with rapamycin doubled lifespan (Weichhart, T., 2018).

Old mice treated with mTOR inhibitors have shown improvement in age-related immunosenescence, which results in an increased rate of infections, such as respiratory tract infections, one of the leading causes of death in the elderly over the age of 85 (Chen et al., 2009). Mannick et al. examined the inhibition of the TORC1 pathway and its impact on ageing by using a low dose mTOR inhibitor in 264 elderly participants aged 65 and above. The results showed that inhibition of the TORC1 pathway decreased the rate of all infections, improved influenza vaccination responses, and up-regulated antiviral immunity. The authors suggest that the results were dose dependent. At higher doses, mTOR inhibitors suppress T cell proliferation, resulting in increased infections, whereas at lower doses, mTOR inhibitors that partially inhibit TORC1 activity enhance immune function and subsequently decrease infection rates in the elderly (Mannick et al., 2018).

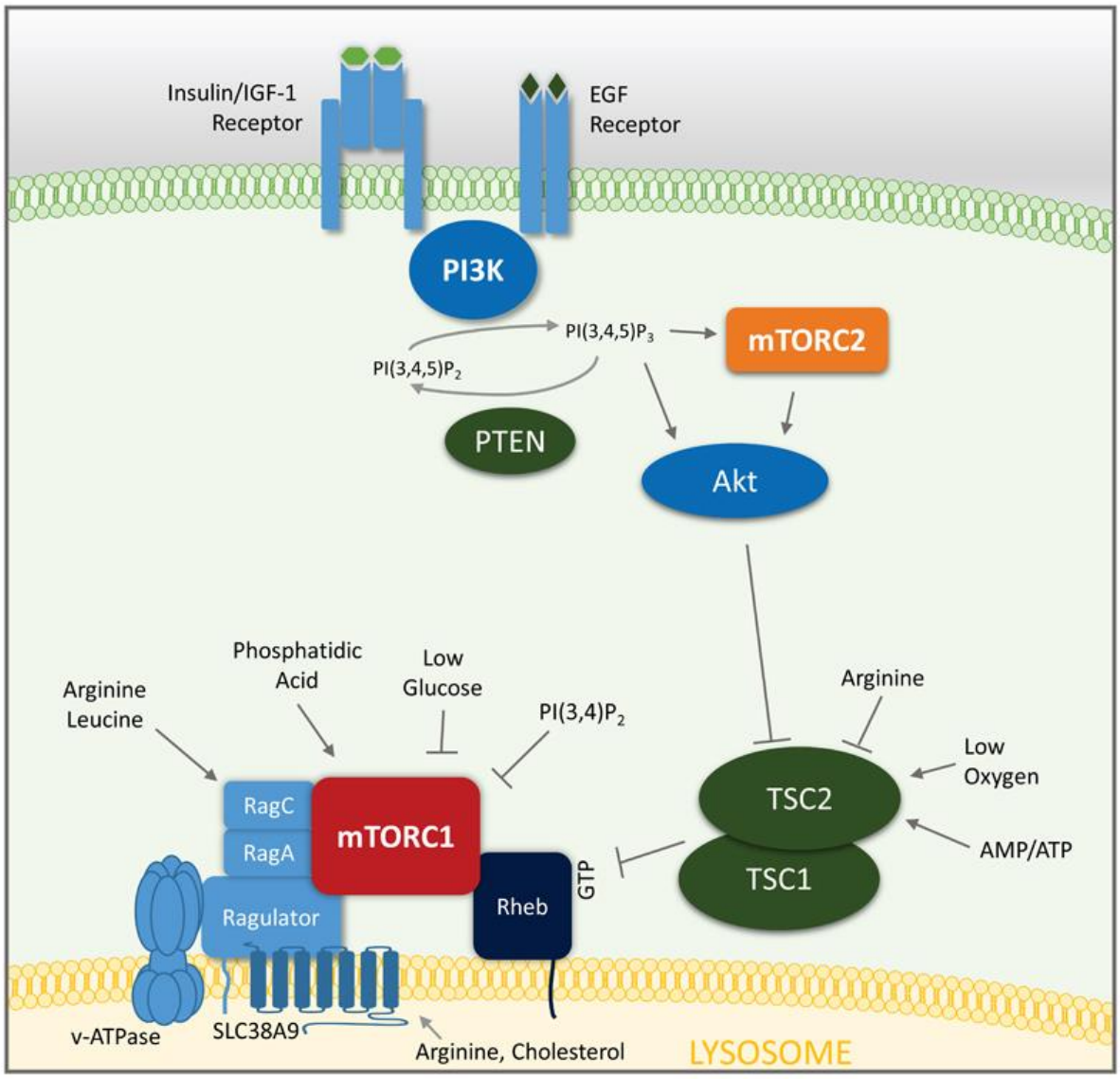


Figure 18. Mechanistic target of Rapamycin (mTOR) signalling pathway. Growth factor receptors insulin/IGF receptor or the epidermal growth factor (EGF) receptor activate tyrosine kinase adaptor molecules. This recruits PI3K which phosphorylates PI(3,4,5)P2 to generate PI(3,4,5)P3. PI(3,4,5)P3 recruits and activates Akt which targets tuberous sclerosis 2 (TSC2) to inhibit mTORC1 activity (Weichhart, T., 2018).

1.5 Relationship between Ageing and Microbiota

1.5.1 *Changes in* microbiota composition with age

The most drastic changes in microbiota composition occur in infancy and early childhood due to the plasticity of the infant microbiota ***(figure.19)*** (Nagpal et al., 2018)**.** This is because early life events related to birth such as the gestational age (full-term or premature) and mode of delivery (vaginal birth or caesarean section) make new-borns more susceptible to environmental invasions. Moreover, the type of feed (breast milk or formula feeds), maternal nutritional status (overweight or undernourished) and the use of antibiotics can all cause changes in the intestinal microbiome of an infant. These changes during the early life development of the new-born microbiota mainly due to maternal microbiota play an important role in maintaining host immune system throughout life and can impact health later in life (Amon, P. and Sanderson, I., 2017).

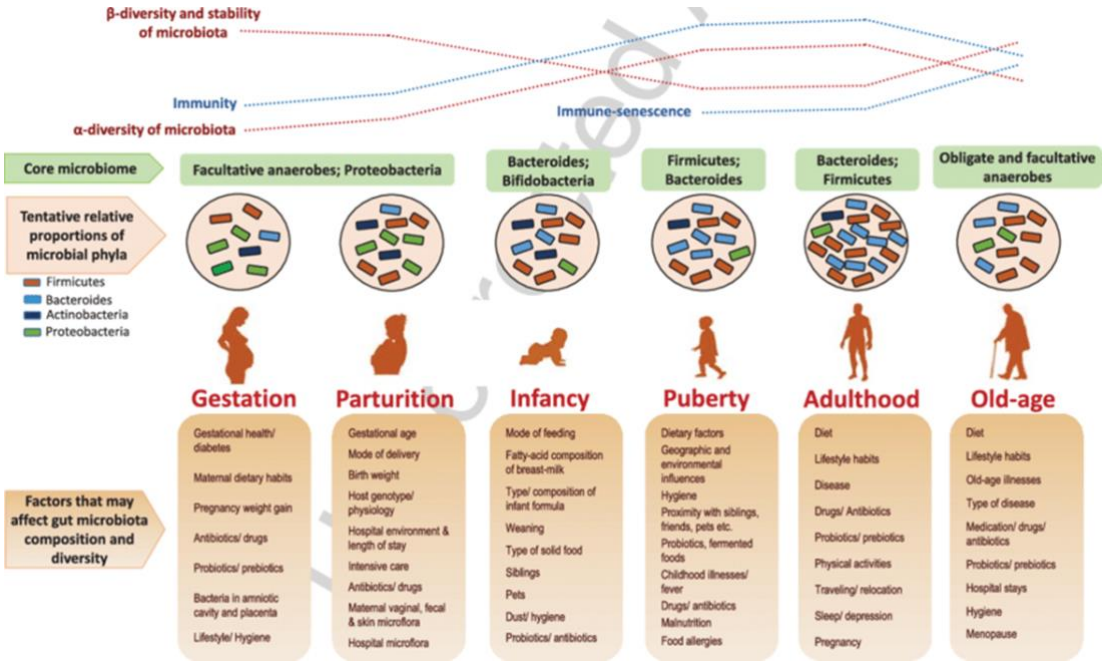


Figure 19. Changes in microbiota composition with age. The human gut microbiota development begins at birth due to microbial exposure from the mother and the environment. The changes in microbiota during early life could also impact health later in life. The human microbiome fluctuates during different stages of life, with prominent changes during old age, which could explain lower immunity at old age (Nagpal et al., 2018).

1.5.2 Microbiota relationship with the immune system

Microbiome is involved in the maturation of the immune system and stimulation of the innate and adaptive immune responses in response to pathogens ***(figure.20)*** (Belkaid, Y. and Harrison, O.J., 2017).

Commensal microbes in the human body have co-evolved to form a host symbiotic relationship which is dependent on the proper functioning of the host immune system. This prevents commensal microbes from over-exploitation of the host resources while maintaining immune tolerance to harmless stimuli. For example, the early life microbial colonisation in the intestine is important for the establishment of immunoregulatory networks which protect from induction of mucosal IgE (allergy susceptibility) (Zheng, D., Liwinski, T. and Elinav, E., 2020). Allergies and autoimmune response are the result of the failure of controlling misallocated immune responses against self and environmental antigens (Belkaid, Y. and Harrison, O.J., 2017).

Furthermore, studies in germ-free animals have demonstrated that the absence of commensal microbes could lead to immune defects such as intestinal defects of lymphoid tissue and microbial colonisation incudes Th17 cells which are absent in GF mice (Zheng, D., Liwinski, T. and Elinav, E., 2020).

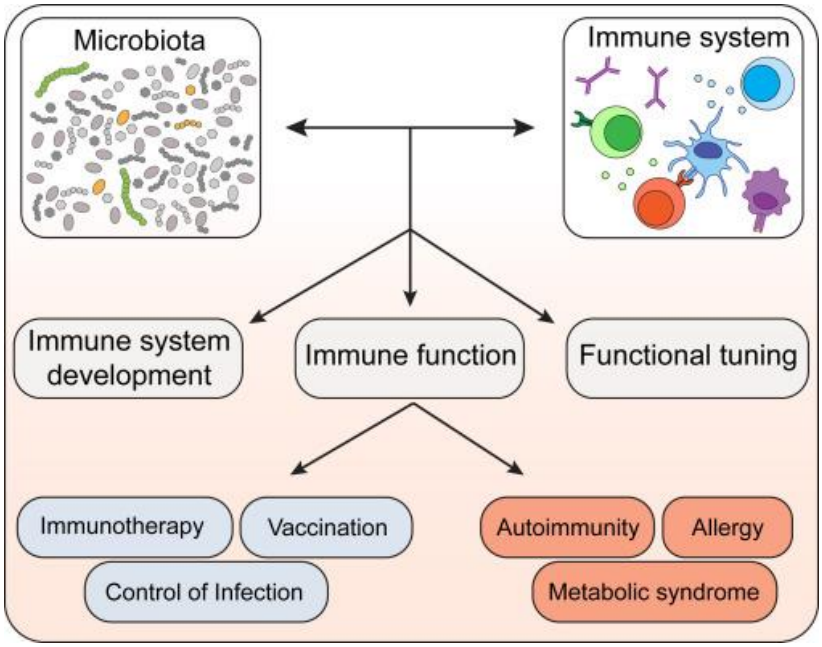


Figure 20. Microbiota is involved in the development and functional tuning of the immune system. Host symbiotic relationship between the microbiota and the human body controls infection and prevents from autoimmunity and allergies (Belkaid, Y. and Harrison, O.J., 2017).

1.5.3 The gut microbiota impacts ageing and stress resistance

Gut microbiota regulates stress responses through gut-brain axis. Animal studies have linked changes in the gut microbiota with several stress-related conditions and the work in GF mice has demonstrated a link between microbiota and anxiety-like behaviour. However, the precise role of the microbiota in the gut-brain signalling and mechanism through which microbiota acts is not fully understood (Foster, J.A., Rinaman, L. and Cryan, J.F., 2017).

The gut microbiota modulates small RNAs involved in hippocampal gene expression to influence the development of hippocampus and therefore is essential for appropriate miRNA control of gene expression in hippocampus. The miRNA expression affected by microbial colonisation and sex, which shows that the gut microbiota may impact CNS in a sex specific manner (Moloney et al., 2017).

Moreover, due to its impact on immunity, microbiome is considered a determinant of healthy ageing. Biagi et al studied the changes in gut microbiota composition with ageing by comparing microbiota analysis of semi-supercentenarians, in comparison to adults, elderly, and centenarians. They showed that the core microbiota of symbiotic bacterial taxa (*Ruminococcaceae, Lachnospiraceae,* and *Bacteroidaceae* families) decreases in abundance with age (Biagi et al., 2016). The interaction between the microbiota and the host affects ageing with the abundance and diversity of microbiota decreasing with age. This decrease in the core microbiota groups is associated with increased frailty which makes the older population susceptible to comorbidities related to the gut bacteria (Wu et al., 2021).

However, increasing evidence suggests that age-related dysbiosis could be corrected with appropriate microbiome-based interventions. Centenarians have shown a considerable increase in *Verrucomicrobia* with a reduction in *Proteobacteria*. Barcena et al were able to transplant *Verrucomicrobia Akkermansia muciniphila* into progeroid mouse models which enhanced health and lifespan in these animals (Barcena et al., 2019).

1.5.4 Gut microbiota and heat stress

A diverse microbial composition is necessary for the optimal regulation of host signalling pathways. Variable temperatures can affect gut microbiota composition, disrupting microbiota-host homeostasis and potentially inducing disease pathology. Heat stress negatively impacts intestinal function by altering microbiota metabolites (Wen et al., 2021).

During heat stress, the body redirects visceral blood flow to the peripheral circulation to dissipate heat. This reduces blood flow to the gastrointestinal tract, resulting in low oxygen levels, which disrupts the balance between ROS production and the antioxidant system, leading to epithelial damage and an inflammatory response. Additionally, it can increase intestinal barrier permeability by damaging tight and adherens junctions, allowing pathogens to enter the bloodstream and activate the inflammatory response (Lian et al., 2020).

1.5.5 Microbiota relationship with the kynurenine pathway

The kynurenine pathway (KP) is the metabolism of tryptophan, an essential amino acid provided through diets, such as fish and milk. L-tryptophan is metabolised in the GI tract by host cells and microbiota leading to the production of antimicrobial compounds and neurotransmitters. Tryptophan is found in two forms, albumin bound or free, which are in equilibrium. Only the free form of tryptophan can cross the blood-brain barrier which is achieved with the help of the gut microbiota (Dehhaghi et al., 2019).

Once acquired, tryptophan can be used as the rate-limiting amino acid for protein synthesis, transformed into the neurotransmitters serotonin and tryptamine and the hormone melatonin, or catabolised through the KP. In humans, 90% to 95% of dietary tryptophan is degraded via the KP mostly at a systemic level in the liver by the enzyme tryptophan 2,3-dioxygenase (TDO) ***(Figure. 21)***. Additional extrahepatic tissue-specific (e.g., in immune cells like dendritic cells, monocytes and macrophages) transformation of tryptophan through the KP occurs via the enzyme indoleamine 2,3-dioxygenase (IDO), which has an independent origin to TDO and greater enzymatic plasticity (Badawy, A.A., 2017). The KP produces intermediate compounds known as kynurenines, which are neuroactive (e.g., Kynurenic Acid (KA), Quinolinic Acid (QA)) and immunomodulatory (e.g., (3-Hydroxykynurenine (3-Hk), Picolinic Acid (PA)) metabolites and oxidized nicotinamide adenine dinucleotide (NAD+), which is a cofactor in many redox reactions in the cell (Castro-Portuguez and Sutphin, 2020; Dehhaghi et al., 2019). Kynurenines have been linked to many biological processes including inflammation, immune responses, and excitatory neurotransmission. Their ability to disperse through circulation suggests that their production can have both local and systemic effects (Allegri et al., 2003; Cervenka et al., 2017).

It is difficult to study the enzyme activity of the KP in humans as it requires isolated tissues. Therefore, the KP is studied in animal models by the administration of L-tryptophan and looking at its urinary metabolites (Allegri et al., 2003).

However, there are differences in enzyme activities and metabolites levels in serum and cerebrospinal fluid of different species, for example, blood-brain barrier permeability of kynurenine metabolites in rats and humans may be higher than that of mice and rabbits (Murakami., 2013). On the other hand, certain enzymes are upregulated in CNS disorders, for example IDO was significantly upregulated in the brain of chronically stressed mice and administration of IDO inhibitor helped with depression.

In *C. elegans*, tdo-2, the first enzyme required for tryptophan degradation in the KP, regulates age-related α-synuclein toxicity. Tdo-2 regulates protein homeostasis and reduction of tdo-2 suppresses toxicity of aggregated proteins such as beta-amyloid. In worms, depletion of tdo-2 causes an increase in tryptophan levels and extends lifespan (Van et al., 2012).

The KP is modulated by ageing, resulting in an increase in kynurenine but a decrease in tryptophan concentration. This is associated with age-related neuropsychiatric symptoms as kynurenine can cause neurotoxicity and neurodegeneration in CNS (Wu et al., 2021).

Sorgdrager et al examined the modulation of the KP with ageing in Parkinson's (PD) and Alzheimer's disease (AD). They looked at the production of systemic and cerebral kynurenine metabolites and their uptake in the brain. Several metabolites produced through the KP, such 3-Hk and quinolinic acid QA, have diverse effects in the brain. KA acts as an endogenous NDMA receptor antagonist and QA agonizes the NDMA receptor leading to neurotoxic properties.

There was no difference in the estimated uptake of kynurenine compared to the control, but the kynurenic acid concentrations were reduced in CFS of AD and PD patients. The metabolites from the KP accumulated in CFS and serum with ageing, in both patients and control subjects (Sorgdrager et al., 2019).

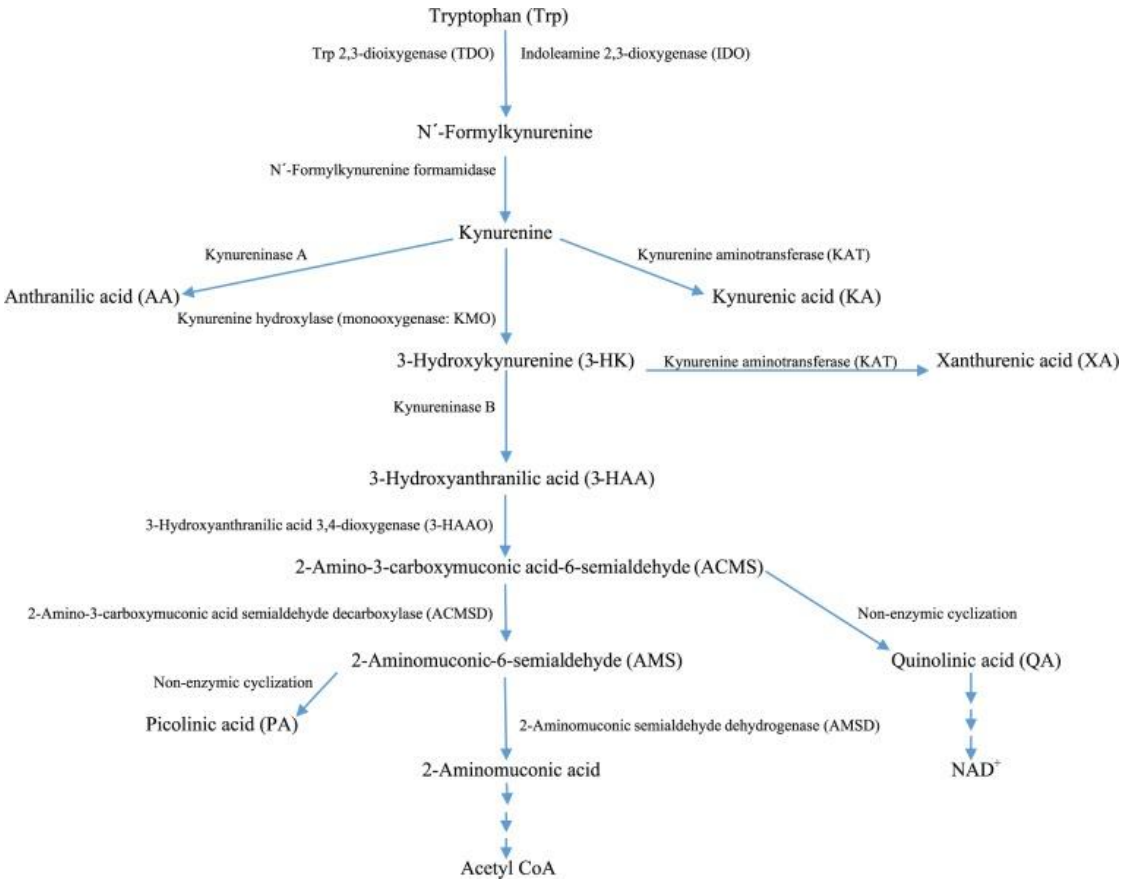


Figure 21. Kynurenine pathway. In humans, 90% to 95% of dietary tryptophan is degraded via the kynurenine pathway mostly at a systemic level in the liver by the enzyme tryptophan 2,3-dioxygenase (TDO) (Badawy, A.A., 2017). Tryptophan is converted to N-formylkynurenine (NFK) by tryptophan 2,3-dioxygenase (TDO) in the liver or by indoleamine 2,3-dioxygenase (IDO) outside the liver. NFK is then hydrolysed to kynurenine by NFK formamidase. Kynurenine can be converted to anthranilic acid by kynureninase or to kynurenic acid by kynurenine aminotransferase. Kynurenine 3-monooxygenase converts kynurenine to 3-hydroxykynurenine (3-HK), which is then converted to 3-hydroxyanthranilic acid (3-HAA) by kynureninase B. 3-hydroxyanthranilate 3,4-dioxygenase converts 3-HAA to 2-amino-3-carboxymuconic acid-6-semialdehyde (ACMS). ACMS can be converted to quinolinic acid, or ACMS decarboxylase can convert it to 2-aminomuconic-6-semialdehyde (AMS), which can be converted to picolinic acid, or to 2-aminomuconic acid by 2-aminomuconic semialdehyde dehydrogenase, which is then converted to acetyl-CoA (Badawy, A.A., 2017).

1.6 Model ageing organisms

Some of the most widely used model organisms to study ageing include *Saccharomyces cerevisiae* (yeast), *C. elegans* (worms), *Drosophila melanogaster* (fruit flies), and *Mus musculus* (mice) ***(figure.22).*** Model organisms are used as they have various advantages, such as short life span allowing parallel experiments, easy maintenance in laboratory, lower ethical restraints and genetic manipulation. However, multiple models may be needed to obtain a comprehensive understanding, as one model alone may not be sufficient to model all ageing phenotypes. For example, yeast cannot be used to study immunosenescence (Taormina et al., 2019).

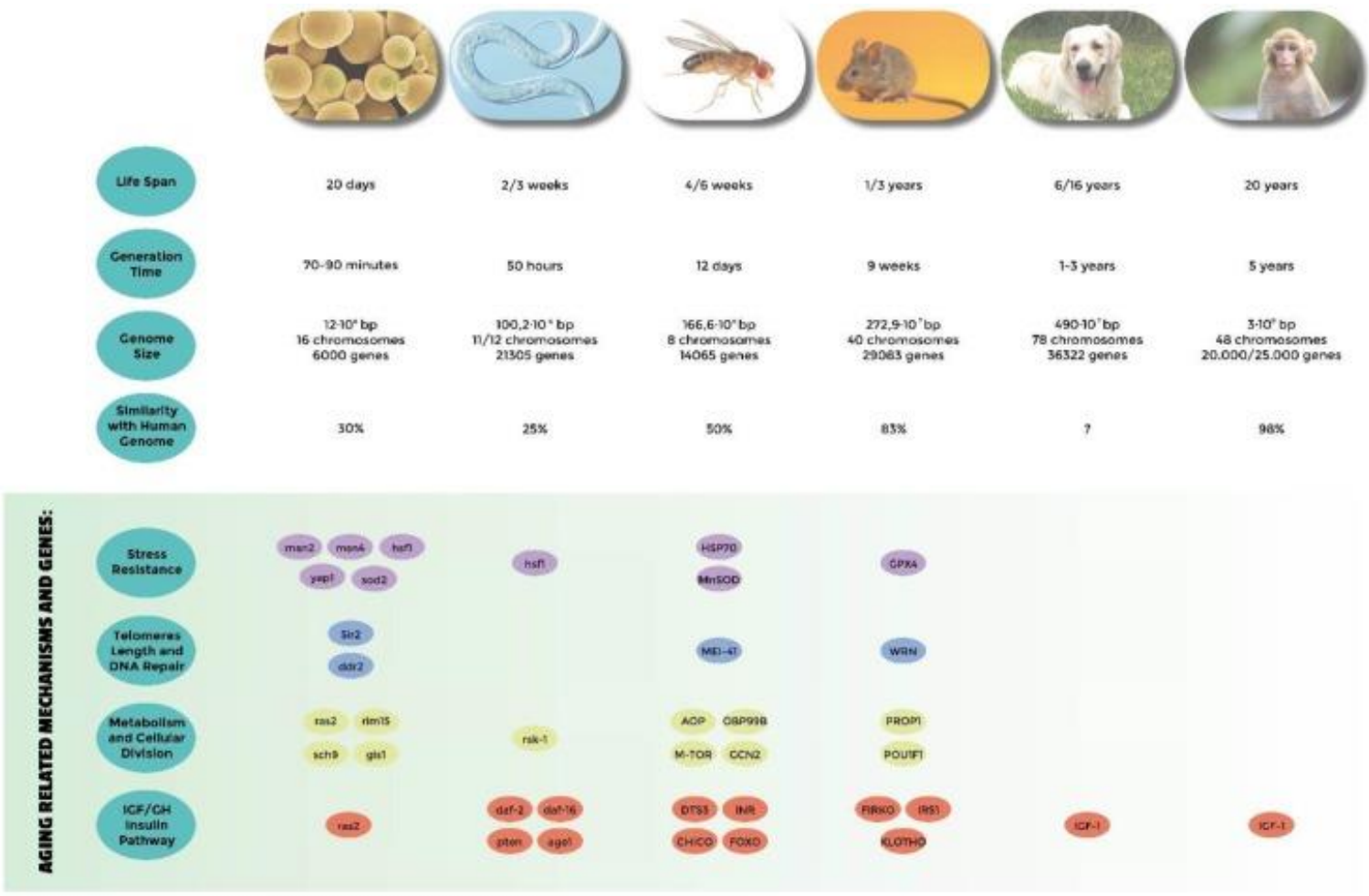


Figure 22. Model organisms to study ageing. S. cerevisiae (yeast), C. elegans (worms), D. melanogaster (fruit flies), M. musculus (mice), domestic dog and non-human primates are some of the most widely used model organisms to study ageing (Taormina et al., 2019).

1.6.1 *Saccharomyces cerevisiae (Yeast)*

Saccharomyces cerevisiae (yeast) is a unicellular fungus used as a model for cellular ageing studies (Zimmermann et al., 2018). It is a facultative anaerobe that generates energy through fermentation (Longo et al., 2012).

The human ageing pathways, such as cell cycle regulation, DNA repair mechanisms, nutrient signalling, mitochondrial homeostasis, protein folding and secretion, lipostasis, proteostasis, stress response, and regulated cell death are conserved in yeast. Approximately, 30% of yeast genome is conserved to humans (Zimmermann et al., 2018).

The two stages of yeast ageing (mitotic and post-mitotic) can be monitored using replicative and chronological lifespan models respectively (Zimmermann et al., 2018). Replicative lifespan (RLS) is the model of undifferentiated stem cells as it refers to number of cell divisions a mother cell can undergo before death. However, this can be time consuming as budded daughter cells need to be separated from the mother cells. On the other hand, chronological life span (CLS) is defined as the survival of non-dividing cells over time, length of time that a cell is viable in a non-dividing quiescent state and can re-enter the cell cycle when stimulated (Zimmermann et al., 2018). In 1959, Mortimer studied RLS by isolating daughter cells on a solid media substrate and, once they started dividing, removed all progeny. His results showed that yeast cells stopped dividing after 20-25 cycles suggesting that cells don't divide forever (Longo et al., 2012). This resembles the Hayflick limit observed in cultured mammalian cells. The deletion of the gene encoding the small G-protein Ras2 in yeast doubles the yeast's lifespan and improves stress resistance. Mutations in the human orthologue of yeast Ras are found in 35% of human cancers (Taormina et al., 2019). Dietary restriction by reducing glucose from 2% to 0.5–0.05% has known to increase lifespan in yeast (Zimmermann et al., 2018).

1.6.2 *Hydra*

Hydra is a cnidarian polyp that can be mass cultured in the laboratory to study ageing Schenkelaars et al., 2018). In humans and mice, the ability of stem cells to regenerate various tissues declines with age (Bellantuono et al., 2015). *Hydra vulgaris* is particularly interesting because it maintains continuous self-renewal through multipotent interstitial stem cells and epithelial stem cells from both the epidermis and the gastrodermis. It shows no signs of ageing

over the years. In contrast, *Hydra oligactis* undergoes rapid ageing under stressful conditions, experiencing senescence after reproduction (Schenkelaars et al., 2018).

Due to Hydra's regenerative ability, it serves as a valuable model for studying stem cell maintenance. *Hydra vulgaris* has been shown to have an estimated lifespan of 1400 years in the laboratory (Bellantuono et al., 2015).

Hydra *oligactis*, when cultivated at 18°C, reproduces asexually by budding, and cultures maintained for years show no signs of mortality. However, when cultivated at 10°C, *Hydra oligactis* initiates sexual reproduction. These animals exhibit mortality, decline in movement, disruption and scattering of the actin fibers, and impaired digestive movement, suggesting that Hydra undergoes ageing following sexual reproduction (Yoshida et al., 2006). Upon a decrease in temperature, both male and female *Hydra oligactis* ceased budding. Males developed testes within three weeks, while females formed ovaries within four weeks. After 30 days, drastic morphological changes occurred in both males and females. In females, the head and foot regions became extremely shortened, while males became motionless in an elongated state, only responding when touched with a glass pipette. In both sexes, the tentacles progressively shortened, and by 60 days, the entire body began to atrophy. By day 150, most animals had completely disintegrated. The reason for Hydra undergoing ageing after sexual reproduction may be due to a directional shift in stem cell differentiation, where stem cells prioritise forming germ cells while differentiation into somatic cells is simultaneously suppressed (Yoshida et al., 2006).

1.6.3 Drosophila melanogaster

Drosophila melanogaster belongs to the family *Drosophilidae* and is commonly known as the fruit fly. The entire genus contains about 1,500 species and is diverse in appearance, behaviour, and breeding habitat. The flies are 3mm short with a short life cycle of about three weeks (Va et al., 2009) and are found worldwide in rotting fruits, vegetables, and plant matter. *Drosophila* has been used in the laboratory since early 1900. Thomas Hunt Morgan pioneered the use of Drosophila as a model organism by demonstrating that chromosomes play a role in hereditary traits for which he was awarded a Nobel prize (Markow, T.A., 2015).

Similar to *C. elegans*, *Drosophila* has low maintenance requirements and can be easily manipulated genetically. These qualities with a short average lifespan of 2–3 months makes *Drosophila* a great genetical tool to study ageing (Sun, Y., et al., 2013). *Drosophila* genome is

about 20-fold smaller than a typical mammalian genome as it contains four pair of chromosomes, 13,000 genes, and about 170 Mbp. But it encodes approximately the same number of gene families, thus making it easier to study gene function (Taormina et al., 2019). The genome of *Drosophila* is fully sequenced and more than 50% of fly genes have homologs in humans. Moreover, more than 75% of known human disease genes, covering a broad range of disorders, have fly homologs (Sun, Y., et al., 2013). Studies have shown that *Drosophila* has a simple microbiome mainly consisting of yeast, *Acetobacter* and *Lactobacillus.* (Arias-Rojas et al., 2022).

Like humans, flies also exhibit an age-related decline in physiological functions. This includes a reduced resting metabolic rate, decreased protein and fat synthesis, a decline in egg-laying, feeding, and stress resistance, as well as impaired learning and memory (Piper, M.D. and Partridge, L., 2018).

After the discovery that the *daf-2* IIS *C. elegans* mutant live twice as long compared to the wildtype worms, IIS mutants in *Drosophila* also showed an extended lifespan. Subsequent genome-wide association studies linked polymorphisms in the insulin pathway transcription factor FOXO with human lifespan, suggesting that reduced insulin signalling increases longevity and that some ageing pathways are evolutionarily conserved (Piper, M.D. and Partridge, L., 2018).

Some studies have shown a correlation between reduced core body temperature and longevity. In 1916, Loeb and Northrop demonstrated that reduced core body temperature increases longevity in *Drosophila*. Flies provided with water only and exposed to 9°C lived for 11.9 days compared to those at 14°C, where they only lived for 8.3 days. Lifespan continued to shorten with increasing temperature: at 19°C, they lived for 4.1 days; at 24°C and 28°C, they lived for 2.4 days; and at 34°C, they lived for 2.1 days. The same correlation was observed in flies provided with a 1% cane sugar solution (Loeb, J. and Northrop, J.H., 1916).

1.6.4 Killifish

The African turquoise killifish (*Nothobranchius furzeri*) lives in seasonal ponds in East Africa. It is a short-lived species, with a natural lifespan ranging from 4 to 9 months. The current laboratory strains include the inbred ‘GRZ’ strain, derived from an original population in 1968, as well as several wild-derived strains. The GRZ strain has the shortest lifespan (9–16 weeks) compared to all other strains of killifish, while long-lived strains have a lifespan of 23–28

weeks. Killifish exhibit rapid sexual maturation, typically within 3–4 weeks in captivity. Female fish in captivity lay approximately 20–40 eggs per day (Kim et al., 2016).

Similar to how *C. elegans* undergoes a dauer stage under stressful conditions, killifish embryos can enter a developmentally arrested state called diapause in the absence of water, allowing them to survive for several months. Whereas, under controlled laboratory conditions, fish usually skip diapause and develop rapidly. The genes regulating diapause in killifish are not yet well characterized (Kim et al., 2016).

With age, killifish show deterioration in fin structure, abnormal spine curvature, reduced fecundity, impaired vision, decreased locomotor activity, and an increased risk of cancer. Several ageing biomarkers can be used to assess the physiological age of killifish and are valuable for ageing research. These include lipofuscin, an autofluorescent pigment that accumulates in the brain and liver with age. Lipofuscin concentration increases with age in several species, including humans. Additionally, Fluoro-Jade B dye can be used to stain neurons undergoing degeneration (Kim et al., 2016).

1.6.5 Mus musculus (Mice)

Mice models provide insight into fundamental mechanisms shared by mammalian systems (Folgueras et al., 2018). After humans, mice were the second mammalian species to have their genome sequenced (Breschi et al., 2017).

Mice genome is 12% smaller than human genome. 40% of the nucleotides in humans are alignable to mice whereas, nucleotide-level insertions and deletions, lineage-specific duplications and lineage-specific deletion of repeated elements from the ancestral genome result in unmatched DNA (Breschi et al., 2017). Other differences include longer telomeres and higher telomerase activity in mice compared to humans.

More than 85% of the genomic sequences between mouse and human are conserved (Hugenholtz, F. and de Vos, W.M., 2018). Additionally, mice can synthesize vitamin C, unlike humans (Vanhooren, V. and Libert, C., 2013).

Studies using mice models can be expensive and time consuming. However, due to the availability of large amount of baseline phenotypic data, genetic manageability of its genome, and ease of breeding, mice are used to study ageing (Kõks et al., 2016).

Over the years, many mouse models that mimic human diseases (diseases that don't naturally occur in mice) such as Alzheimer's and cystic fibrosis, have been created using CRISPR–Cas9 technology (Breschi et al., 2017).
Reducing insulin signalling has shown to increase lifespan in several mouse models, such as PI3K (Pik3caD933A/+), Pten-overexpressing (Tg-Pten), and Akt1 heterozygous (Akt1+/−) (Folgueras et al., 2018).

1.6.6 Naked mole rat

The naked mole-rat (*Heterocephalus glaber*) is the longest-living rodent, with a lifespan of around 30 years. It serves as an intriguing model for ageing research due to its delayed ageing phenotype and resistance to age-related functional decline (Liang et al., 2010). NMRs also do not show significant changes in body composition or metabolic profiles with age, but they do exhibit an age-related decline in activity (Kerepesi et al., 2022).
Cancer is a major age-related disease in humans and accounts for 23% of human mortality. In mice, cancer-related mortality is even higher. However, in comparison, NMRs show an unusual resistance to cancer and do not develop spontaneous neoplasms. Anticancer mechanisms in mammals include cell cycle checkpoints, apoptosis, and replicative senescence, controlled by a network of tumour suppressor genes such as p53 and Rb. Normal cells stop proliferating when they come into contact with each other. This is called contact inhibition. This growth arrest is signalled by membrane proteins and is mediated by elevated levels of the cyclin-dependent kinase inhibitor p27, which binds cyclin-CDK complexes and arrests cells in the G1 phase of the cell cycle. NMRs are hypersensitive to contact inhibition which causes them to undergo early contact inhibition resulting in fibroblasts becoming contact inhibited at a very low cell density. This early contact inhibition requires the activity of both the Rb and p53 pathways (Seluanov et al., 2009).
The epigenetic ageing clock of NMRs can successfully predict their lifespan and shows that, like other mammals, NMRs also undergo epigenetic changes with age. While mice have long telomeres, NMRs possess short telomeres. Compared to mice, the age-related methylation pattern of *Tert* (the catalytic subunit of telomerase reverse transcriptase) in NMRs was remarkably different. In NMRs, there was increased methylation of the *Tert* promoter with age and *Tert* expression remained stable over time. Moreover, telomere length did not shorten with

increasing age in NMRs. An age-related increase in methylation was also observed in the promoter of *Prpf19*, a gene involved in double-strand break repair. Similar age-related changes were also observed at clock sites conserved between mice and NMRs. For example, methylation at CpG sites within the *Mab21l2* gene (involved in retina and ventral body wall formation) increased with age in both species. (Kerepesi et al., 2022).

1.6.7 Dogs

Dogs share several age-related diseases with humans, such as neurodegeneration, diabetes, and cancer. They also share the human environment and are exposed to similar environmental factors, making them a useful model for studying ageing. The lifespan of dogs varies greatly and it's been observed that the size of dog may be inversely correlated with its lifespan, as such that the larger breeds live almost six to seven years, while smaller breeds can reach up to 16 years. The domestic dog's genome is well-annotated and consists of 38 pairs of autosomes and one pair of sex chromosomes (Taormina et al., 2019).

The Dog Ageing Project (DAP) is a comprehensive, long-term project aimed at understanding the biological and environmental factors that influence ageing in dogs. It collects data on genetics, lifestyle, and health by following tens of thousands of dogs over their lifetimes (Creevy et al., 2022). Around 50,000 companion dogs are enrolled in DAP (dogagingproject.org). Within DAP, the Test of Rapamycin in Ageing Dogs (TRIAD) study is being conducted, as previous research has shown that rapamycin can extend lifespan in mice. The TRIAD study is a double-blind, placebo-controlled clinical trial involving the administration of oral rapamycin once weekly for 12 months to healthy medium to large, large, or senior companion dogs. The study aims to assess the impact of rapamycin on lifespan and healthspan, including physical and neurological function, as well as cardiovascular health (Coleman et al., 2025).

1.6.8 Rhesus monkey

Rhesus macaques (*Macaca mulatta)* are the most studied type of non-human primate in ageing research. They share approximately 92% genetic homology with humans and exhibit a similar

ageing process. Rhesus monkeys have an average lifespan of 25 years and can live up to 40 years in captivity. Monkeys aged 15–22 are considered middle-aged, while those over 30 are considered old. However, there are several challenges associated with studying ageing in monkeys, such as their expensive maintenance and considerable individual variation among monkeys. The age-related conditions take years to develop making lifespan studies lengthy (Mattison et al., 2017).

Around 30–40% of neuroscience research is conducted using mice and rats, and many diseases can be studied through brain lesions and genetic manipulation in these models. However, it can be argued that the failure of new treatments for brain disorders in clinical trials may be due to the low translational value of rodent models. This limitation may stem from the underdeveloped brain regions in rodents, such as the prefrontal cortex. In contrast, Rhesus monkeys offer greater translational value for studying neurodegenerative disorders. For instance, the dorsolateral prefrontal cortex (associated with memory, decision-making, and executive function), and commonly affected in age-related human brain diseases, is well developed in monkeys. Additionally, rodents do not naturally develop age-related amyloid or tau pathologies; these are typically mimicked using transgenic models. Whereas, Rhesus monkeys over the age of 30 have been shown to exhibit similar age-related cognitive decline to humans (Plagenhoef et al., 2020).

Table 1 shows the strengths and limitations of different model organisms for studying gut microbiota, stress, ageing, and health. This includes, yeast, hydra, drosophila, killifish, mice, NMR, dog and monkey.

Table 1. Strengths and limitations of different model organisms for studying gut microbiota, stress, ageing, and health

Organism	Genome size and complexity	Similarity to humans	Lifespan	Strengths	Limitations
Yeast (*Saccharomyces cerevisiae)*	12.07 Mb 6000 genes	30% of yeast genome is conserved to humans	20 days	Cheap, rapid growth, high throughput, easy genetic manipulation, can study replicative senescence, protein folding, intracellular trafficking, cell cycle, identification of ageing genes	Unicellular, Lacks the multicellular complexity of humans, cannot provide information on tissue and organ ageing mechanisms
Hydra	0.9-1.05 Gb 20,000	Share around 6000 genes with humans	1400 years in lab	Cheap, easy genetic manipulation, can use it to study stem cell renewal and pathways that lead to its longevity, can study shift in stem cell differentiation after sexual reproduction, can study differences in lifespan of the same genus	Doesn't reflect human ageing due to its unique stem cell dynamics and lack of senescence, Lacks the multicellular complexity of humans
Drosophila	170 Mbp 14,000 genes	50% of fly genes have homologs in humans	4-6 weeks	Cheap, well annotated genome, conserved ageing pathways, ease of genetic manipulation, short lifespan - can observe several generations, mutagenesis screens, RNAi tools, clone libraries, microarrays	Simpler immune system, some physiological functions are not conserved, such as immune system, lack of complex organs and systems

Killifish (*Nothobranchius furzeri*)	1.24 Gb 26,000 genes	Share 60% of genes with humans	9–16 weeks	Cheap, rapid life cycle, share similar age-related decline in functions (reduced fecundity, impaired vision, decreased spontaneous locomotor activity) and an increased risk of cancer, can study neurodegeneration using biomarkers such as lipofuscin	lack of complex organs and systems, can't study late age-related diseases
Mice *(Mus musculus)*	2.5 Gbp 22,000 genes	Around 85% of the genomic sequences between mouse and human are conserved	1-3 years	Lots of data, genetic manipulation, can mimic human disease models, have an immune, endocrine and digestive system similar to humans	Expensive, time consuming, smaller brood size and increased generation time (8-10 weeks) compared to simple models, such as *C.elegans*, underdeveloped brain regions
Naked mole rat (*Heterocephalus glaber)*	2.7 Gbp 23,000	93 %	30 years	Can study to look for protective mechanisms that may delay aging phenotype and resistance to age-related functional decline	Studying them might not translate into humans due to their unusual ageing process
Dog	4900 Mbp	share over 17,000 similar genes	7-16 years	Share human environment/lifestyle, well-annotated genome, share similar age-related diseases as humans (cancer, cardiovascular diseases), cognitive decline, can conduct observational longitudinal studies	Variability in ageing rate among different breeds, long and time consuming, ethical considerations

Rhesus Monkey (*Macaca mulatta*)	3146 Mbp	93% genetic homology with humans	25-40 years	similar inter-individual variability to humans, share physiology, sleeping and eating behaviour, endocrinology, immunology- can use it to study host-microbiota interaction, similar age-related cognitive decline to humans- can use it to study neurodegeneration, many similar age-related diseases (cancer, cataracts, cardiovascular diseases, osteoporosis), can fully control the environment and diet, have access to medical history	Expensive, requires specialised care, considerable individual variation among monkeys, long lifespans, small sample size, genetic manipulation is not possible due to ethical considerations

1.7 *C. elegans* as a model organism

1.7.1 Origin of the model

C. elegans (non-parasitic nematode) is a saprophytic species, found in soil, leaf-litter environment and is often carried by gastropods and other smaller organisms in soil habitats (Leung et al., 2018). It was chosen as a model organism in 1963 by molecular biologist Sydney Brenner due to its relatively simple examples of animal biology, which means it can used as a genetic model to study various areas in biology (Meneely et al., 2019).

It's simplicity and versatility makes it an excellent experimental model. This includes worm's small size (1mm), rapid life cycle (about 3 days), transparency, well-annotated genome, ease of maintenance (*Escherichia coli* diet), and the option of long-term storage at −80°C (Corsi et al., 2015). In addition, despite having a limited number of cells (<1000), *C. elegans* have highly differentiated tissues, such as skin, nerves, muscles, gut tissues and reproductive organs (Smith et al., 2015).

The outcomes in *C. elegans* are predictive in higher eukaryotes, as basic physiological processes and stress responses observed in humans are conserved in *C. elegans* (Ahringer, J., 2006). Therefore, *C. elegans* survival assays are used to study ageing and stress resistance. Due to the short life span of worms, most stress resistance and lifespans assays take around several days and weeks, respectively. The temperature of experiments ranges from 15°C to 25°C and most assays use solid and liquid media systems with bacterial food (Park et al., 2017). However, there are several limitations of using *C.elegans* for studying gut microbiota, stress, ageing, and health ***(Table 2)***. This includes lack of complex organs/tissues and systems, such as circulatory and respiratory systems that exist in humans. and being evolutionarily distant from humans. Some physiological functions are not conserved, such as immunity, as *C.elegans* don't have an adaptive immune system and specialised immune cells (Melnikov et al., 2023).

Table 2. Strengths and limitations of C.elegans for studying gut microbiota, stress, ageing, and health

Strengths of *C.elegans* as a model ageing organism	**Limitations of *C.elegans* as a model ageing organism**
Cheap, small size, 100 Mb 20,000 genes, rapid life cycle (2-3 weeks), transparency which can allow tracking of cells in vivo over time, fluorescent tags, well-annotated genome, 50% of genes are conserved in the human genome, Self-fertilizing hermaphrodite, ease of maintenance, long-term storage at −80°C, lifespan and stress assays, evolutionary conserved molecular pathways, easy gene knockout by RNAi, easy gene overexpression (transgenics) tissue specific knockouts, age associated changes similar to humans such as movement decline	lack of complex organs and systems, some physiological functions are not conserved, such as immune system, evolutionary far from humans and many important ageing genes in worms do not have human homologs

1.7.2 C. elegans reproduction

Most *C. elegans* are self-fertilising hermaphrodites. However, hermaphrodite germline does infrequently (0.1%) produce males through mating and the frequency of males can increase up to 50%. Overall, there are much more hermaphrodites than males, and it is easy to generate a synchronised isogenic progeny as self-producing hermaphrodites generate a large number of worms ***(figure.23)*** (Park et al., 2017).

Most laboratories use the N2 wild-type (isolated in 1965) strain as the reference. N2 was cultured in the laboratory for many years using *E. coli* as a food source. *C. elegans* are bacterivores and form a symbiotic relationship with microbes as their food source which provide the worms with essential amino acids (Kingsley et al., 2021). In nature, *C. elegans* primarily feed on different species of bacteria, such as *Comomonas sp., Pseudomonas medocina* and *Bacillus megaterium* (soil bacteria), *Acetobacteriacea*e and *Enterobacteriaceae* found in rotting fruits (Zečić et al., 2019).

In the laboratory, *C. elegans* are grown on a single strain of *E. coli*, which then inhabits its gut in a commensal relationship (Kingsley et al., 2021). *E. coli* was used as the model organism as it was already available in many laboratories and not because it was associated with wild *C. elegans*. Over the years, N2 has been adapted to laboratory conditions and the regular bleaching treatment (with hypochlorite) means that the strain does not carry any microbes in its gut or on its surface (Dirksen et al., 2016).

C. elegans hermaphrodites exhibit a considerable post reproductive lifespan, which is similar to female humans that undergo menopause around age 50, and generally live until around age 80. In worms, adult lifespan is typically around day 15 of adulthood and peak progeny production is achieved on adult day 2, when worms lay the most eggs. Following this peak, there is a rapid decline in progeny production, which ceases by day 8 of adulthood. Additionally, in worms, oocyte quality declines with ageing, similar to the increasing likelihood of birth defects as maternal age increases in humans (Scharf at al., 2021).

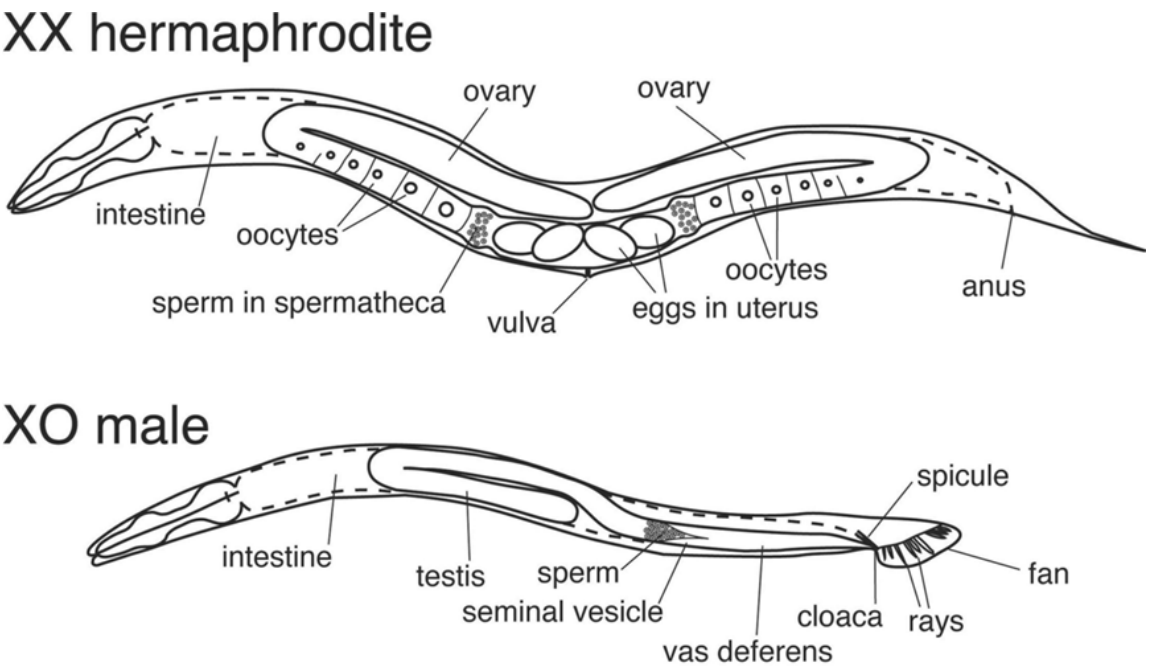


Figure 23. Male and hermaphrodite C. elegans (Zarkower, D., 2006).

1.7.3 C. elegans life cycle

The first stage of development in *C. elegans* (L1) following completion of embryonic development is the emergence of larvae from the eggshell. Larvae pass through the four larval stages (L1, L2, L3 and L4) before developing into a reproductive adult ***(figure.24).*** However, in hostile environmental conditions, such as limited food and increased population density, L1 larvae enter dauer (development arrest) stage which can survive 4-8 times the normal 3-week life span. Dauer are non-feeding but explore the environment for food sources and upon finding a food source resume reproductive development. They develop into L4 larvae and subsequently into reproductive adults.

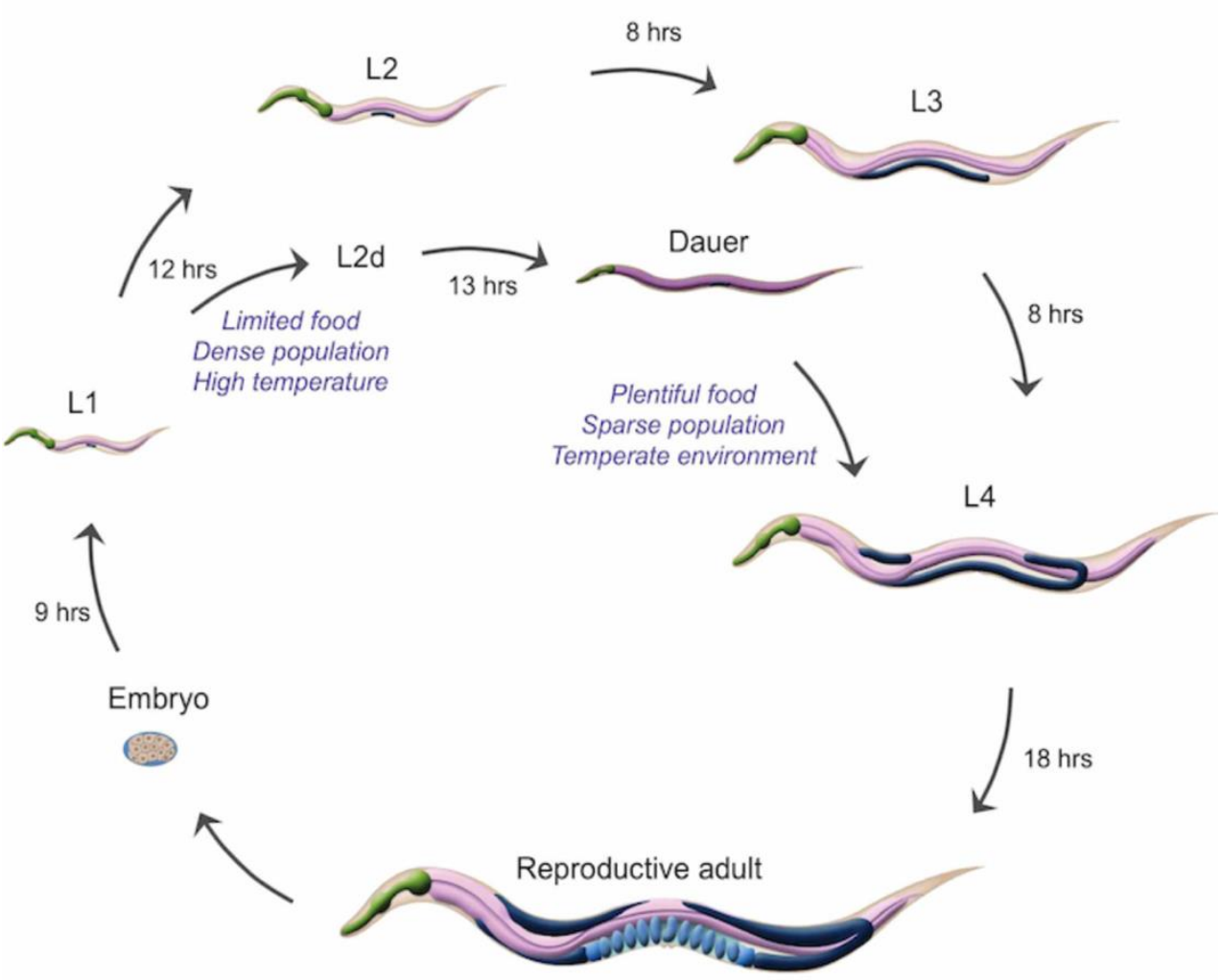


Figure 24. C. elegans life cycle. C. elegans larvae emerge from the eggshell and pass through the four larval stages (L1, L2, L3 and L4) before developing into a reproductive adult. In stressful conditions, such as limited food availability worms undergo development arrest/dauer stage and upon finding a food source resume development (wormatlas.org, 2018).

1.7.4 Relationship between ageing and stress resistance in C. elegans

1.7.4.1 Heat and oxidative stress responses

Organisms experience fluctuating environments and changes in intracellular homeostasis, which means that cellular components are prone to stress associated damage. This includes detrimental effects on the proteome and physiology of the organism (Bar-Ziv et al., 2020). Proteins, nucleic acids, and membranes are extremely sensitive to heat stress. This is due to the structural stability of large molecules being fundamental for processes depending on a molecule–molecule recognition, such as substrate-enzyme binding, proteins assembly into multiprotein complexes, and the interactions between gene regulatory proteins and their DNA targets (Somero, G.N., 2020).

Therefore, adequate stress responses are required to maintain cellular homeostasis and organisms have evolved targeted and specific stress responses to repair damage and maintain homeostasis (Bar-Ziv et al., 2020). These mechanisms include the unfolded protein response of the mitochondria, the heat shock response and the oxidative stress response ***(Figure.25)*** (Hoppe, T. and Cohen, E., 2020).

In *C. elegans*, heat shock proteins (HSPs) are produced in response to external stresses such as heat stress. HSPs are controlled by the transcription factor Heat shock factor-1 (HSF-1) (Prithika et al., 2016). Heat exposure leads to protein aggregation in the cytosol triggering the heat-shock response. Cytosolic chaperones, HSP70s recognise protein aggregates and translocate transcription factor HSF1 into the nucleus. Once inside the nucleus, HSF-1 regulates the expression of chaperones and proteostasis regulators, and the resulting transcripts migrate to the cytosol to maintain homeostasis. This includes the formation of stress granules, alternative splicing, and abnormal transcriptional termination (Hoppe, T. and Cohen, E., 2020).

Oxidative stress can mediate mitochondrial unfolded protein response. HSP70 chaperone HSP-6 activates transcription factors ATFS-1 and DVE-1 which translocate to the nucleus and upregulate stress-responsive genes, including mitochondrial chaperones and proteases (Hoppe, T. and Cohen, E., 2020).

The oxidative stress response in *C. elegans* is mediated by a single CNC transcription factor, SKN-1, which translocates to the nucleus upon oxidative stress and activates the transcription of over 2,000 antioxidant genes. These include antioxidant genes and genes involved in detoxification, such as those encoding glutathione S-transferases (e.g., *gst-4*, *gst-12*, and *gst-30*). γ-Glutamyl cysteine synthetase (*gcs-1*) is the rate-limiting enzyme involved in glutathione

synthesis. GSTs conjugate glutathione to xenobiotic toxins to detoxify them. On the other hand, the WDR-23 protein, which is part of a CUL-4–DDB-1 ubiquitin ligase complex, negatively regulates SKN-1 by reducing its nuclear accumulation and thus decreasing its transcriptional activity (Crombie, T.A et al., 2016).

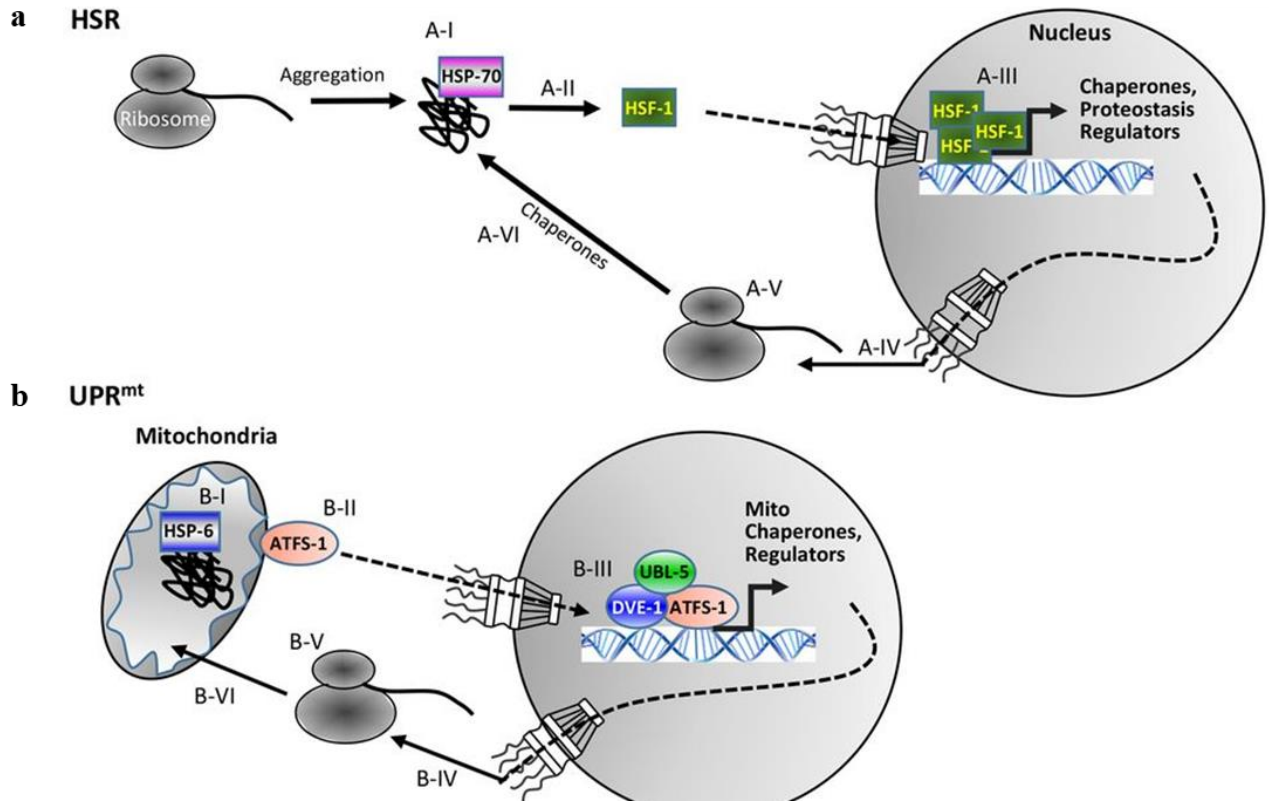


Figure 25. Stress responses. a) Heat shock response – Heat stress causes the accumulation of protein aggregates which are recognised by cytosolic chaperones, HSP70s. This causes the translocation of Transcription factor HSF1 into the nucleus. Once inside the nucleus, HSF-1 regulates the expression of chaperones and proteostasis regulators and the resulting transcripts migrate to the cytosol to maintain homeostasis. b) Mitochondrial unfolded protein response - oxidative stress accumulates protein aggregates, HSP70 chaperone HSP-6 activates transcription factors ATFS-1 and DVE-1 which translocate to the nucleus and upregulate stress responsive genes, mitochondrial chaperones and proteases (Hoppe, T. and Cohen, E., 2020).

1.7.4.2 In C. elegans oxidative stress resistance is extensively studied

Oxidative stress is the imbalance between the production of ROS and antioxidant defences which can lead to oxidative modifications and damage to carbohydrates, proteins and DNA ***(figure.26).*** This could be due to the overproduction of ROS or by dysfunctional antioxidant systems (Selman et al., 2012).

The oxygen demands of different tissues vary depending on metabolic needs, for example the brain represents only 2% of the body weight but accounts for 20% of the total oxygen consumption (Gandhi, S. and Abramov, A.Y., 2012). The high metabolic rate results in the increased amount of omega-three polyunsaturated fatty acids in brain tissue which are susceptible to peroxidation. Brain tissues also contain more iron and copper which makes the brain more susceptible to oxidative damage. Therefore, oxidative stress is associated with neurogenerative disorders (Garbarino et al., 2015).

Consequently, various studies have used antioxidants as potential targets for improving health. However, accumulating evidence from randomised controlled trials has highlighted the negative impact of antioxidant supplements on health. Long-lived *C. elegans* mutants such NU0-6 and SOD-12345 have higher oxidative stress and mice that underwent genetic manipulation of their oxidant defence system do not show a change in lifespan (Belenguer-Varea et al., 2020).

Therefore, it is now accepted that ageing is a much more complex phenomenon involving several factors and low levels of ROS could be beneficial for health. This is because organisms have evolved protective mechanisms to prevent damage from abnormally high ROS levels and therefore exposure to lower levels of ROS can initiate these protective measures (Ewald, C.Y., 2018). For example, Yang and Hekimi analysed ROS levels in *C. elegans* ISP-1 and NUO-6 mutants. These proteins are directly involved in mitochondrial electron transport and lead to a dramatic increase in *C. elegans* lifespan. However, superoxide levels were not low as expected but instead were elevated in these mutants, which proposed that gradual molecular damage during ageing triggers a progressive protective superoxide response (Yang and Hekimi., 2010).

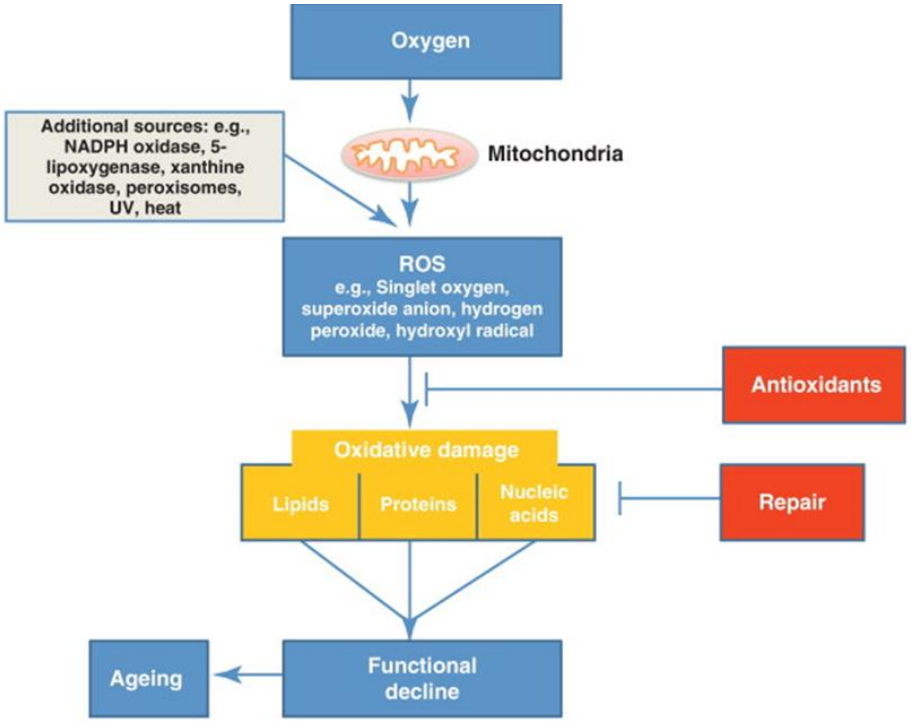


Figure 26. Oxidative stress theory. Imbalance between the production of reactive oxygen species ROS (by-products of mitochondrial respiration) and antioxidant defences damage carbohydrates, proteins and DNA leading to functional decline and ageing (Selman et al., 2012).

1.7.4.3 Stress resistance and longevity

Stress resistance is associated with a long lifespan. Studies have demonstrated that short-term exposure to severe toxic stress can increase resistance to subsequent exposure to the same stress and in some cases may increase lifespan. Zhou et al demonstrated that early life heat experience establishes epigenetic memory by histone acetyltransferase CBP-1 and chromatin remodelling complex SWI/SNF. This promotes long-lasting stress responses to slow down the ageing process (Zhou et al., 2019).

Worms with increased stress resistance are better able to survive acute exposures to stress such as *daf-2* and die later compared to the wild-type worms. *daf-2* is a long-lived mutant and many long-lived genetic mutants also exhibit high resistance to multiple stresses suggesting that enhanced resistance to stress may promote longevity. However, it is also possible that common genetic pathways control stress resistance and longevity such that there is an associative, rather than causative, relationship (Dues et al., 2019).

Dues et al examined the relationship between stress resistance and lifespan in *daf-2* worms by exposing the worms to different stresses. Survival assays included 37°C heat stress, 4 mM paraquat oxidative stress, anoxic stress, exposure to pathogenic bacteria *P. aeruginosa*, and osmotic stress 700 mM NaCl. Compared to the wild-type, *daf-2* mutants showed increased stress resistance associated with an increase in lifespan (Dues et al., 2019).

Heat shock factor 1 (hsf-1) transcription factor responds to heat stress by inducing the expression of various heat shock proteins. The IIS mediates heat shock response through HSF-1 and DAF-16. In *C. elegans*, DAF-16 is more active during adulthood, whereas HSF-1 is more active during early larvae development. DAF-16-dependent longevity genes, DDL-1 and DDL-2, regulate the activity of HSF-1, and the inhibition of DDL-1/2 increases longevity and thermotolerance (Rodriguez et al., 2013). In *C. elegans*, HSF-1 and FOXO together increase lifespan when IIS is reduced (Son et al., 2018).

daf-2 mutants were crossed with *hsf-1* mutants to assess the contribution of stress resistance in *daf-2*. This showed that *hsf-1* mutation causes a decrease in heat stress resistance in *daf-2* worms but not affect sensitivity to other types of stresses. Additionally, lifespan was also decreased in *daf-2;hsf-1* worms compared to *daf-2* (Dues et al., 2019).

On the other hand, increasing resistance to oxidative stress *isp-1* worms through deletion of sod-3 or sod-5 was found to decrease lifespan, demonstrating that oxidative stress resistance and longevity can be experimentally dissociated. Thus, concluding that oxidative stress resistance can be experimentally dissociated from longevity (Dues et al., 2019).

In 1999, Van Voorhies et al. reported that a decrease in temperature by 5°C (from 20°C to 15°C and 25°C to 20°C) can increase lifespan of *C. elegans* by 75 % (Van Voorhies et al., 1999).

TRPA-1 channel in *C. elegans* is a cold-sensitive channel that detects a drop in temperature and induces calcium influx into the cell, activating protein kinase C, which in turn activates DAF-16/FOXO. TRPA-1 remains closed at 25°C but is activated at 20°C. Mutants lacking TRPA-1 were cultivated at 15°C, 20°C, and 25°C. The results showed that *trpa-1* null mutant worms had a significantly shorter lifespan compared to the wild type at 15°C and 20°C but not at 25°C, suggesting that TRPA-1 may promote lifespan at lower temperatures but not at warmer ones. Expression of TRPA-1 in intestinal cells and neurons increased lifespan, but not in muscles or hypodermal cells, suggesting that TRPA-1 functions in intestinal cells and neurons to modulate lifespan (Xiao, R et al., 2013).

1.7.5 Death in C. elegans

1.7.5.1 Cell death mechanisms

Cell death is categorised into programmed (apoptosis and autophagy) and unprogrammed (necrosis) processes ***(figure. 27)*** (Montaseri et al., 2020).

Apoptosis is a programmed process (fastest and irreversible cell death) that is required for the elimination of unwanted cells during embryologic development, after birth and during adulthood. There are two pathways (extrinsic and intrinsic apoptotic pathways) to eliminate cells damaged beyond repair. Through the extrinsic apoptotic pathway, death ligands such as tumour necrosis factor-α (TNF-α) and Fas bind to their homologous receptors triggering cell death. This initiates several caspases causing apoptosis. On the other hand, the intrinsic pathway works through the mitochondria by the formation of a megapore (formed of proteins complex permeability transition pore) in the inner and outer membranes of the mitochondria. This releases mitochondrial proteins such as cytochrome C (Cyto C) into the cytosol. Cyto C combines with apoptosis protease activating factor-1 to form apoptosome complex which mediates the cleavage and activation of downstream caspases to dismantle the cell. Apoptosis features the shrinkage of the cytoplasm, DNA fragmentation, blebbing of the plasma membrane, and eventually the formation of the apoptotic body (Chen et al., 2020).On the other hand, necrosis is an unprogrammed cell death that occurs due to lethal damage such as high temperature and therefore does not have any specific signalling pathways. Secondary necrosis may also occur in the late stages of apoptosis or autophagy in case cells cannot be removed by phagocytosis (Chen et al., 2020).

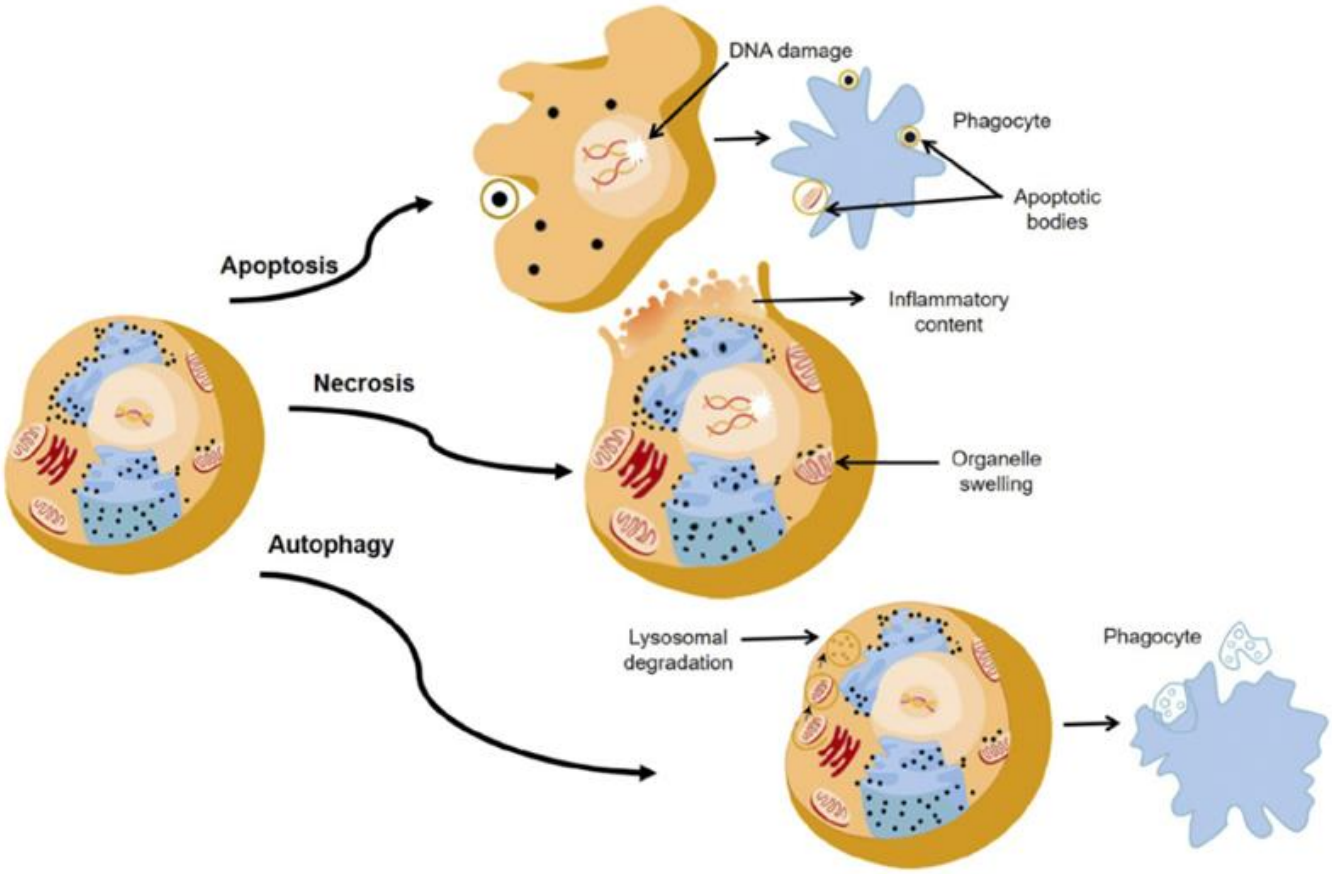


Figure 27. Cell death pathways. Cell death can be programmed (apoptosis and autophagy) or unprogrammed (necrosis) (Montaseri et al., 2020).

1.7.5.2 Death in ageing C. elegans

Zhao et al demonstrated two forms of (late and early) deaths in ageing *C. elegans* by examining corpses of worms that had died from old age. The early deaths with a swollen pharynx were dubbed as 'P death' (big P) and later deaths with an atrophied pharynx were dubbed as 'p death' (small p) (Zhao et al., 2017).

Worms develop various pathologies with increasing age, such as deterioration of the pharynx, neurons and intestine. Therefore, wild-type adult hermaphrodites with pathologies were monitored over time to assess the correlation between pathology severity and the age at death. It was found that in *C. elegans* pathology of the pharynx could be life-limiting as the pharynx pumping span correlated with longevity. Pharyngeal pumping mediates food uptake in worms.

‘P death’, the immediate cause of pharyngeal swelling, might be due to the invasion and proliferation of bacteria due to damage induced by increased pharyngeal pumping during early adulthood. The rate of pharynx pumping did not differ between worms that underwent P and p deaths. However, rapid pumping in early adulthood causes mechanical damage to the pharyngeal cuticle, allowing the invasion of bacteria which may explain the cause of early death (Zhao et al., 2017).

1.7.5.3 Necrotic cell death in C. elegans

Necrotic cell death is described as a complete collapse of cellular homeostasis, which may occur due to extreme stimuli such as heat shock and is followed by morphological changes such as osmotic swelling of most organelles, increased cell volume, and eventually the rupture of the plasma membrane ***(figure.28)*** (Nikoletopoulou, V. and Tavernarakis, N., 2014). The intestine of *C. elegans* contains lysosome-like organelles known as gut granules. Gut granules contain anthranilate glucosyl esters (glycosylated forms of anthranilic acid), which are tryptophan-derived compounds produced by the KP. When worms die, de-quenching of anthranilic acid conjugates releases an endogenous burst of blue fluorescence in the intestine of the worm, called death fluorescence. The anterior to posterior wave of intestinal cell death is propagated via the innexin INX-16 (gap-junction subunit), likely by calcium influx (Coburn et al., 2013).

Stresses such as heat shock increase intracellular Ca^{2+} levels through influx from plasma membrane channels or release from endoplasmic reticulum stores. Ca^{2+} then activates calpain proteases in the cytoplasm, which break down lysosomal membrane proteins and damage lysosomal integrity. This causes the lysosomes to rupture, releasing hydrolytic enzymes such as cathepsin proteases (Nikoletopoulou, V. and Tavernarakis, N., 2014).

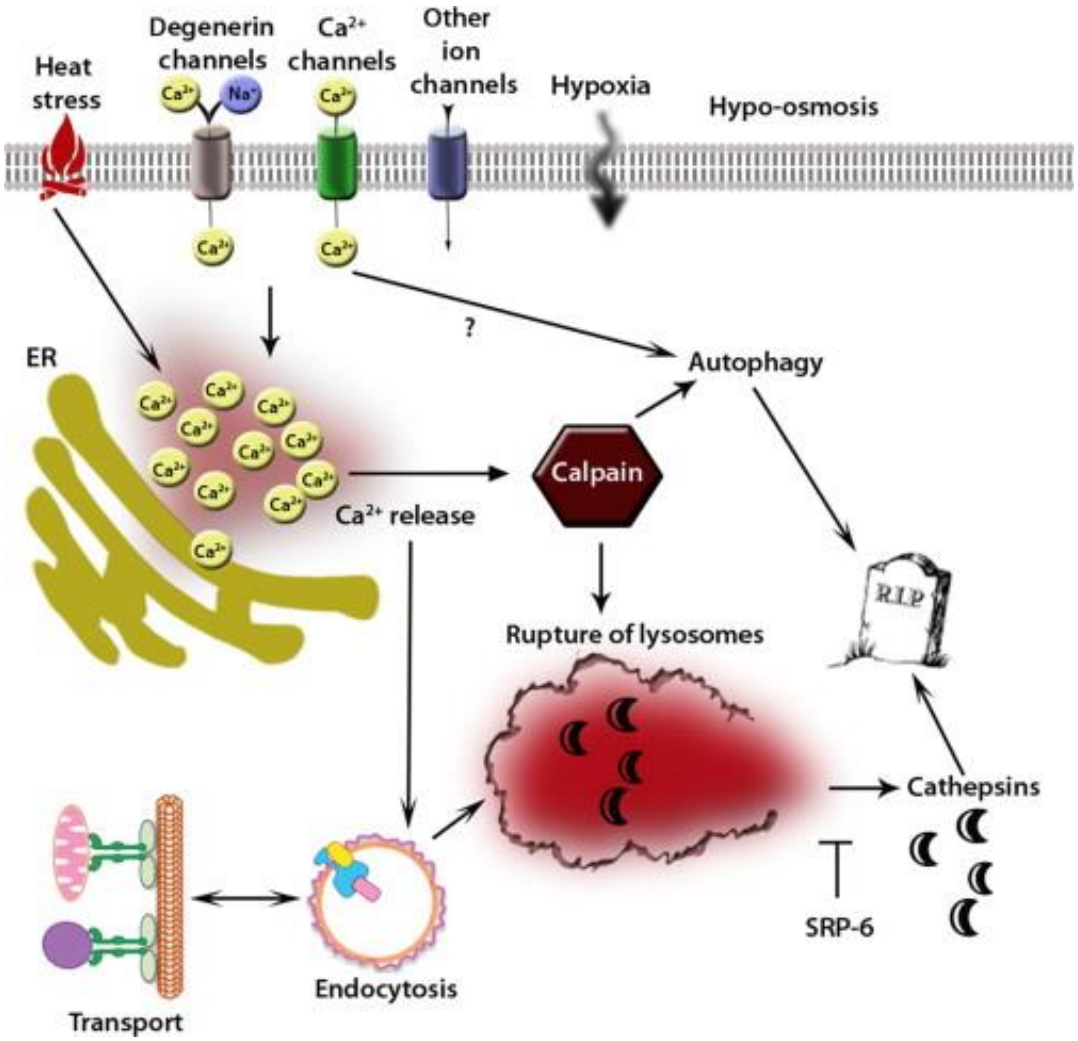


Figure 28. Necrotic cell death in C. elegans. Necrotic cell death refers to the complete collapse of cellular homeostasis in response to extreme stimuli. Heat stress increases intracellular Ca^{2+} levels by facilitating influx through plasma membrane channels or release from endoplasmic reticulum stores. Ca^{2+} activates calpain proteases in the cytoplasm, which break down lysosomal membrane proteins, leading to lysosome rupture (Nikoletopoulou, V. and Tavernarakis, N., 2014).

1.7.5.4 Measuring time of death in C. elegans

Lifespan assays usually require manual scoring based on the movement of worms. This requires the manual prodding of worms using a worm pick, as worms become less responsive and move less with age. This manual analysis of inspecting live and dead worms is time-consuming as it requires counting large number of worms under a dissection microscope (Puchalt et al., 2021). Worms also need to be transferred regularly onto fresh plates to avoid

environmental contamination. Moreover, adult worms must be separated from their progeny, as newly hatched worms become indistinguishable from adults in around 48 hours. To avoid this problem, adult worms are transferred no less than every 48 hours for the first several days of their lives (Felker et al., 2020). Worms can also be sterilised using fluorodeoxyuridine (FUdR) which blocks DNA synthesis by inhibiting thymidylate synthetase, preventing the development of progeny. However, FUdR treatment is no longer the preferred method as it can alter the lifespan of some worm mutants, such as *tub-1* and *exo-3,* leading to inaccurate results (Felker et al., 2020). These limitations make large scale screening of worms difficult.

Therefore, various automated artificial vision analysis systems have been developed in recent years. But, there are several limitations to automated systems, such as changes in media culture, blocked zones or condensation of the petri dishes, all of which can result in false negatives or false positives (Puchalt et al., 2021).

Other scoring systems include microfluidic devices which provide high throughput data. The design of the device includes areas for worm stay and examination, inlets for adding fresh liquid media and food, and outlets for waste removal, eggs, and progeny. A movement tracker can be used to track the movement of a single worm over time. Another system is a flatbed scanner in which an agar plate with worms is flipped upside-down and placed in the scanner to allow for image capture for a specified time duration. Flatbed scanners take time-lapse images of worms and by depicting worm movement between images, can show which worms are alive (Puchalt et al., 2021). Therefore, there remains a need for an easy to implement, versatile and high throughput system to pinpoint the time of death.

Using autofluorescence as a marker of death, label-free automated survival scoring assays (LFASS) have been developed. This has notably enabled using 96 and 384-well plate formats to simultaneously measure time of death across multiple conditions, using fluorescence plate-readers. In these assays, raw fluorescence data is measured over time and median time of death is calculated from blue fluorescence dynamics (Benedetto et al., 2019).

1.8 Approach to project

With *C. elegans* longevity correlating with severe heat resistance rather than oxidative stress resistance, and the recent recognition of a key role for the gut microbiota in defining worm healthspan, this project will explore how *C. elegans* gut microbes found in its natural environment may interact with the worm physiology to mediate stress resistance and healthy ageing. I will follow complementary approaches to form a better understanding of the role of gut microbiota-host genetics interactions in animal health.

1.8.1 Establishing differential effects of gut microbiota in C. elegans severe stress resistance to identify gut microbes promoting healthy ageing

C. elegans young gravid adults will be exposed to natural gut microbiotas in monoxenic conditions and assayed for heat and oxidative stress resistance using LFASS (Label-Free Automated Survival Scoring) assays. Once beneficial bacteria are identified from the above steps, they will be further studied in monoxenic and cocktail conditions for their impact on lifespan, brood size, and locomotion, as proxies for fitness and health.

1.8.2 Testing the involvement of known gut-brain axis host genetic pathways in mediating gut microbiotas effects on C. elegans severe stress resistance

To determine if microbial effects on stress resistance involve known gut-brain axis pathways, LFASS assays will be conducted on Insulin/IGF-1 Signalling (IIS) and Kynurenine Pathway (KP) *C. elegans* mutants exposed to the same gut microbial isolates.

1.8.3 Identifying novel C. elegans genes involved in severe heat resistance and possibly healthy ageing

To identify new host genetic pathways potentially mediating gut microbiotas effects on severe stress resistance and healthy ageing, I will conduct a genome-wide RNAi screen on young adult *C. elegans* exposed to severe heat stress.

1.8.4 Summary

This thesis will describe the experimental methodologies employed, and the results of the approaches described above, including the optimisation of a new implementation of the LFASS protocol for microbial screening, which has underpinned most of the experimental work carried out here. As further investigations are required to form a coherent and comprehensive picture of the findings, I will also discuss limitations of my work, emerging hypotheses and how they might be tested.

2 Methods

2.1 Reagents and Media

All reagents were purchased from Thermo Fisher Scientific unless otherwise stated. Solutions for bacterial media are listed in **Table 3** (*E. coli* OP50, Terrific Broth, LB Broth and LB agar plates) and solutions for worm media are listed in **Table 4** (M9, worm bleaching solution and NGM plates). **Table 5** shows preparation of buffers.

Table 3 Solutions for Bacterial Cultures/plates

***E. coli* OP50 stock solution (1 L)**	
Bactotryptone	5 g
Yeast extract	2.5 g
H_2O	1 L
Autoclave and cool to 55 °C	
LB agar plates (1L)	
Bactotryptone	10 g
Yeast Extract	5 g
NaCl	10 g
H_2O	1 L
LB Agar	17 g
Autoclave and cool to 55 °C	
LB Broth (1L)	
Bactotryptone	10 g
Yeast Extract	5 g
NaCl	10 g
H_2O	1L
Autoclave and cool to 55 °C	
Terrific Broth (1L) for growing RNAi bacteria	
H_2O	900 mL
Bactotryptone	12 g
Yeast Extract	24 g

Glycerol	4 mL
Autoclave and cool to 55 °C	
KH_2PO_4	17 mL
K_2HPO_4	72 mL
Ampicillin (100 mg/mL)	500 µL
IPTG (1 M)	500 µL

Table 4 Solutions for worm cultures/plates

Contents of M9 solution (1L)	
Na_2KPO_4. $2H_20$	7 g
KH_2PO_4	3 g
NaCl	5 g
Autoclave and cool to 55 °C	
$MgSO_4$ (1M)	1 mL
NGM Agar plates (900 ml)	
NaCl	2.7 g
Agar/ agarose	15.3 g
Tryptone	2.25 g
H_2O	1 L
Autoclave and cool to 55 °C	
KH_2PO_4	22.5 ml
$MgSO_4$ (1M)	0.9 ml
$CaCl_2$ (1M)	0.9 ml
Cholesterol in Ethanol (5mg/ml)	0.9 ml
NGM-agarose plates (250 ml) for RNAi bacteria	
NaCl	0.75 g
Agarose	4.25 g
Tryptone	0.625 g
H_2O	250 mL
Autoclave and cool to 55 °C	
KH_2PO_4	6.25 mL

$MgSO_4$ (1M)	250 µl
$CaCl_2$ (1M)	250 µl
Cholesterol in Ethanol (5mg/ml)	250 µl
IPTG (1M)	1200 µl
Carbenicillin (50 µg/mL)	300 µl
Contents for bleaching solution (10 mL)	
NaOH (4M)	5 mL
NaClO	5 mL

Table 5 Salts

1M KH_2PO_4 (1L)	
KH_2PO_4	136g
MiliQ	1 L
Adjust pH to 6.0 with KOH tablets. *It should be approximately 14g of KOH to adjust the pH.	
Autoclave and cool to 55 °C	
1M $CaCl_2$	
$CaCl_2 \times 2H_2O$	29.40g
MiliQ water	200mL
Autoclave and cool to 55 °C	
1M MgSO4	
$MgSO_4 \times 7H_2O$	49.30g
MiliQ water	200mL
Autoclave and cool to 55 °C	
5mg/mL Cholesterol	
Cholesterol (-20°C)	150mg
100% Ethanol	30mL
Let all cholesterol dissolve and filter sterilise mixture into falcon tube.	

2.2 Bacterial and *C. elegans* strains used

The worm and bacterial strains used here are listed in **Table 6** and **7**, respectively (Cgc.umn.edu. 2021, Uniprot.org. 2021). All CeMbio+ (extended gut microbial strains) were gifted by Schulenburg laboratory. Asterisk (*) next to the bacterial strains represents the twelve members of the CeMbio collection.

Table 6 Worm Strains

Strain name	**Genotype**	**Description**
N2CGC	Wild-type	Wild-type worms have a generation time of about 3 days. The brood size is 350.
N20W	Wild-type	
GA1928	*daf-2 (e1370)*	Long-lived strain
HT1890	*daf-16(mgDf50)I; daf-2(e1370)*	Short-lived and dauer-defective
PJ1	*cad-1(j1)*	Gut transparent worms. Worms are small and have 90% reduced cathepsin. Egg retainers that have little to no reproduction at 25°C.
JT3	*aex-2(sa3)*	Constipated
JT73	*itr-1(sa73)*	Worms appear scrawny and grow slightly slower. Worms have long defecation cycle period, reduced brood size, especially at 25°C.
CB66	*unc-22(e66)*	Twitcher

CB156	*unc-25(e156)*	Worms appear slightly small and shrink when prodded by contracting both dorsally and ventrally.
CB113	*unc-17(e113)*	Worms coil. Lannate sensitive.
TQ1101	*lite-1(xu7)*	Defective phototaxis (light avoidance)
ABA004	*unc-17(e113)/ lite-1(xu7)*	Coiler worms that exhibit defective phototaxis
ABA006	*unc-25(e156)/ lite-1(xu7)*	Smaller and shrinker worms that exhibit defective phototaxis
DA1116	*eat-2 (ad1116)*	Worms eat less due to reduced pumping rate. Long lived worms. Show embryonic lethality in a significant proportion of worms that could be due to a mutation in an essential gene linked to eat-2.
IK777	*egl-8(nj77)*	EMS mutagen. The mutagen induces mutations in the sperm and oocytes of hermaphrodites.
OW478	*kmo-1(tm4529)*	Encodes kynurenine 3-monooxygenase
NL2099	*rrf-3(pk1426)*	Homozygous rrf-3 deletion allele. Increased sensitivity to RNAi when compared to WT animals

Table 7 Bacterial Strains

Bacterial strains	**Species**	**Lab of origin**
OP50 (CeMbio)	*Escherichia coli*	Schulenburg lab, University of Kiel
OP50 (CGC/ABA)	*Escherichia coli*	CGC/ABA
B. longum 1	*Bifidobacterium longum*	Schulenburg
B. longum 2	*Bifidobacterium longum*	Schulenburg
BIGb0170*	*Sphingobacterium multivorum*	Wild *C. elegans* from rotting apple (Samuel)
BIGb0172*	*Comamonas piscis*	Wild *C. elegans* from rotting apple (Samuel)
BIGb0393*	*Pantoea nemavictus*	Wild *C. elegans* from rotting plant stem (Samuel)
CEent1*	*Enterobacter hormaechei*	*C. elegans* N2 from mesocosm (Shapira)
JUb19*	*Stenotrophomonas indicatrix*	Rotting pear with wild *C. elegans* (Félix)
JUb44*	*Chryseobacterium scophthalmum*	Rotting apple with wild *C. elegans* (Félix)

JUb66*	*Lelliottia amnigena*	Rotting apple with wild *C. elegans* (Félix)
JUb134*	*Sphingomonas molluscorum*	wild *C. elegans* from rotting plant stem (Félix)
MSPm1*	*Pseudomonas berkeleyensis*	*C. elegans* N2 from mesocosm (Shapira)
MYb10*	*Acinetobacter guillouiae*	Wild *C. elegans* from compost (Schulenburg)
MYb11*	*Pseudomonas lurida*	Schulenburg
MYb21	*Comamonas*	Schulenburg
MYb49	*Ochrobactrum*	Schulenburg
MYb58	*Ochrobactrum*	Schulenburg
MYb69 (a)	*Comamonas*	Schulenburg
MYb69 (b)	*Comamonas*	Schulenburg
MYb71*	*Ochrobactrum vermis*	Wild *C. elegans* from compost (Schulenburg)
MYb115	*Pseudomonas*	Schulenburg

MYb121	*Erwinia*	Schulenburg
MYb158	*Acinetobacter*	Schulenburg
MYb174	*Enterobacter*	Schulenburg
MYb176	*Enterobacter*	Schulenburg
MYb177	*Acinetobacter*	Schulenburg
MYb181	*Sphingobacterium*	Schulenburg
MYb186	*Enterobacter*	Schulenburg
MYb191	*Acinetobacter*	Schulenburg
MYb25 (b)	*Chryseobacterium*	Schulenburg
MYb264	*Chryseobacterium*	Schulenburg
MYb317	*Chryseobacterium*	Schulenburg
MYb328	*Chryseobacterium*	Schulenburg
MYb330	*Pseudomonas*	Schulenburg
MYb331	*Pseudomonas*	Schulenburg

MYb371	*Pseudomonas*	Schulenburg
MYb375	*Erwinia*	Schulenburg
MYb382	*Sphingobacterium*	Schulenburg
MYb388 (a)	*Sphingobacterium*	Schulenburg
MYb396 (a)	*Comamonas*	Schulenburg
MYb396 (b)	*Comamonas*	Schulenburg
MYb398	*Pseudomonas*	Schulenburg
MYb416 (a)	Unknown	Schulenburg
MYb526 (a)	*Stenotrophomonas*	Schulenburg
MYb526 (b)	*Stenotrophomonas*	Schulenburg
MYb535 (b)	*Erwinia*	Schulenburg
MYb536	*Stenotrophomonas*	Schulenburg
MYb541	*Pseudomonas*	Schulenburg

2.3 Worm maintenance

C. elegans were frozen following established procedures. Starved L1 and L2 young larvae were frozen in 15% glycerol and *C. elegans* freezing solution, by gradually cooling the vials in a Styrofoam container at -80°C (Wormbook.org., 2021). Frozen worm strains were kept frozen at -80°C until needed, at which they were thawed at 20°C, and the vial content was poured onto NGM plates seeded with *E. coli* OP50. Then the following day, 10-30 live larvae were individually transferred to fresh OP50-NGM plates to propagate the worms (Stiernagle, T., 1999) and maintained at 15°C following standard procedure as previously described (Brenner 1974, Stiernagle 2006).

Worms were kept at 15°C, so they exit the dauer stage quicker. This is because higher temperatures, such as at 25°C, are more stressful and cause worms to remain as dauer. Worms were then picked and transferred onto plates using a worm picker. The worm pick was made by mounting a 1-inch piece of 32-gauge platinum wire into the tip of a Pasteur pipette and then the tip of the wire was flattened like a scooping spatula. Platinum wire can be sterilised between worm transfers using a flame to avoid contamination. The flattened end of the pick was touched onto OP50 paste from OP50 seeded NGM plate to form a blob of bacterial paste. This was then used to gently pick worms as they stick to the bacterial paste. Worms were then transferred quickly to OP50-NGM plates ensuring they don't desiccate and die as the bacterial paste dries (Stiernagle, T., 1999).

2.3.1 Growing large numbers of C. elegans on OP50

For LFASS and high-throughput RNAi screen, a large number of worms were required. Therefore, initially, a large number of *C. elegans* hermaphrodites of all stages were grown on NGM plates. Once the plates had enough gravid adult worms, they were bleached to achieve a synchronised population. This approach avoided unnecessary dietary changes, such as using alternate faster-growing *E. coli* strains like NA22, or richer growth media to remain as close as possible to the standard growth conditions that are still widely used.

The worms were propagated by cutting agar chunks measuring half a centimetre with a scalpel from the recently starved NGM worm plate. These chunks containing the worms were then

transferred onto eight 6 cm Petri dishes inoculated with 100 µL of OP50 and grown at 20°C. The worms were incubated at 20°C until they finished the food (approximately three days). The worms were then collected from these plates using sterile M9 buffer into a 15 mL conical tube and centrifuged at 142 rcf for 2 minutes. After centrifugation, the supernatant was removed, and the worms were transferred onto a 15 cm NGM plate inoculated with 3mL OP50 and 0.5 mL of concentrated OP50 culture. The worms were propagated on the plate for 3-4 days at 15°C by feeding them with 0.5 mL of concentrated OP50 every day. Once the worms had consumed their food, they were transferred onto additional plates and further propagated at 20°C until approximately 95% of the population consisted of gravid adults. For the N2 and NL2099 strains this took about 24 hours. The plates full with gravid adults were then bleached.

2.3.2 Worm Bleaching/synchronisation

The gravid adult worms were bleached using a 1:1 ratio of 4 molar Sodium hydroxide (NaOH) with Sodium hypochlorite (NaClO) solution. First, the worms were collected in Milli-Q water into 15 mL conical tubes. They were washed twice in Milli-Q water by centrifuging at 1500 rpm for 2 minutes. After centrifugation, the supernatant was removed, and 8 mL of Milli-Q water was added, followed by the addition of 2 mL of the bleach solution. The worm tubes were vortexed gently for a few seconds, and after seven minutes, the tubes were centrifuged. The supernatant was removed, and the worms were washed twice with M9 solution to remove the bleach. After supernatant removal and washing, the eggs were transferred onto unseeded 15 cm NGM plates and incubated for 24 hours at 20°C to allow L1 larvae to hatch. Worms were then collected in M9 and tubes were centrifuged as described earlier. Supernatant was discarded and worms were transferred onto NGM plates seeded with OP50. Worms were propagated on the plate for 3 days at 20°C by feeding them with 0.5 mL of concentrated OP50 every day until they reached the L4 stage.

2.4 Bacterial maintenance

E. coli grows on inexpensive carbon sources. The starting OP50 bacterial colonies were isolated on LB agar streak plates. LB is a rich medium in which bacteria grow faster (Stiernagle, T., 1999). White/beige colonies were formed, and a single bacterial colony was picked from the streak plate and inoculated to grow in 10mL liquid OP50 medium overnight at 37°C in a shaking incubator. The next day, bacteria were tested for contamination by growing a small 100µl bacterial lawn at room temperature. After testing, large NGM plates were seeded with 3ml OP50.

Concentrated *E. coli* (cOP50) was also prepared to grow a large number of worms required for the experiment. This was prepared by growing 10ml of OP50 culture in 1 litre of LB broth for 4 hours at 37°C, 160 rpm shaker incubator. Bacteria were then centrifuged at 3500 rpm, 20°C for 15 minutes. The supernatant was removed, and the pellet was resuspended with OP50 medium. Large plates seeded with 3ml OP50 were seeded with an additional 1ml of cOP50.

2.4.1 Preparation of CeMbio+ bacteria for LFASS assays

CeMbio+ bacteria were streaked on 6 cm LB plates and grown for 48 hours at 20°C. After this, all the bacterial mass grown on LB agar plates was collected using plastic loops and transferred into 1.5ml Eppendorf tubes containing 1ml of M9 solution ***(figure.29).*** Tubes were vortexed and then centrifuged at 1000 rpm for 10 minutes at 4 °C to form pellets. The supernatant (300 µl) was removed, and the bacterial pellet was resuspended by vortexing. Bacteria were then transferred onto a sterile 96 well plate, from which 10 µl of bacterial suspension was transferred onto 4 96-well NGM-agarose plates using a multichannel pipette (plates were made the day before using 150 µl NGM-agarose). NGM-agarose plates with bacterial suspensions were kept at 25°C for 24 hours for bacteria to grow on NGM overnight. On the other hand, the 96-well plate from which bacteria were transferred was sealed with parafilm and kept at 15°C to be used later in the experiment to feed the worms.

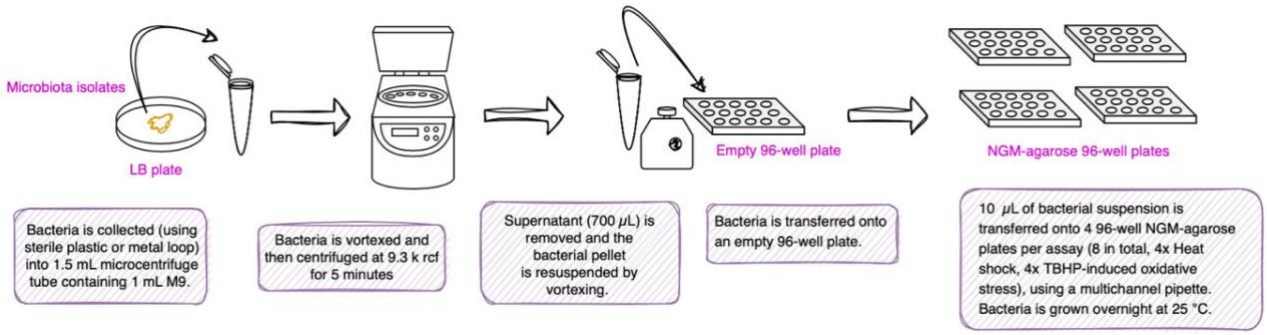


Figure 29. CeMBio+ collection. Bacterial mass grown on LB agar plates was collected and transferred into 1.5ml Eppendorf tubes containing 1ml of M9 solution. Tubes were vortexed and centrifuged at 1000 rpm for 10 minutes at 4 °C to form a pellet. The supernatant (300 µl) was removed, and the bacterial pellet was resuspended by vortexing. Bacteria were then transferred onto 96-well NGM-agarose plates using a multichannel pipette. Plates were kept at 25°C for 24h for bacteria to grow on NGM overnight.

2.5 LFASS Heat shock and oxidative stress assays

For death mutants, adults were picked and transferred daily onto new OP50-seeded NGM plates to synchronise egg laying every 24h. 4-5 days later, L4s were picked from these plates and transferred onto OP50 NGM plates kept at 25°C for 24 and 48 hours, to assess differences in stress resistance after day 1 and day 2 of adulthood, respectively ***(figure.30).*** 20-30 worms per well were then picked manually and processed for LFASS assays as previously described (Benedetto, 2019).

To assess the impact of microbiota on stress resistance in selected worm strains (N2OW, OW478, N2CGC, GA1928 and HT1890), 96-well NGM-agarose plates were seeded with CeMbio+ bacteria and kept at 25°C for 24h, as described earlier. Hermaphrodites from all strains were maintained at 20°C on NGM plates seeded with OP50. Large worm populations were grown for a week and age-synchronised by bleaching gravid adults. Synchronised worm populations were grown at 15°C on NGM agar plates seeded with OP50, until they reached the L4 stage. L4s were then washed and collected in M9 solution. 120-150 worms were transferred onto each well of the bacterial plates using a distriman pipette holding a 1250uL DistriTip (Gilson). Worms were kept on CeMbio+ strains for 36 hours at 25°C, after which LFASS assays were performed.

For both oxidative and heat stress assays, worms were transferred onto 384-well plate seeded with CeMbio+. Heat stress assay was performed using a plate reader set at 42°C and for oxidative stress, worms were subjected to 0.002% (7% by volume) tert-butyl hydroperoxide (t-BHP) with plate reader set at 25°C. The assays were performed as previously described (Benedetto 2019). All experiments included 4 biological replicates and were repeated on 3-4 different days (12 to 16 biological replicates in total) to account for experimental variability and variability between worm cohorts.

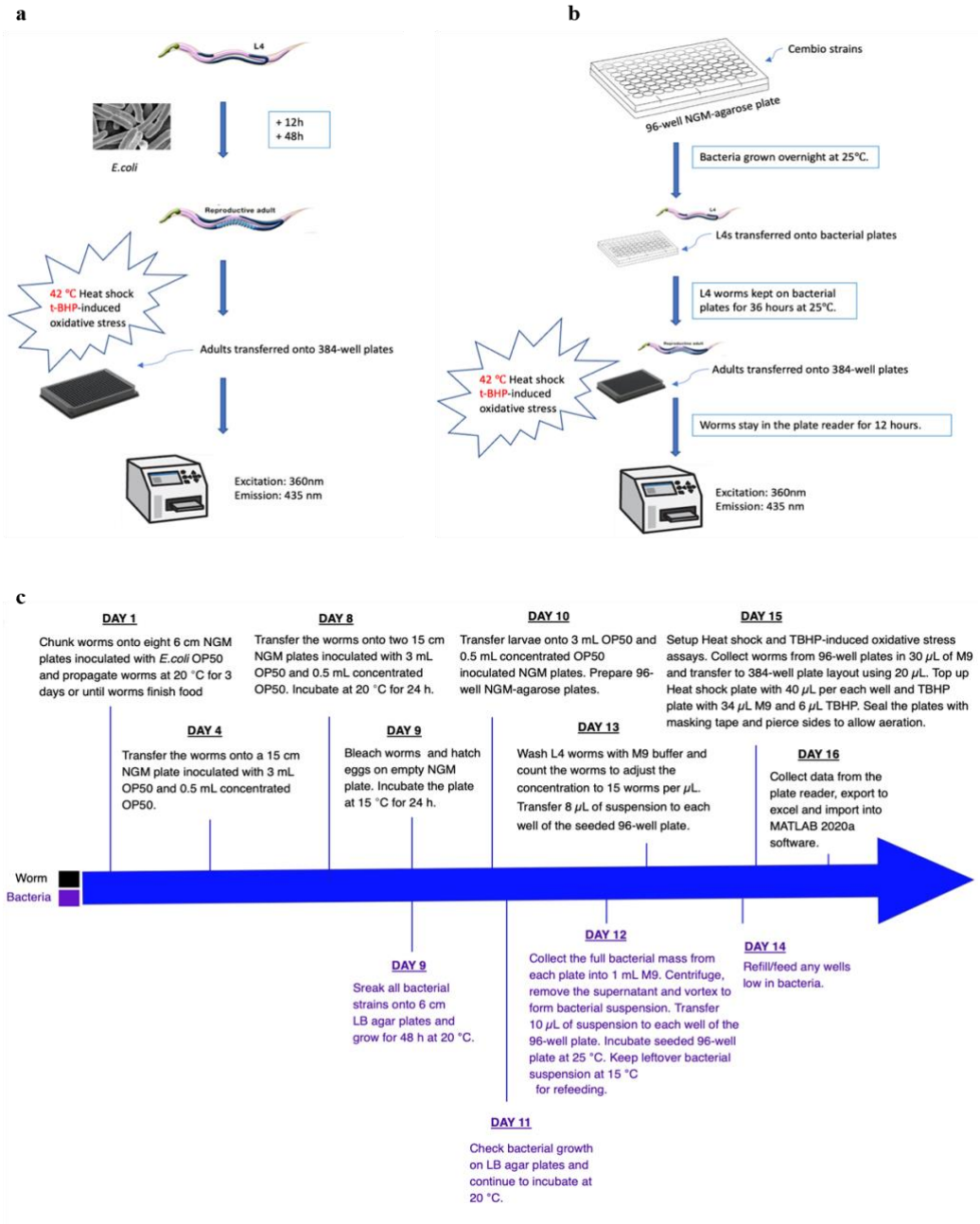


Figure 30. Heat and oxidative stress assays. a) Death mutants- L4+24h and L4+ 48 hours worms were transferred into 384-well plates and exposed to 42°C heat shock and 7% tertbutyl hydroperoxide induced oxidative stress in a plate reader. b) Microbiota - L4 worms were transferred and kept onto

NGM-agarose plates seeded with CeMbio+ strains for 36 hours at 25°C. Worms were transferred into 384-well plates and exposed to 42°C heat shock and t-BHP induced oxidative stress in a plate reader. c) Timeline of experiments- The experiment timeline outlines the experimental setup, the process of worm propagation, transfer onto bacteria for experimentation, and the duration of each stage (Ali et al., 2022).

2.6 CeMbio+ bacteria preparation for lifespan, movement and brood-size assays

Bacteria were streaked onto LB agar plates using sterile loops, followed by incubation at either 25°C for 24 hours or 20°C for 48 hours to promote bacterial growth. Afterward, a single colony was selected from each agar plate and transferred into 10 ml of OP50 growth medium. Liquid cultures were then incubated overnight at 20°C in a rotating incubator.

The following day, bacterial cultures were tested for contamination by inoculating plates with 100 µl of each culture. These plates were incubated at 20°C for a few days while being carefully examined under a microscope to ensure the bacterial lawn remained clean and free from contamination.

Once it was established that bacterial cultures were clean, small NGM plates were inoculated with 100 µl of the bacterial culture and dried before being stored at 4°C. Once plates were needed, they were brought to room temperature to ensure the temperature was suitable for the worms before transferring worms onto them.

Figure. 31 shows CeMbio+ bacteria preparation for lifespan, movement and brood-size assays. Certain bacterial strains from the CeMbio+ collection, like JUb44, MYb10, and MYb49, show enhanced growth compared to other isolates such as OP50. Therefore, these bacteria can be diluted with clean OP50 medium before seeding the plates, with dilution ratios potentially reaching up to 5 times. This ensures better visibility of worms, as some bacteria strains can be too dark and potentially interfere with accurate worm counting.

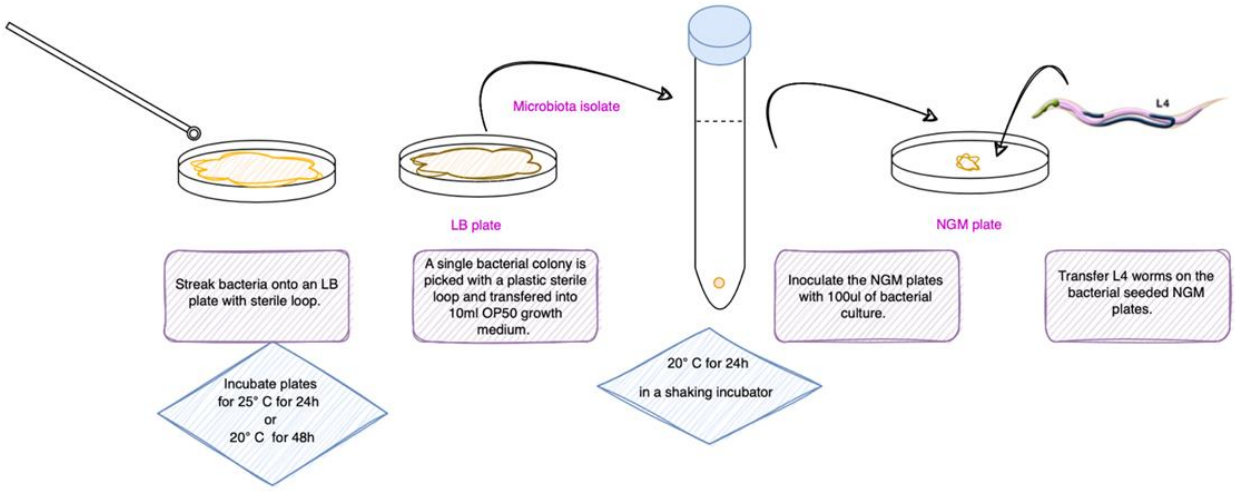


Figure 31. CeMBio+ bacteria preparation for lifespan, movement and brood-size assays. Bacteria is streaked onto LB plates using a sterile loop, followed by incubation at either 25°C for 24 hours or 20°C for 48 hours. After bacterial growth, a single colony is selected with a sterile loop and transferred into OP50 growth medium. The culture is then shaken in a rotating incubator for 24 hours until the bacteria are fully grown. Next, NGM plates are inoculated with 100 µl of the bacterial culture. L4 worms are transferred onto these seeded NGM plates for the experiment.

2.6.1 Brood size assays

The brood size experiment was conducted by transferring one L4 stage worm per plate ***(figure.32).*** One L4 worm was transferred onto each of the 12 NGM plates seeded with OP50 bacteria. The process was repeated for the CeMbio+ bacteria, enabling the brood size counting of 12 worms per strain. The plates were kept at 25°C overnight.

The following day, adult worms were transferred onto fresh plates and maintained at 25°C. The previous plates/egg plates were moved to 20°C for approximately 48 hours or until they reached the L4 stage. These plates were monitored closely, as certain bacteria may have altered the development rate of the worms. The objective was to allow the eggs to hatch and reach the L3/L4 stage, ensuring they were sufficiently large and easily countable. Meanwhile, the original 12 worms per strain were transferred onto fresh plates every 24 hours, maintaining them at 25°C, until day 5 or until the worms stop laying eggs. Afterward, the number of worms present on each plate were counted.

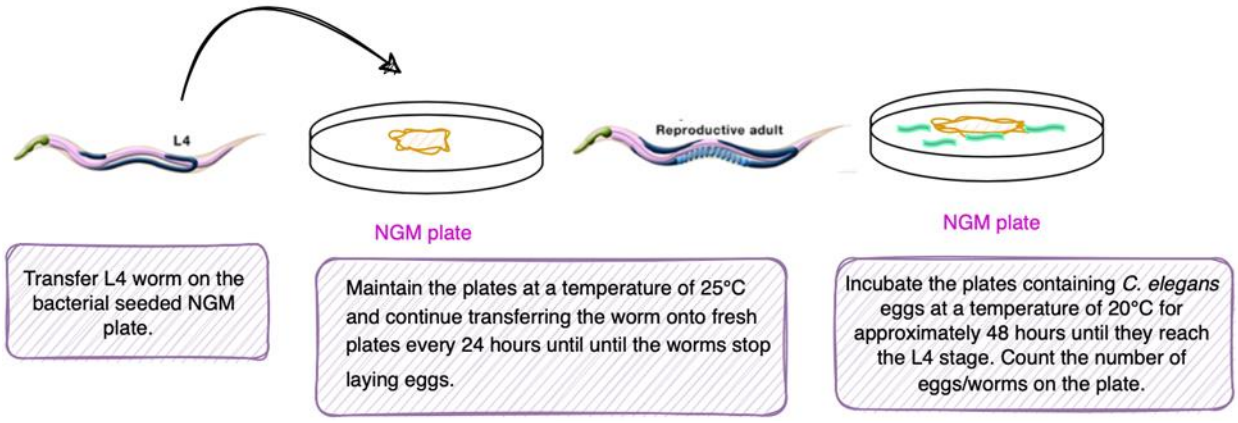


Figure 32. Brood size experiment. L4 worms are transferred onto bacterial seeded NGM plates. Maintain the plates at a temperature of 25°C and continue transferring the worms onto fresh plates every 24 hours until day 5 or until the worms stop laying eggs. Incubate the plates containing C. elegans eggs at a temperature of 20°C for approximately 48 hours until they reach the L4 stage. Afterward, proceed to count the number of worms present on each plate.

2.6.2 Lifespan and ABC scoring

Thirty *C. elegans* worms at the L4 stage were transferred onto each of the four NGM plates containing OP50 bacteria (control) resulting in a total of 120 worms. The process was repeated for CeMbio+ bacteria, thus allowing 120 worms per condition compared to the control. Distributing the worms across multiple plates facilitates counting and reduces the chances of contamination.

The worms were maintained at a constant temperature of 25°C throughout the experiment. The worms were transferred onto new plates every 24 hours until day 5 or until the worms ceased laying eggs, and old plates were discarded to prevent the original population from mixing with the progeny.

The worms were monitored daily, counting both dead and alive individuals until all the worms died. Any worms accidentally killed during handling or those that escaped the plate and desiccated were recorded as censored data. **Figure 33** shows Lifespan and ABC scoring method.

Worm movement can serve as an indicator of healthspan. Therefore, worm movement was also examined in parallel to lifespan analysis. The movement classification was as follows:

Category A: Worms that exhibited a fast sigmoidal shape while moving. These worms demonstrated healthy movement patterns, indicating good motor function and overall vitality.

Category B: Worms that could still move their bodies but moved in a straight shape and were slightly slower. Although they showed some decline in movement compared to Category A, they still retained the ability to move and were considered to be in a reasonably healthy state.
Category C: Worms that had lost the ability to move their bodies and only moved their heads when prodded with a pick. These worms had severely compromised movement and were experiencing a decline in healthspan.

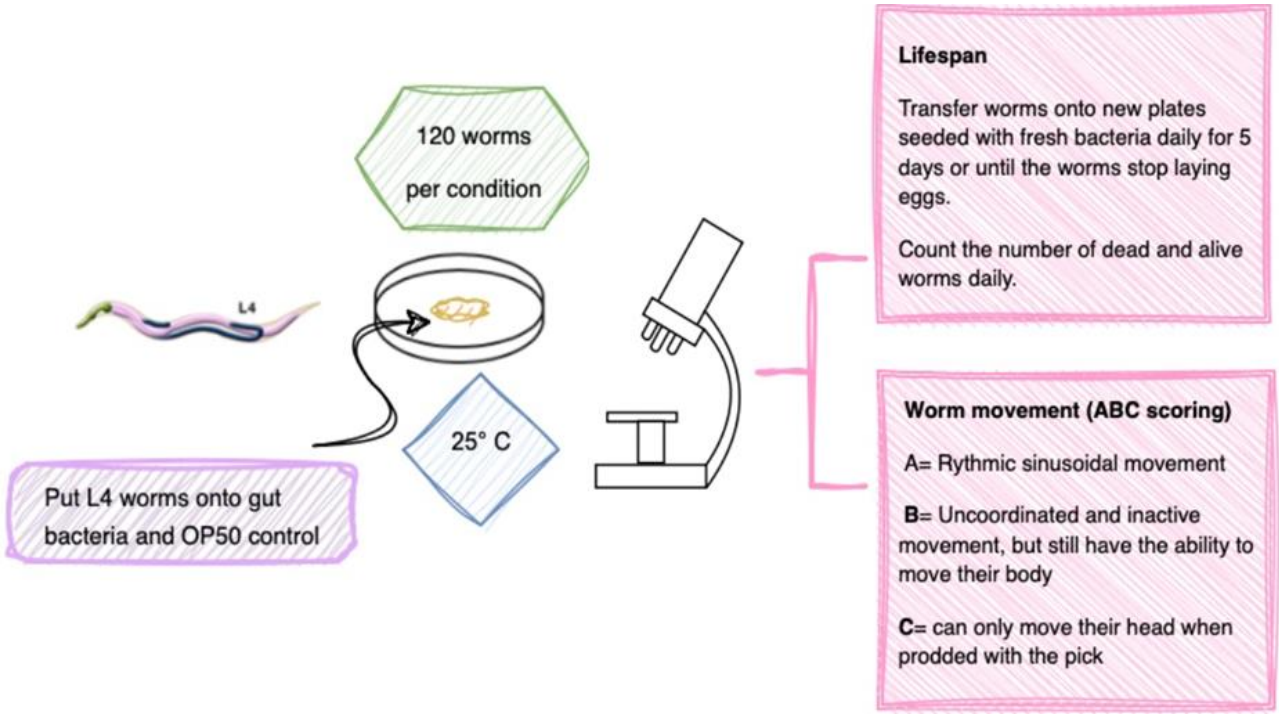


Figure 33. Lifespan and ABC scoring. i) Transfer worms onto new plates seeded with fresh bacteria daily until the worms stop laying eggs. Count the number of dead and alive worms daily. ii) ABC scoring. A- Rhythmic sinusoidal movement. B- Uncoordinated and inactive movement but still have the ability to move their body. C- Worms can only move their head when prodded with a pick.

2.7 CeMbio+ combinations for heat shock assay

Combination of five promising microbial isolates BIGb170, JUb19, CEent1, MYb21, and MYb71, with E. coli OP50 as the control, were used to determine the effects of the combination of microbial isolates on heat stress resistance. ***Figure. 34*** shows the layout of the 96 well plate with different bacterial combinations.

CeMbio+ bacteria were streaked on 6 cm LB plates and grown for 48 hours at 20°C. The next day, bacterial mass grown on LB agar plates was collected using plastic loops and transferred into 1.5 ml Eppendorf tubes containing 1 ml of OP50 medium. 96-well NGM-agarose plates was suspended with 20 µL bacteria in each well. Plates were then dried under the fume hood.

These experiments were conducted in parallel to the RNAi screen and therefore the same worm strain (NL2099) was used to be more time efficient. A synchronised population of L4 worms was washed and collected in M9 solution (refer to 2.8.2 for worm synchronisation protocol). 120-150 worms were distributed into each well using a DistriTip holding a 1250 µL DistriTip (Gilson). Worms were dried, kept on CeMbio+ strains for 24 hours at 25°C, after which LFASS assays were performed.

a

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	B+C+J+21+71	B+C+J+21+71	B+C+J+21+71	B+C+J+21+71	B+C	B+C	B+J	B+J	B+21	B+21	B+71	B+71	C+J	C+J	C+21	C+21	C+71	C+71	J+21	J+21	J+71	J+71	21+71	21+71
B	B+C+J+21+71	B+C+J+21+71	B+C+J+21+71	B+C+J+21+71	B+C	B+C	B+J	B+J	B+21	B+21	B+71	B+71	C+J	C+J	C+21	C+21	C+71	C+71	J+21	J+21	J+71	J+71	21+71	21+71
C	B+C+J+21+71	B+C+J+21+71	OP50	OP50	B+C	B+C	B+J	B+J	B+21	B+21	B+71	B+71	C+J	C+J	C+21	C+21	C+71	C+71	J+21	J+21	J+71	J+71	21+71	21+71
D	B+C+J+21+71	B+C+J+21+71	OP50	OP50	B+C	B+C	B+J	B+J	B+21	B+21	B+71	B+71	C+J	C+J	C+21	C+21	C+71	C+71	J+21	J+21	J+71	J+71	21+71	21+71
E	OP50	OP50	OP50	OP50	B+C	B+C	B+J	B+J	B+21	B+21	B+71	B+71	C+J	C+J	C+21	C+21	C+71	C+71	J+21	J+21	J+71	J+71	21+71	21+71
F	OP50	OP50	OP50	OP50	B+C	B+C	B+J	B+J	B+21	B+21	B+71	B+71	C+J	C+J	C+21	C+21	C+71	C+71	J+21	J+21	J+71	J+71	21+71	21+71
G	B	B	B	B	B+C+J	B+C+J	B+C+21	B+C+21	B+C+71	B+C+71	B+J+21	B+J+21	B+J+71	B+J+71	B+21+71	B+21+71	C+J+21	C+J+21	C+J+71	C+J+71	C+21+71	C+21+71	J+21+71	J+21+71
H	B	B	B	B	B+C+J	B+C+J	B+C+21	B+C+21	B+C+71	B+C+71	B+J+21	B+J+21	B+J+71	B+J+71	B+21+71	B+21+71	C+J+21	C+J+21	C+J+71	C+J+71	C+21+71	C+21+71	J+21+71	J+21+71
I	C	C	C	C	B+C+J	B+C+J	B+C+21	B+C+21	B+C+71	B+C+71	B+J+21	B+J+21	B+J+71	B+J+71	B+21+71	B+21+71	C+J+21	C+J+21	C+J+71	C+J+71	C+21+71	C+21+71	J+21+71	J+21+71
J	C	C	C	C	B+C+J	B+C+J	B+C+21	B+C+21	B+C+71	B+C+71	B+J+21	B+J+21	B+J+71	B+J+71	B+21+71	B+21+71	C+J+21	C+J+21	C+J+71	C+J+71	C+21+71	C+21+71	J+21+71	J+21+71
K	J	J	J	J	B+C+J	B+C+J	B+C+21	B+C+21	B+C+71	B+C+71	B+J+21	B+J+21	B+J+71	B+J+71	B+21+71	B+21+71	C+J+21	C+J+21	C+J+71	C+J+71	C+21+71	C+21+71	J+21+71	J+21+71
L	J	J	J	J	B+C+J	B+C+J	B+C+21	B+C+21	B+C+71	B+C+71	B+J+21	B+J+21	B+J+71	B+J+71	B+21+71	B+21+71	C+J+21	C+J+21	C+J+71	C+J+71	C+21+71	C+21+71	J+21+71	J+21+71
M	21	21	21	21	B+C+J+21	B+C+J+21	B+C+J+71	B+C+J+71	B+C+21+71	B+C+21+71	B+J+21+71	B+J+21+71	C+J+21+71	C+J+21+71	B+C+J+21	B+C+J+21	B+C+J+71	B+C+J+71	B+C+21+71	B+C+21+71	B+J+21+71	B+J+21+71	C+J+21+71	C+J+21+71
N	21	21	21	21	B+C+J+21	B+C+J+21	B+C+J+71	B+C+J+71	B+C+21+71	B+C+21+71	B+J+21+71	B+J+21+71	C+J+21+71	C+J+21+71	B+C+J+21	B+C+J+21	B+C+J+71	B+C+J+71	B+C+21+71	B+C+21+71	B+J+21+71	B+J+21+71	C+J+21+71	C+J+21+71
O	71	71	71	71	B	B	C	C	J	J	21	21	71	71	B+C+J+21	B+C+J+21	B+C+J+71	B+C+J+71	B+C+21+71	B+C+21+71	B+J+21+71	B+J+21+71	C+J+21+71	C+J+21+71
P	71	71	71	71	B	B	C	C	J	J	21	21	71	71	B+C+J+21	B+C+J+21	B+C+J+71	B+C+J+71	B+C+21+71	B+C+21+71	B+J+21+71	B+J+21+71	C+J+21+71	C+J+21+71

b

	Combination of 2 strains	Combination of 3 strains	Combination of 4 strains
	B+C	B+C+J	B+C+J+71
OP50	B+J	B+C+21	B+C+21+71
B= BIGb170	B+21	B+C+71	B+J+21+71
J= JUb19	B+71	B+J+21	C+J+21+71
C= CEent1	C+J	B+J+71	
21= MYb21	C+21	B+21+71	
71= MYb71	C+71	C+J+21	
	J+21	C+J+71	
	J+71	C+21+71	
	21+71	J+21+71	

Figure 34. 96-well plate layout for CeMbio+ combination assay: combinations of BIGb170, JUb19, CEent 1, MY21, and MYb71 with OP50 as the control were used. B= BIGb170, J= JUb19, C=CEent1, 21=MYb21 and 71=MYb71.

2.8 RNAi Bacteria Preparation

RNAi clones were frozen at -80°C and then slowly thawed at room temperature by placing the 96-well RNAi library plates on ice. Using a 96-well plastic replicating pad, clones were transferred onto LB plates containing tetracycline and ampicillin. The plates were then incubated at 37°C for 24 hours. Subsequently, the clones were inoculated and grown in 96-well deep plates containing Terrific broth supplemented with 100 mg/mL ampicillin and 1 M IPTG overnight at 37°C in a shaking incubator.

The 96-well deep plates were centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded, and 96-well agarose plates (containing 1 M IPTG and 50 µg/mL carbenicillin) were inoculated with bacteria using a multichannel pipette. The plates were dried and then incubated at 20°C for 24 hours. L4 worms were then transferred onto the plates.

2.9 Data Plotting and Analysis

2.9.1 LFASS Data acquisition and processing

LFASS data were acquired with a Tecan MNano+ and a Tecan Spark (Tecan, Austria) equipped with enhanced fluorescence optics and performing bottom fluorescence readings at 365nm excitation (UV light) and 430nm emission (blue light) every 2 min for 12 hours. Raw fluorescence data obtained were exported to Excel worksheets and imported into MATLAB 2020a software, as previously described (Benedetto, 2019) ***(figure. 35)***. Briefly, the LFASS routine fits a sigmoid curve onto normalised smoothed fluorescence data. A noise threshold is set, chosen above the fluorescence values measured in empty wells and below the peak blue fluorescence. Higher and lower boundaries for fluorescence curve fitting, as well as time intervals to fit minimum and peak signal amplitude, are set by the user after a quick visual scanning of the data. The first few time points (up to 12min) are excluded from the fitting as early fluorescence values can fluctuate due to worm thrashing. The peak of the curve corresponds to the median time of death. The LFASS routine return the estimated median time of death for each well in a .csv file. Values are then imported into GraphPad Prism 9.0 for statistical analysis and figure preparation. For death wave amplitude measurements (Fig22b), only the first 2 hours of data were considered, and the minimum fluorescence intensity was subtracted to the maximum peak fluorescence intensity.

For determining the influence of organismal death mutations on stress resistance, a one-way ANOVA followed by Dunnett's multiple comparison test was performed using GraphPad Prism 9.0, assuming significance for $p<0.05$. To investigate the contributions of worm genetic background (mutants) versus bacterial isolate in stress resistance, two-way ANOVA were performed with Dunnett's multiple comparison post-hoc analysis for multiple comparisons, comparing mutants with wildtype for each bacterial isolate in each LFASS assay performed, and comparing for each genotype of how CeMBio+ isolates impact stress resistance compared to OP50 control. We considered that significance was reached for adjusted $p<0.05$.

For RNAi, Excel files were analysed using LFASS to determine the median time of death. The plate average was taken and used as the control. Promising RNAi candidates were chosen based on how much they differed from the control.

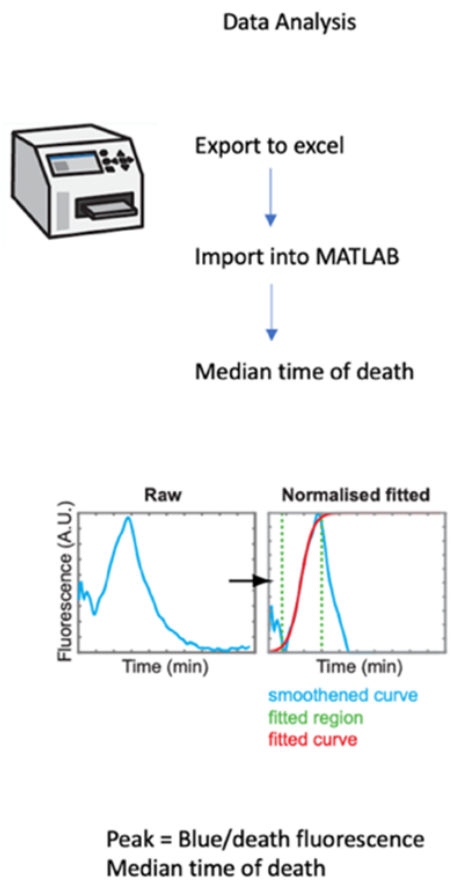


Figure 35. Data analysis. Data was analysed using MATLAB software following LFASS guidelines (Benedetto et al; 2019).

2.9.2 Lifespan and Brood size data analysis

For lifespan analysis, the number of dead, censored, and alive worms was counted manually. GraphPad Prism was used to plot Kaplan-Meier survival curves based on the collected data and the curves were compared using Log-rank (Mantel-Cox) test.

For brood-size analysis, the total number of eggs laid on each bacterial diet was collected and used to draw a bar graph on GraphPad Prism. Additionally, the number of eggs laid on each day was also plotted. One-way ANOVA was performed using OP50 (CGC/ABA) as the control versus gut microbiota to statistically analyse the results.

Worms were categorised into different movement categories (ABC) throughout their lifespan by visual observation. Worms from all categories were plotted on a line graph, illustrating their movement changes over time.

3. Results

3.1 Overview of the project and optimisation of LFASS assay

Figure. 36 shows a simplified scheme of the experiments that were conducted. The first step was to optimise the LFASS assays to make sure that the accurate median time of death is recorded. This approach relies on applying stressors, such as heat shock and oxidative stress that lead to the death of the animal, allowing the recording of the time of death. An animal will be deemed more resistant if it dies later. Therefore, it was first investigated whether the impairment of the organismal death pathway genes (*itr-1,lite-1,cad-1,aex-2,unc-22,unc-25,unc-17,eat-2, and egl-8)* involved in the generation of the autofluorescence affects the outcomes of heat and oxidative stress assays. Secondly, the death fluorescence amplitude of different CeMbio+ bacteria was recorded to determine whether the bacteria affected the autofluorescence generated by the worms and whether it impacted the death readouts.

Once the assay was deemed fit and reliable, the role of *C. elegans'* natural gut microbiota in health was examined by comparing it to *E. coli* OP50. It was also investigated whether the effects of the microbiota were due to the modulation of molecular pathways, specifically IIS and KP. Promising beneficial gut bacterial strains were selected to assess their impact on lifespan, brood size, and worm movement. Furthermore, combinations of microbial isolates were used to determine their impact on heat stress resistance. Finally, an RNAi screen was conducted to identify genes that may promote heat stress resistance.

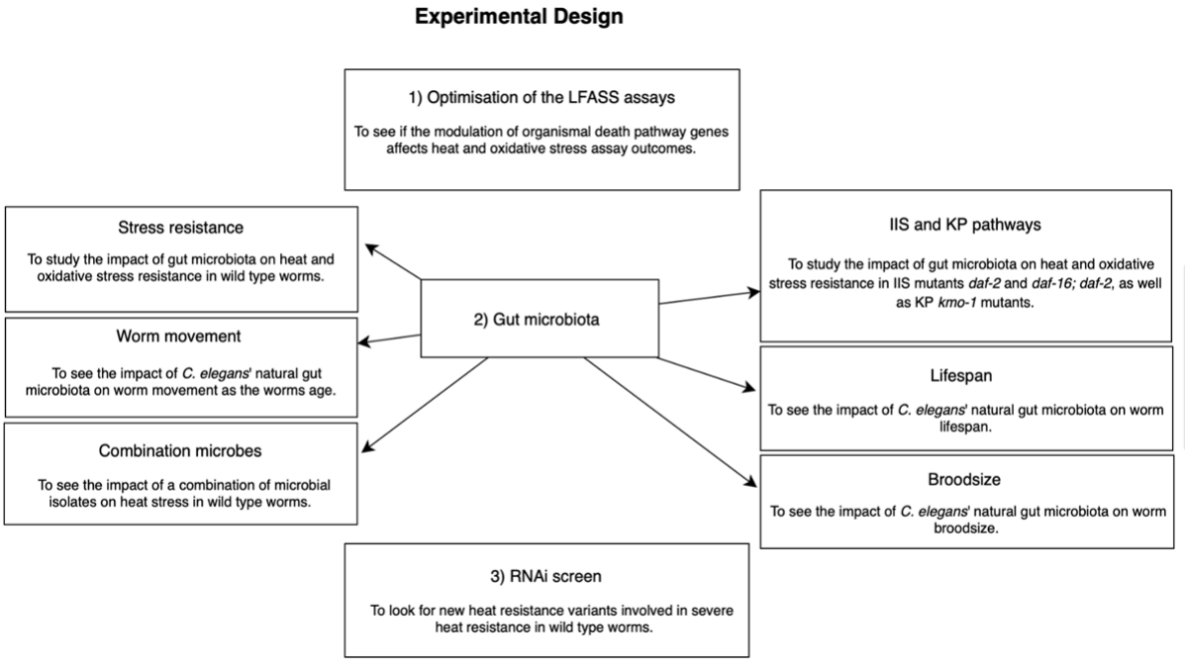


Figure 36. Experimental design. The diagram shows several different assays that were conducted. These include heat and oxidative stress assays using IIS and KP mutants, worm movement analysis, lifespan and brood size assays using CeMBio+ bacteria, and an RNAi screen to identify genes involved in heat stress.

3.1.1 Modulation of organismal death pathway genes do not dramatically affect heat and oxidative stress LFASS assay outcomes

First, I checked if the LFASS assay produced reliable results by checking if timing of death is delayed by impairment in the organismal death pathway.

The LFASS assay relies on the accumulation of blue autofluorescence in the worm gut as a death signal. However, this signal develops over time when most of the worms have died in the assay and is not an exact measure of the time of death, but rather a best estimate. Worms are considered more resistant if they die later, as determined by the timing of the autofluorescence peak.

Any endogenous process unrelated to stress resistance that delays or confounds the visualisation of the death fluorescence may affect the interpretation of LFASS assays. Therefore, it was necessary to ensure that the timing of death was not significantly biased by other processes involved in the generation of the death wave.

Because neuronal necrosis requires Ca^{2+} release from ER stores, Coburn et al. tested mutants in the ryanodine receptor *(unc-68),* inositol-1,4,5-triphosphate receptor *(itr-1),* and the ER Ca^{2+}-binding protein calreticulin (*crt-1)* that suppress neuronal necrotic cell death. They found

that these mutations, along with the mutations in the calpain TRA-3 mutant *(tra-3)* and the cathepsin mutant *(cad-1)* significantly reduced death fluorescence. *daf-2* mutants also had reduced death fluorescence (Coburn et al., 2013). This could lead to a delay in death readout, that could potentially be misinterpreted as increased resistance to stress.

Thus, I first investigated whether modulation of death wave gene activity could compromise the effectiveness of LFASS to screen for stress resistance variants, by comparing wild-type and *daf-2* worms with mutants involved in the death pathway, *lite-1, cad-1, aex-2, itr-1,unc-22,unc-25,unc-17,eat-2, and egl-8.* To do this, approximately 120 day 1 and day 2 adults per condition were exposed to heat and oxidative stress.

The results showed that worms die quicker when 42°C heat shock is applied (within 40-130 minutes approximately) compared to 7% tertbutyl hydroperoxide oxidative stress (approximately within 90-240 minutes). One-way ANOVA was performed using wild-type worms as the control followed by Dunnett's multiple comparison test ($p < 0.05$).

The results showed that most of the mutants involved in the organismal death pathway were not that critically different from the wild-type in their response to heat and oxidative stresses on both days, suggesting that impairment of the organismal death pathway does not critically impact the timing of death in this stress assay ***(figure. 37)***.

Compared to the wild-type, there was a slight significant increase in heat stress timing of death in *daf-2, itr-1* and *unc-25* on day 1 and in *lite-1*, *daf-2*, and *unc-25* on day 2 ($p < 0.05$). There was a decrease in *lite-1*, *daf-16;daf-2* and increase in *itr-1* response to oxidative stress in day 1 worms but a decrease in *aex-2* response on day 2 ($p < 0.05$).

As expected, compared to the wild-type, *daf-2* mutants were critically more resistant to both stresses on both days. There were also no critical differences in response to stress between day 1 and day 2 adults.

Once it was shown that the modulation of the death pathway genes does not dramatically impact the death readouts in worms, the assay was considered to provide reliable results. I then sought to determine whether different bacteria influence the amplitude of the blue autofluorescence generated by the worms, and whether this could impact the readout.

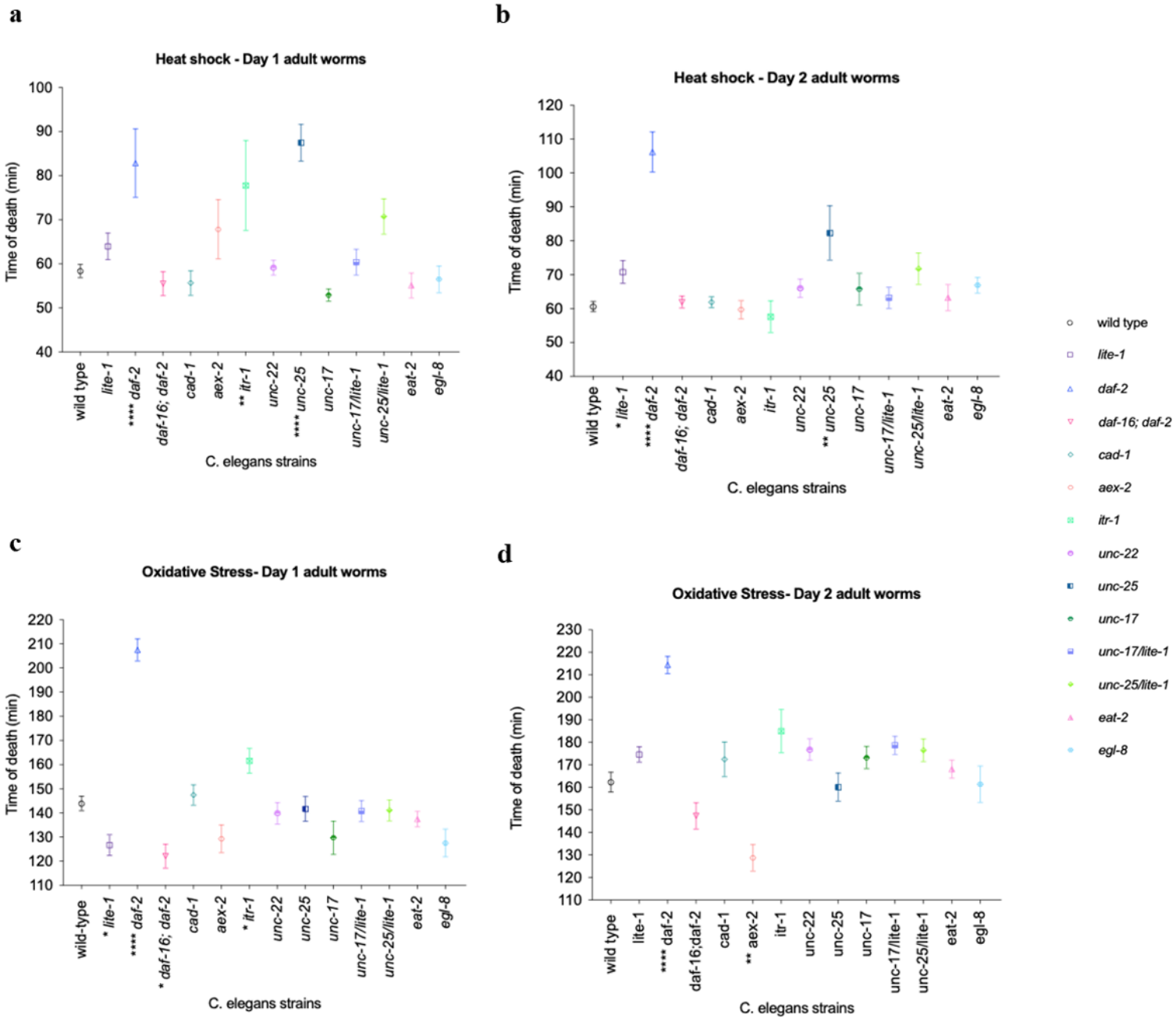


*Figure 37. Effect of organismal death genes on young adult worm resistance to severe stress. (a) Severe heat stress resistance (42°C) of day 1 adult mutant vs control worms; (b) Severe heat stress resistance of day 2 adult mutant vs control worms; (c) Severe oxidative stress resistance (7% tertbutyl hydroperoxide) of day 1 adult mutant vs control worms; (d) Severe oxidative stress resistance of day 2 adult mutant vs control worms. Control worms are wild-type; positive control worms are daf-2(e1370). Error bars represent the standard error of the mean (SEM). * indicates statistical significance at p=<0.05 with one-way ANOVA followed by Dunnett's multiple comparison test. 120 worms per condition were used.*

3.1.2 Death fluorescence readouts are impacted by the bacterial species worms feed on

Once I confirmed that modulation of the death pathway genes do not dramatically impact the death readouts in worms, I then checked whether bacteria influence the amplitude of the autofluorescence.

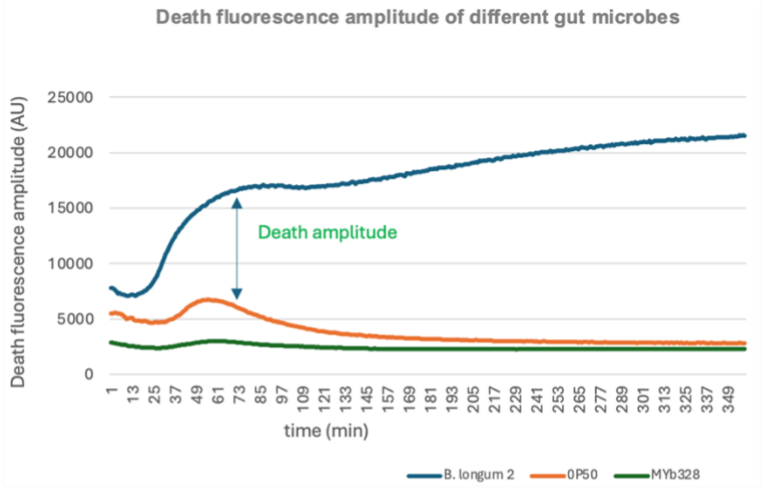
Bacteria can influence the amplitude of the blue autofluorescence generated by worms which means that the death autofluorescence levels change depending on the bacteria. Therefore, I looked at the amplitude of the blue autofluorescence generated by worms fed on different bacteria and compared with the blue autofluorescence peak to determine whether these amplitude differences impacted the death readout. 120 worms per condition were used.

Compared to the OP50, *B. longum 2* has increased, whereas MYb328 has decreased death autofluorescence in wild-type worms ***(figure. 38)***. These differences in death fluorescence amplitude across different microbes could be due to different anthranilate levels and accordingly different KP activities.

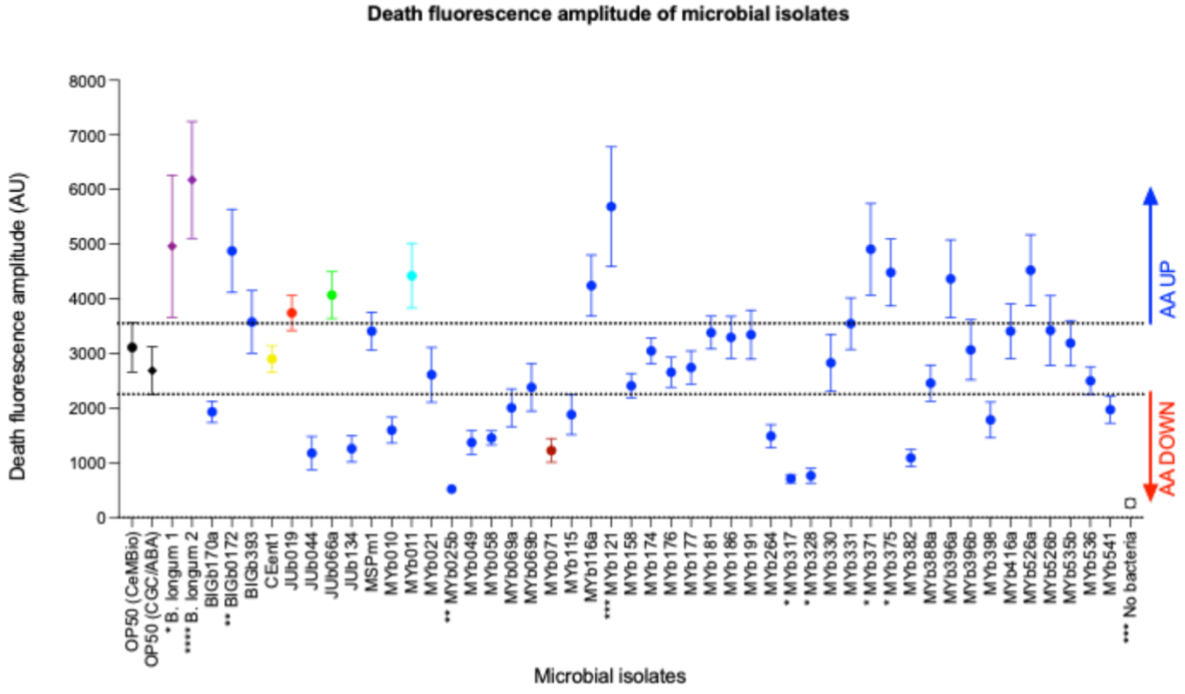
The one-way ANOVA followed by Dunnett's multiple comparison test showed that *B. longum* 1, *B. longum* 2, BIGb0172, MYb121, MYb371 and MYb375 have increased death amplitude compared to OP50, whereas MYb25b, MYb317 and MYb328 have decreased death amplitude compared to OP50, in wild-type worms ($p<0.05$).

Despite the difference in amplitudes, death waves are well-defined due to significant death peaks and therefore we can determine the time of death accurately. This means that it is possible to precisely pinpoint times of death and compare them across bacterial strains. Once I confirmed that the death readout was reliable, I used LFASS to study the influence of *C. elegans*' natural gut microbiota composition on adult worms' heat and oxidative stress tolerance, as well as overall health (brood size and movement).

a



b



c

Bacteria with increased Death amplitude compared with OP50	Bacteria with decreased Death amplitude compared with OP50
B. longum 1	MYb25b
B. longum 2	MYb317
BIGb0172	MYb328
MYb121	
MYb371	
MYb375	

*Figure 38. Death fluorescence amplitude of different gut microbe isolates. a) Death fluorescence amplitude of OP50, B. longum 2 and MYb328 in wild-type worms. b) Average death fluorescence amplitude of worms on Cembio gut microbes. c) One-way ANOVA followed by Dunnett's multiple comparison test was performed using OP50 (CGC/ABA) as the control, statistically significant = * (<0.05). 120 worms per condition were used.*

3.1.3 Summary

My first aim was to make sure that the LFASS provides reliable results. Therefore, I looked at the genes which are involved in organismal death pathway as the impairment of the organismal death pathway could affect death-associated fluorescence and consequently delay the timing of death, which might be misinterpreted as increased stress resistance. I found that most mutants involved in the organismal death pathway were not critically different from the wild-type in their response to heat and oxidative stresses, and *daf-2* mutants (positive control) had higher median time of death compared to wild-type and death mutants, suggesting that the impairment of death pathway does not delay the death signal too much.

However, I did make a note of *unc-25* mutants which on day 1 of the heat shock assay, showed an increased time of death (by five minutes) compared to *daf-2*. This was not observed in day 2 heat shock animals or in the oxidative stress assay. We expect some delay in the timing of death in our assay, as organismal death processes that lead to death fluorescence can significantly influence the screening results. This will also help in defining a threshold to consider when selecting top and bottom candidate genes in the RNAi screen (results chapter 3).

Because, I intended to use gut microbes for heat and oxidative stress assays, I also looked at the influence of gut microbes on the amplitude of autofluorescence generated by worms and if it affects the timing of death in the assay. I found that despite the difference in amplitudes, death waves are well-defined due to significant death peaks and therefore we can determine the time of death accurately.

3.2 Influence of gut microbiota composition on *C. elegans* adult heat tolerance, oxidative stress handling, and health (brood size, movement)

3.2.1 Gut microbial isolates differentially affect oxidative and heat stress resistance in C. elegans

To study the impact of gut microbiota on stress resistance, adult wild-type worms were exposed to heat (42°C) and oxidative stress (7% tBHP). 120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicates.

The results showed that gut microbial isolates differentially affect oxidative and heat stress resistance in wild-type *C. elegans* ***(figure.39).***

The two-way ANOVA (CGC/ABA OP50 compared to the gut microbiota) followed by Dunnett's multiple comparison test showed that MYb021, critically improved resistance to heat stress, whereas JUb066 (a), MYb011, MYb025, MYb264, MYb375, MYb398, MYb416, MYb526 (a), and MYb535 (b) sensitised wild-type worms to heat stress ($p < 0.05$).

Two-way ANOVA (followed by Dunnett's multiple comparison test) using CeMbio+ OP50 as the control with the gut microbiota was also performed. The results showed that *B. longum* 2, JUB44, MYb021, MYB317, and MYb396(a), critically improved resistance to heat stress in wild-type worms, whereas MYb416 (a), MYb526(a), and MYb535 (b) sensitised wild-type worms to heat stress ($p < 0.05$).

For the rest of the experiment, I used CGC/ABA OP50 as the control, to keep results comparable to our lab.

These reason for gut microbial isolates differentially affecting oxidative and heat stress resistance in *C. elegans* could be due to the modulation of the IIS and KP pathways by CeMbio+ bacteria. Therefore, in the next step, I examined IlS (*daf-16; daf-2*) and KP (*kmo-1*) mutants.

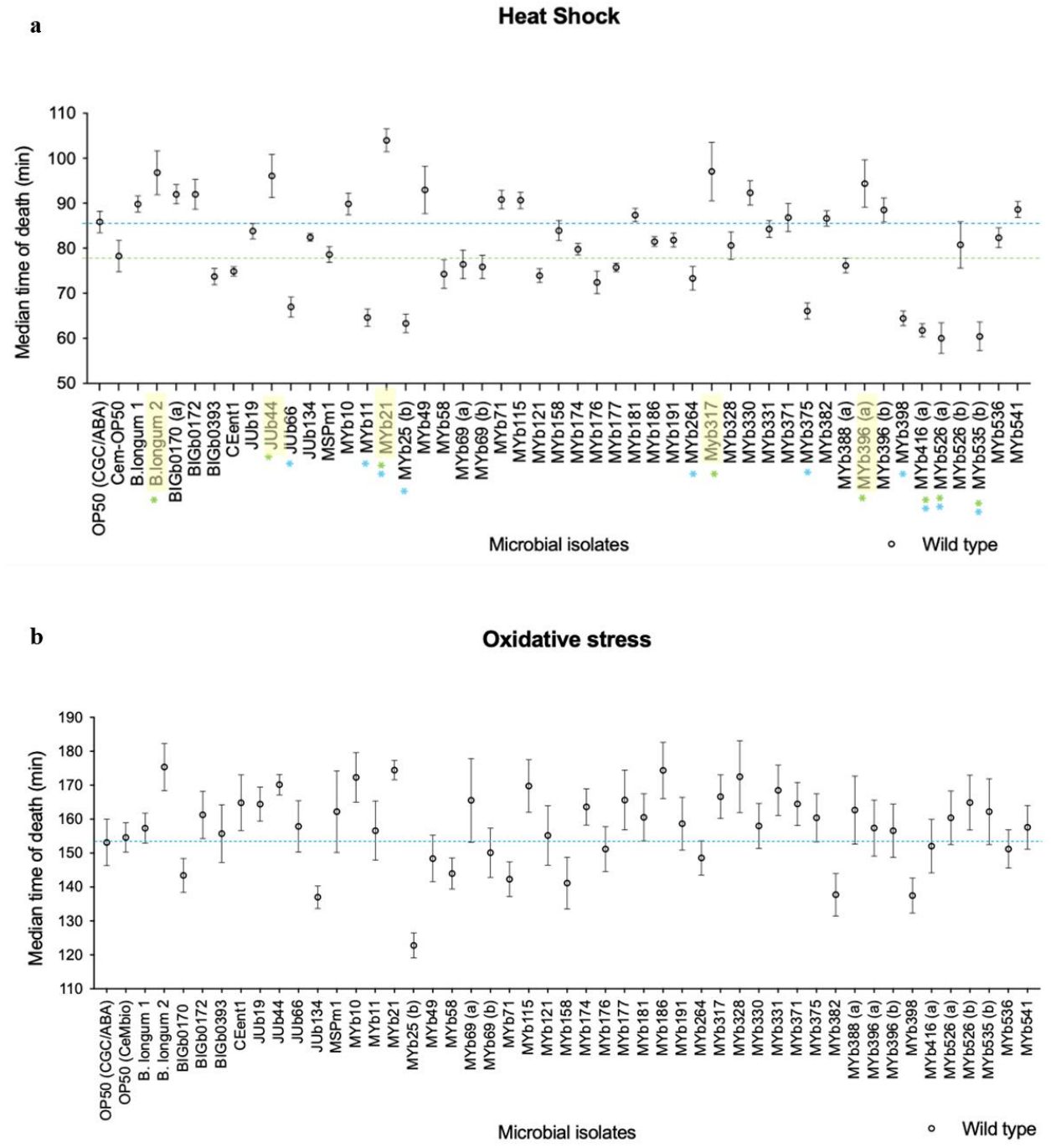


*Figure 39. Wild-type worms on the gut microbiota. a) Severe heat stress (42°C) resistance of wild-type adult worms. b) Severe oxidative stress resistance (7% tertbutyl hydroperoxide) of wild-type adult worms. Error bars represent the standard error of the mean (SEM). * indicates statistical significance at p<0.05 with two-way ANOVA followed by Dunnett's multiple comparison test. Blue * = OP50 CGC/ABA, * Green = OP50 CemBio+ control. 120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicates.*

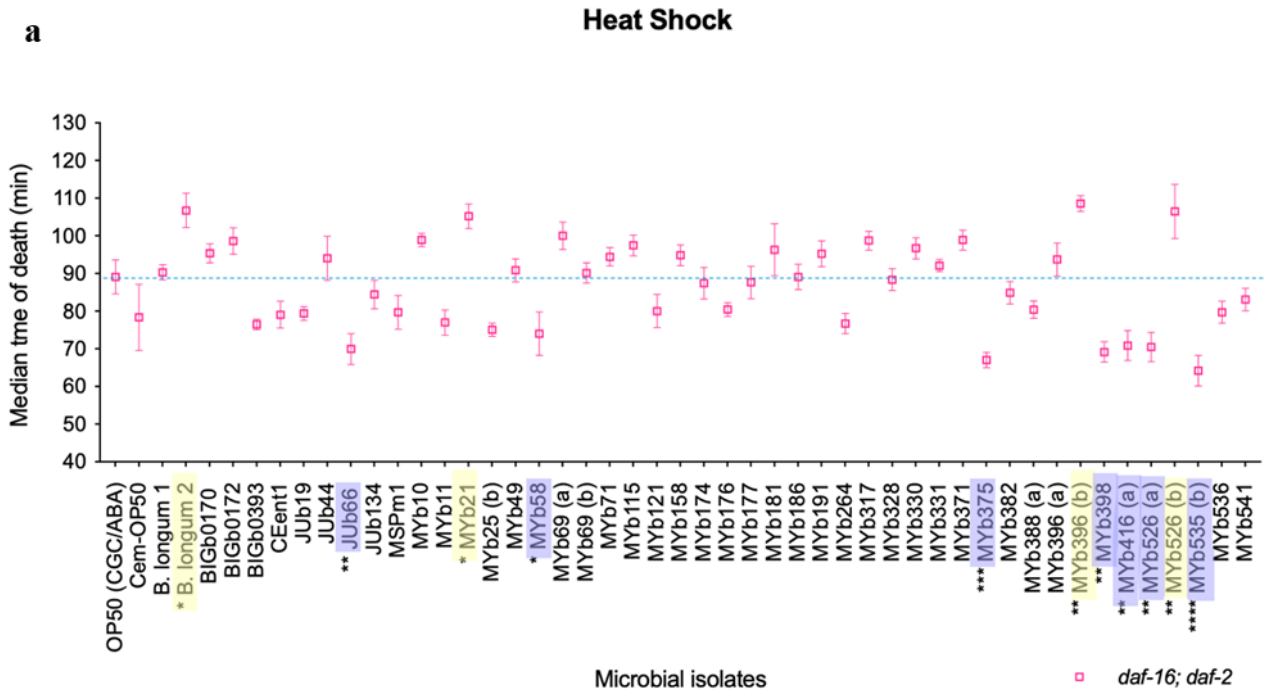
3.2.2 IIS mediates some of the shifts in stress resistance seen with some gut microbiota isolates

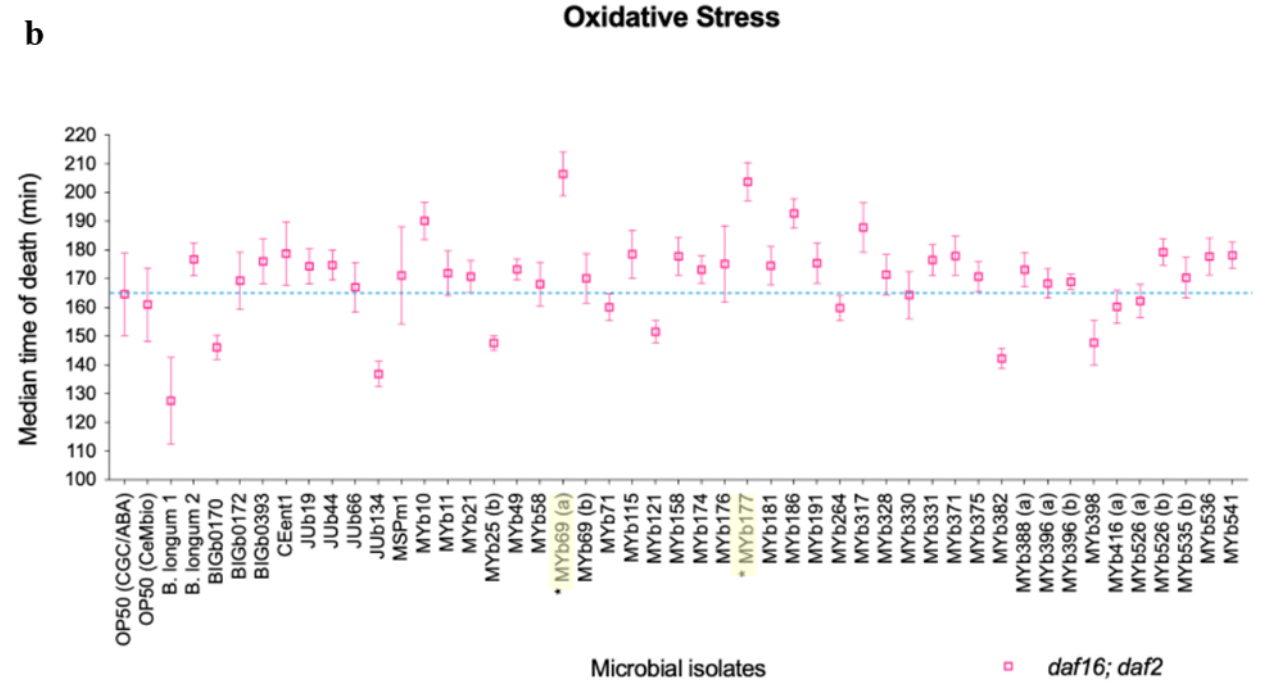
Next, to test whether IIS mediates some of the microbiota effects on worm stress response, IIS mutants *daf-2* and *daf16;daf-2* were exposed to CeMbio+ microbes before being exposed to heat and oxidative stress. Then, two-way ANOVA followed by Dunnett's multiple comparison test was performed using OP50 (CGC/ABA) as the control.

120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicates. The results showed that most gut bacteria influence stress response (increase or decrease) in IIS dependent or independent manner (***figure.40***). The way to determine if the changes are IIS dependent or independent is to observe the changes in both wildtype and *daf-2* worms. If there is an increase/decrease in both control and *daf-2* worms, the increase/decrease is IIS independent. However, if the increase/decrease is only in wild-type worms, then changes are due to IIS signalling.

In *daf-16;daf-2* worms, *B. longum* 2, MYb21, MYb396(b), and MYb526 (b) significantly improved resistance to heat stress and MYb69 (a), and MYb177 significantly improved resistance to oxidative stress when compared to the control OP50 (ABA/CGC). JUb66, MYB58, MYb375, MYb398, MYb416 (a), MYb526 (a), and MYb535 (b) sensitised *daf-16;daf-2* worms to heat stress ($p < 0.05$).

daf-2 is a long-lived stress resistant strain, and as expected, the time of death in OP50 is higher in *daf-2* when compared to the wild-type. In *daf-2* mutants, *B. longum* 2, JUb44 and MYb21 significantly increased resistance to heat stress, whereas BIGb0393, JUb19, JUb66, MYb11, MYb69(b), MYb174, MYb176, MYb375, MYb398, MYb416(a), MYb526(a), MYb535 (b), and MYb536 sensitised the worms to heat stress.


a
Heat Shock
Median tme of death (min)
130
120
110
100
90
80
70
60
50
40
OP50 (CGC/ABA)
Cem-OP50
B. longum 1
* B. longum 2
BIGb0170
BIGb0172
BIGb0393
CEent1
JUb19
JUb44
** JUb66
JUb134
MSPm1
MYb10
MYb11
* MYb21
MYb25 (b)
MYb49
* MYb58
MYb69 (a)
MYb69 (b)
MYb71
MYb115
MYb121
MYb158
MYb174
MYb176
MYb177
MYb181
MYb186
MYb191
MYb264
MYb317
MYb328
MYb330
MYb331
MYb371
*** MYb375
MYb382
MYb388 (a)
MYb396 (a)
** MYb396 (b)
** MYb398
** MYb416 (a)
** MYb526 (a)
** MYb526 (b)
**** MYb535 (b)
MYb536
MYb541
Microbial isolates
daf-16; daf-2


b
Oxidative Stress
Median time of death (min)
220
210
200
190
180
170
160
150
140
130
120
110
100
OP50 (CGC/ABA)
OP50 (CeMbio)
B. longum 1
B. longum 2
BIGb0170
BIGb0172
BIGb0393
CEent1
JUb19
JUb44
JUb66
JUb134
MSPm1
MYb10
MYb11
MYb21
MYb25 (b)
MYb49
MYb58
* MYb69 (a)
MYb69 (b)
MYb71
MYb115
MYb121
MYb158
MYb174
MYb176
* MYb177
MYb181
MYb186
MYb191
MYb264
MYb317
MYb328
MYb330
MYb331
MYb371
MYb375
MYb382
MYb388 (a)
MYb396 (a)
MYb396 (b)
MYb398
MYb416 (a)
MYb526 (a)
MYb526 (b)
MYb535 (b)
MYb536
MYb541
Microbial isolates
daf16; daf2

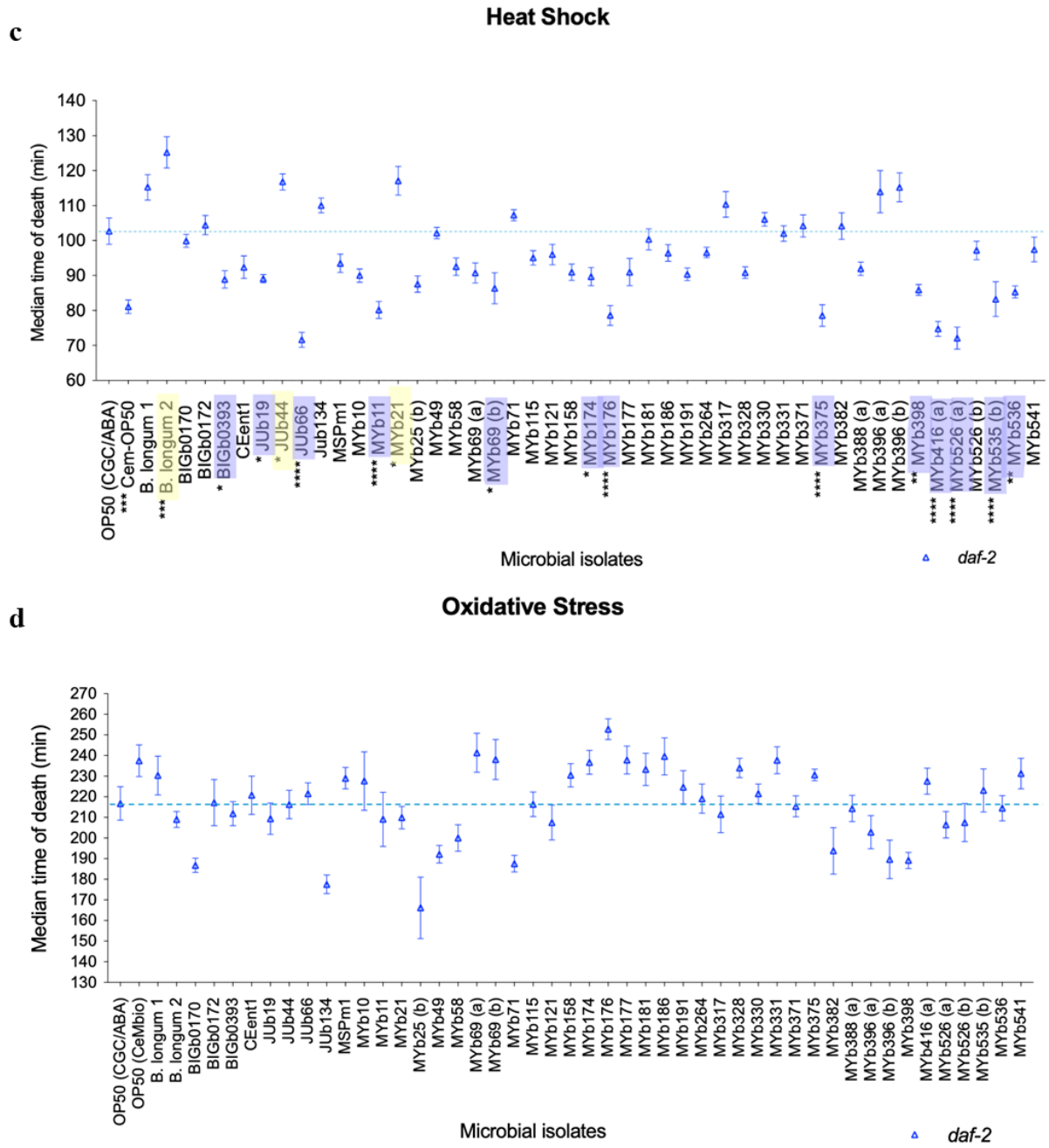


*Figure 40. IIS mutants (daf-16;daf-2 and daf-2) on the gut microbiota. a) Severe heat stress (42°C) resistance of daf-16;daf-2 on adult worms. b) Severe oxidative stress resistance (7% tertbutyl hydroperoxide) of daf-16;daf-2 on adult worms. c) Severe heat stress (42°C) resistance of daf-2 on adult worms. d) Severe oxidative stress resistance (7% tertbutyl hydroperoxide) of daf-2 on adult worms. Error bars represent the standard error of the mean (SEM). * indicates statistical significance at p<0.05 with two-way ANOVA followed by Dunnett's multiple comparison test. Yellow = increase in stress resistance, purple = decrease in stress resistance. 120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicates.*

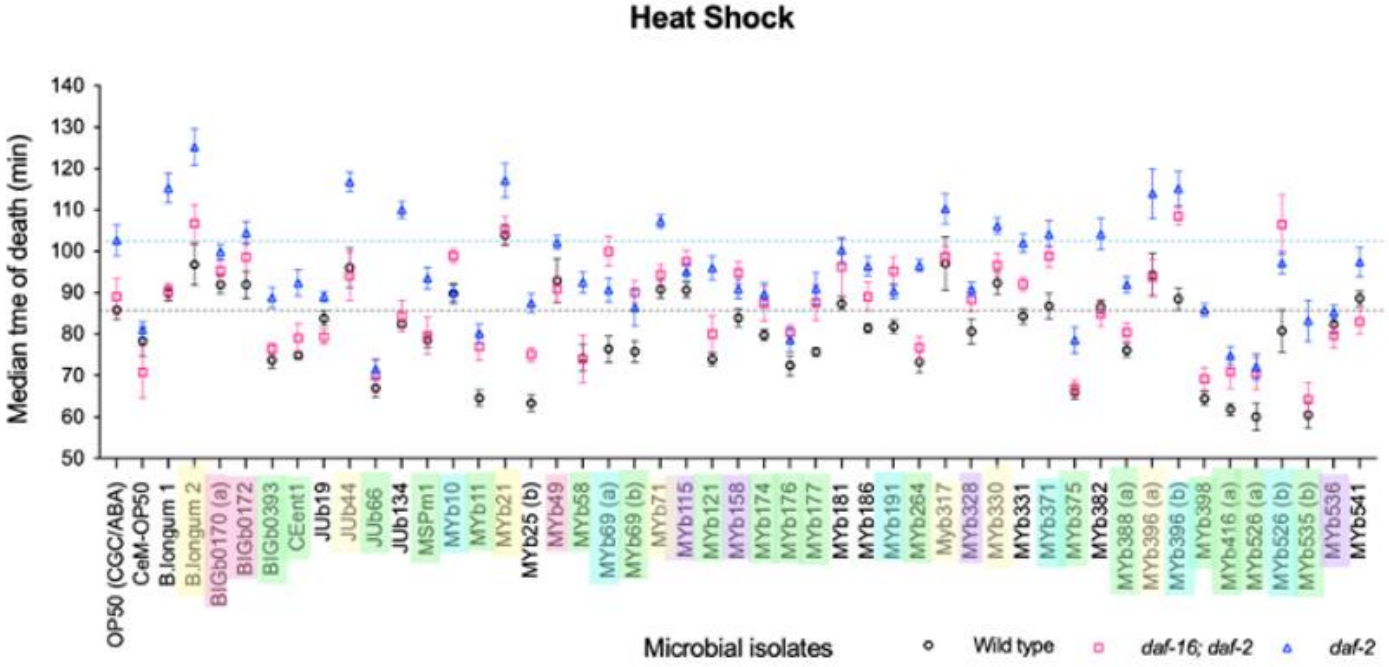
3.2.3 Gut microbiota influences response to stress in IIS dependent or independent manner

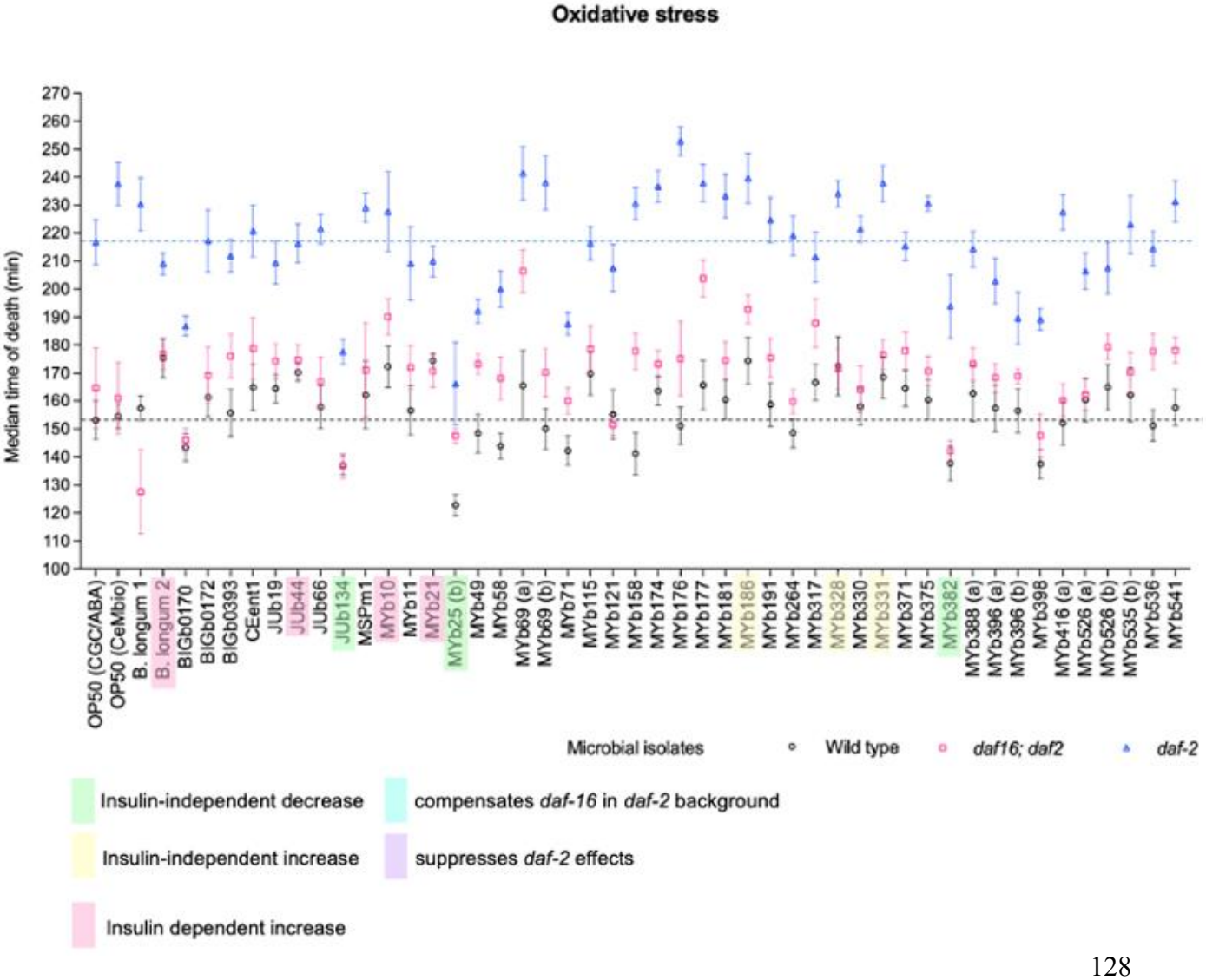
Some gut bacteria influenced stress response in IIS dependent manner (changes in wild-type worms but not in *daf-2* mutants) such as BIGb170, BIGb172 and MYb49 improved resistance to heat stress, in an IIS dependent manner, whereas *B. longum* 2, JUb44, MYb21, MYb317, MYb330 and MYb396 (a) led to an IIS independent increase to heat stress. On the other hand, BIGb0393, CEent1, JUb66, MSPm1, MYb11, MYb58, MYb69 (b), MYb121, MYb174, MYb176, MYb177, MYb264, MYb375, MYb388 (a), MYb398, MYb416 (a), MYb526 (a) and MYb535 (b) led to an IIS independent decrease in heat stress.

Some bacteria such as MYb10, MYb69 (a), MYb191, MYb371, MYb396 (b) and MYb526 (b) compensated the *daf-16* in *daf-2* background, whilst MYb115, MYb158, MYb328 and MYb536 suppressed *daf-2* effects to heat stress.

B. longum 2, JUb44, MYb10 and MYb21 improved resistance to oxidative stress in an IIS dependent manner, whereas MYb186, MYb328, and MYb331 improved oxidative stress resistance in an IIS independent manner. However, JUb134, MYb25 (b), and MYb382 led to an IIS independent reduction in oxidative stress resistance ***(figure 41).***

After the results suggested that certain bacteria may enhance stress resistance in worms through IIS dependent or independent manner, I selected a subset of these strains for their potential probiotic effects. These selected strains included *B. longum* 2, JUb44, and MYb21, which improved resistance to both heat and oxidative stress; BIGb170, BIGb172, MYb49, MYb71, MYb317, MYb330, and MYb396(a), which improved resistance to heat stress; and MYb10, MYb186, MYb328, and MYb331, which improved resistance to oxidative stress. I aimed to perform lifespan, brood size, and movement assays on worms using as many of the selected strains as time permitted, in order to gain a broader understanding of their effects. It could also be that some microbes might be modulating another molecular pathway, such as KP, to exert these responses; therefore I next looked at KP mutants.


Heat Shock
Median tme of death (min)
140
130
120
110
100
90
80
70
60
50
OP50 (CGC/ABA)
CeM-OP50
B.longum 1
B.longum 2
BIGb0170 (a)
BIGb0172
BIGb0393
CEent1
JUb19
JUb44
JUb66
JUb134
MSPm1
MYb10
MYb11
MYb21
MYb25 (b)
MYb49
MYb58
MYb69 (a)
MYb69 (b)
MYb71
MYb115
MYb121
MYb158
MYb174
MYb176
MYb177
MYb181
MYb186
MYb191
MYb264
Myb317
MYb328
MYb330
MYb331
MYb371
MYb375
MYb382
MYb388 (a)
MYb396 (a)
MYb396 (b)
MYb398
MYb416 (a)
MYb526 (a)
MYb526 (b)
MYb535 (b)
MYb536
MYb541
Microbial isolates
Wild type
daf-16; daf-2
daf-2


Oxidative stress
Median time of death (min)
270
260
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
OP50 (CGC/ABA)
OP50 (CeMbio)
B. longum 1
B. longum 2
BIGb0170
BIGb0172
BIGb0393
CEent1
JUb19
JUb44
JUb66
JUb134
MSPm1
MYb10
MYb11
MYb21
MYb25 (b)
MYb49
MYb58
MYb69 (a)
MYb69 (b)
MYb71
MYb115
MYb121
MYb158
MYb174
MYb176
MYb177
MYb181
MYb186
MYb191
MYb264
MYb317
MYb328
MYb330
MYb331
MYb371
MYb375
MYb382
MYb388 (a)
MYb396 (a)
MYb396 (b)
MYb398
MYb416 (a)
MYb526 (a)
MYb526 (b)
MYb535 (b)
MYb536
MYb541
Microbial isolates
Wild type
daf16; daf2
daf-2
Insulin-independent decrease
compensates daf-16 in daf-2 background
Insulin-independent increase
suppresses daf-2 effects
Insulin dependent increase

c) Gut microbiota influences response to stress in IIS dependent or independent manner

Insulin-independent decrease	**Insulin-independent increase**	**Insulin dependent increase**	**Compensates *daf-16* in *daf-2* background**	**Suppresses *daf-2* effects**
Heat stress				
BIGb0393	B. longum 2	BIGb170	MYb10	MYb115
CEent1	JUb44	BIGb172	MYb69 (a)	MYb158
JUb66	MYb21	MYb49	MYb191	MYb328
MSPm1	MYb71		MYb371	MYb536
MYb11	MYb317		MYb396 (b)	
MYb58	MYb330		MYb526 (b)	
MYb69 (b)	MYb396 (a)			
MYb121				
MYb174				
MYb176				
MYb177				
MYb264				
MYb375				
MYb388 (a)				
MYb398				
MYb416 (a)				
MYb526 (a)				
MYb535 (b)				
Oxidative stress				
JUb134	MYb186	B. longum 2		
MYb25 (b)	MYb328	JUb44		
MYb328	MYb331	MYb10		
		MYb21		

d) Bacteria with potential probiotic effects

Bacteria	Improved resistance to heat stress	Improved resistance to oxidative stress
B. longum 2	✓	✓
BIGb0170	✓	
BIGb172	✓	
JUb44	✓	✓
MYb10		✓
MYb21	✓	✓
MYb49	✓	
MYb71	✓	
MYb186		✓
MYb317	✓	
MYb328		✓
MYb330	✓	
MYb331		✓
MYb396(a)	✓	

Figure 41. Wild-type and IIS mutants (daf-16;daf-2 and daf-2) on the gut microbiota. A) Severe heat stress (42°C) resistance of adult worms. B) Severe oxidative stress resistance (7% tertbutyl hydroperoxide) of adult worms. 120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicates. C) Table showing which gut microbes influence response to stress in IIS dependent or independent manner during heat shock and oxidative stress. D) Bacteria with potential probiotic effects based on their ability to improve resistance to heat or oxidative stress.

3.2.4 The KP mediates some of the shifts in stress resistance seen on gut microbiota isolates

To test whether KP mediates some of the microbiota effects on worm stress response, I exposed *kmo-1* mutants to CeMbio+ microbes before exposing them to heat and oxidative stress. 120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicate.

After exposing the adult wild-type and *kmo-1* mutant worms to heat and oxidative stress, the results showed the involvement of KP in stress resistance (***figure.42***). The two-way ANOVA followed by Dunnett's multiple comparison (OP50 compared to the gut microbiota) showed that BIGb172, BIGb393, JUb066 (a), MYb011, MYb25 (b), MYb069 (a), MYb121, MYb176, MYb25 (b), MYb371, MYb375, MYb388 (a), MYb398, MYb416 (a), MYb526 (a), MYb526 (b), and MYb535 (b) critically decreased resistance to heat stress in wild-type worms (p= <0.05). Whereas, in *kmo-1* mutants, CEent1, JUb066, MSPm1, MYb011, MYb49, MYb69 (a), MYb158, MYb176, MYb177, MYb375, MYb388 (a), MYb398, MYb526 (a), MYb526 (b) and MYb535 (b) decreased resistance to heat stress (p= <0.05).

The results from the two-way ANOVA Šídák's multiple comparisons test (wild-type compared to *kmo-1* mutants) were significant for *B. longum* 1, BIGb0393, CEent1, JUb19, MSPm1, MYb21, MYb49, MYb69 (b), MYb158, MYb174, MYb176, MYb177, MYb181, MYb191, MYb328, MYb396 (a), and MYb536.

Overall, on OP50 (control diet), *kmo-1* mutants generally survived oxidative stress better but were more susceptible to heat stress, compared to the wild-type. Compared to OP50, only, *B. longum* 2 increased resistance in both wild-type and *kmo-1* mutants, whereas MYb21 and MYb396 (a) increased heat stress resistance in only wild-type.

However, oxidative stress showed more variation which resulted in larger error bars and less statistical significance. The two-way ANOVA showed no significance in wildtype and *Kmo-1* worms fed on OP50 vs other microbial isolates. However, two-way Anova test showed Jub44 and MYb396(a) results were significant between wildtype and *Kmo-1* worms.

Compared to OP50, some bacteria improved oxidative stress resistance in both wild-type and *kmo-1* worms, such as, MYb010, MYb069 (a), MYb176, MYb186, MYb330, and MYb526 (a), whereas MYb25 (b) decreased resistance to oxidative stress.

On the other hand, some bacteria, such as, MYb071, MYb181 and MYb382 decreased oxidative stress resistance in *kmo-1* worms, and some decreased resistance in wild-type worms such as BIGb0170, MYb264, MYb388, and MYb398.

MYb396 (a) improved oxidative stress resistance in *kmo-1,* but not in wild-type. JUb044 and MYb382, and MYb396(b) increased oxidative stress resistance in wild-type worms but decreased oxidative stress resistance in *kmo-1* mutants.

Once I found that the bacteria modulate IIS and KP to improve or desensitise the worms to heat or oxidise stress, I next looked whether the combination of bacteria with probiotic effects could improve resistance to heat stress.

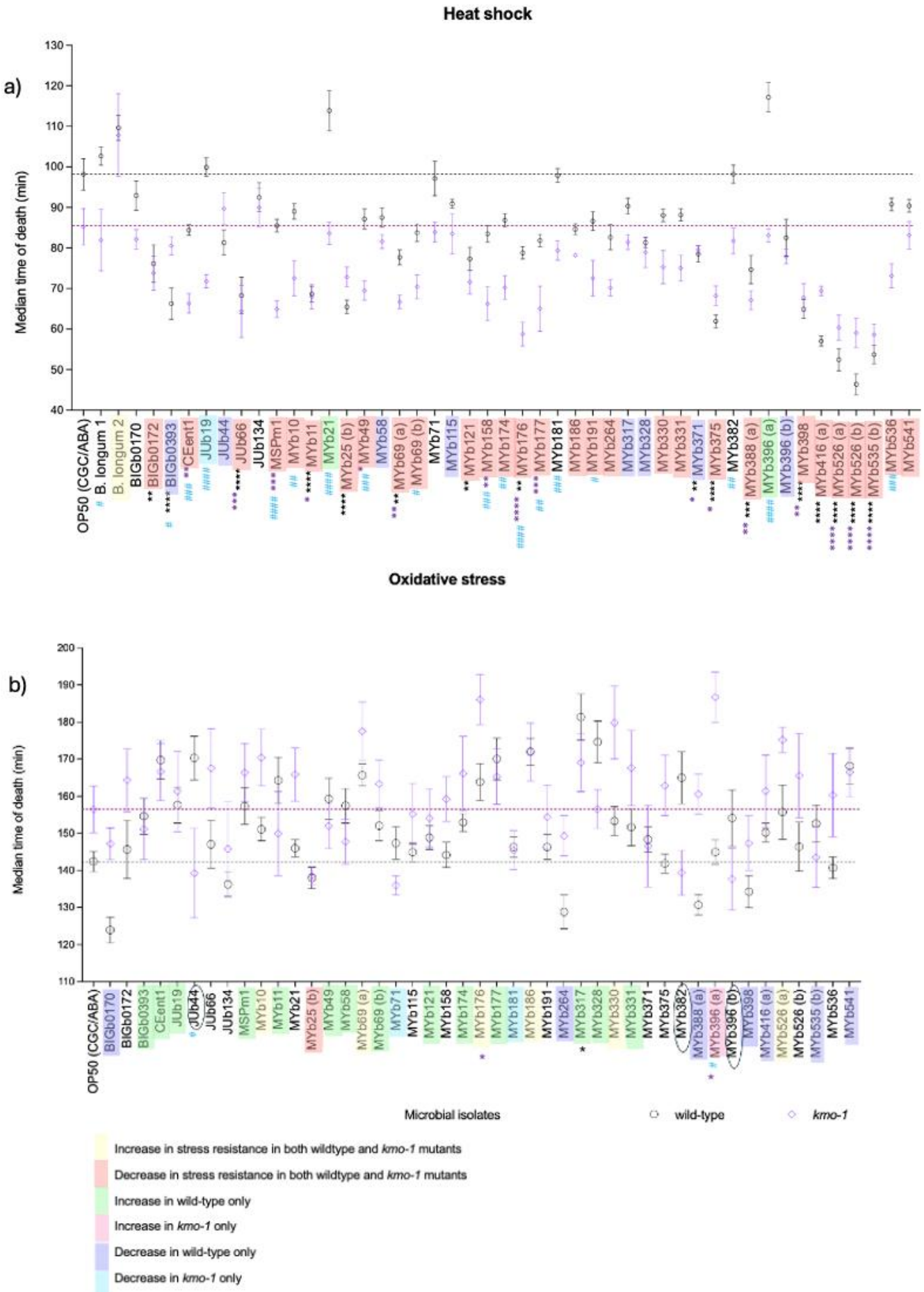

Heat shock
a)
Median time of death (min)
OP50 (CGC/ABA)
B. longum 1
B. longum 2
BIGb0170
BIGb0172
BIGb0393
CEent1
JUb19
JUb44
JUb66
JUb134
MSPm1
MYb10
MYb11
MYb21
MYb25 (b)
MYb49
MYb58
MYb69 (a)
MYb69 (b)
MYb71
MYb115
MYb121
MYb158
MYb174
MYb176
MYb177
MYb181
MYb186
MYb191
MYb264
MYb317
MYb328
MYb330
MYb331
MYb371
MYb375
MYb382
MYb388 (a)
MYb396 (a)
MYb396 (b)
MYb398
MYb416 (a)
MYb526 (a)
MYb526 (b)
MYb535 (b)
MYb536
MYb541
Oxidative stress
b)
Median time of death (min)
Microbial isolates
wild-type
kmo-1
Increase in stress resistance in both wildtype and kmo-1 mutants
Decrease in stress resistance in both wildtype and kmo-1 mutants
Increase in wild-type only
Increase in kmo-1 only
Decrease in wild-type only
Decrease in kmo-1 only

*Figure 42. Wild-type and kmo-1 mutants on the gut microbiota. a) Severe heat stress (42°C) resistance of wild-type and kmo-1 adult worms. b) Severe oxidative stress resistance (7% tertbutyl hydroperoxide) of wild-type and kmo-1 adult worms. Two way ANOVA followed by Dunnett's multiple comparison was performed using OP50 (CGC/ABA) as the control versus gut microbiota in wild-type and kmo-1 mutants, statistically significant = * ($p < 0.05$). (Black* = wildtype OP50 vs CEMbio+, purple* = kmo-1 OP50 vs CEMbio+). Two-way ANOVA (Šídák's multiple comparisons test) was also performed using wildtype as the control versus kmo-1 mutants, statistically significant = Blue#). Error bars represent the standard error of the mean (SEM). (120-150 worms were analysed in four sets of samples, each experiment was performed in quadruplicate). Due to bacterial growth issues data could not be acquired for B.longum 1 and B.longum 2 in kmo-1 mutants for oxidative stress assay.*

3.2.5 Combination of microbial isolates improves heat stress resistance

Combination of five promising microbial isolates BIGb170, JUb19, CEent1, MYb21, and MYb71, with E. coli OP50 as the control, were used to determine the effects of the combination of microbial isolates on heat stress resistance ***(figure. 43).*** These experiments were conducted in parallel to the RNAi screen and therefore, the same worm strain (NL2099) was used in order to be more time efficient. Three experiments were conducted, each using 120-150 worms per condition, with each condition repeated eight times in each experiment.

When compared to the control, CEent1, MYb21, and MYb71 showed improved heat stress resistance, while BIGb170 reduced it and JUb19 was similar to the control. These results were comparable to the wild-type strain, apart from BIGb170, which highlights strain differences as previous experiments have shown that BIGb170 increases heat stress resistance in wildtype and insulin signalling mutants. However, it seems to make NL299 worms more susceptible to heat stress.

The combined effects of all the strains were similar to the control, potentially indicating a balance of different strain effects. Additionally, all combinations of BIGb170 and JUb19 showed higher median time of death compared to the individual results of BIGb170 and JUb19. The highest median time of death was observed in the combination of BIGb0170, MYb21, and MYb71. This was higher than their individual impacts, suggesting that a combination of these bacteria increases resistance to heat stress. While BIGb170 has a negative impact on its own, MYb21, MYb71, and their combination increase heat stress resistance. However, the

combination of all three is the highest, indicating that the effects of MYb21 and MYb71 are enhanced, suggesting synergistic effects.

One way ANOVA followed by Dunnett's multiple comparisons test showed that compared to OP50, MYb21, (BIGb170+MYb21), (CEent1+ MYb21), (CEent1+ MYb71), (JUb19 + MYb21), (MYb21+ MYb71), (BIGb170+ CEent1+ MYb21), (BIGb170+ CEent1+ MYb71), (BIGb170+ JUb19+ MYb21), (BIGb170+ JUb19+ MYb71), (BIGb170+ MYb21+ MYb71), (CEent1+ JUb19+ MYb21), (CEent1+ JUb19+ MYb71), (CEent1+ MYb21+ MYb71), (JUb19+ MYb21+ MYb71), (BIGb170+ CEent1+ JUb19+ MYb21), (BIGb170+ CEent1+ JUb19+ MYb71), (BIGb170+ CEent1+ MYb21+ MYb71), (BIGb170+ JUb19+ MYb21+ MYb71), and (CEent1+ JUb19+ MYb21+ MYb71) increased resistance to heat stress in NL2099 worms ($p = <0.05$).

After I found which bacteria and bacterial combinations improve resistance to heat stress, I then looked if they also lead to an increase in lifespan.

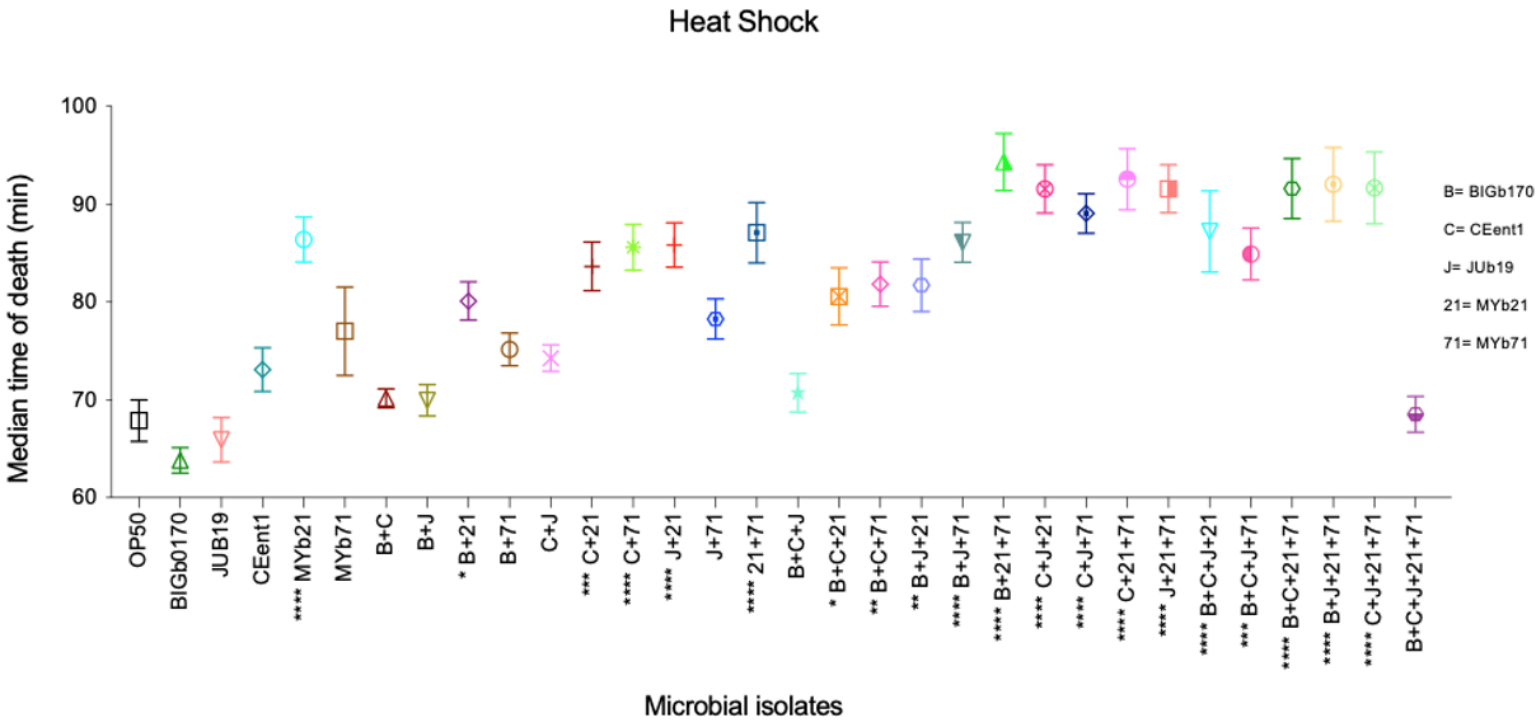


*Figure 43. Wild-type worms on combination of gut microbial isolates. Combination of BIGb170, JUb19, CEent1, MYb21, and MYb71, with E. coli OP50 as the control, were used to determine the effects of the combination of microbial isolates on heat stress resistance. One way ANOVA followed by Dunnett's multiple comparisons test was performed using OP50 (CGC/ABA) as the control versus gut microbiota in wild-type worms, statistically significant = * ($p < 0.05$). Error bars represent the standard error of the mean (SEM). Three experiments were conducted, each using 120-150 worms per condition, with each condition repeated eight times in each experiment.*

3.2.6 CeMbio+ strains do not increase lifespan in wildtype worms

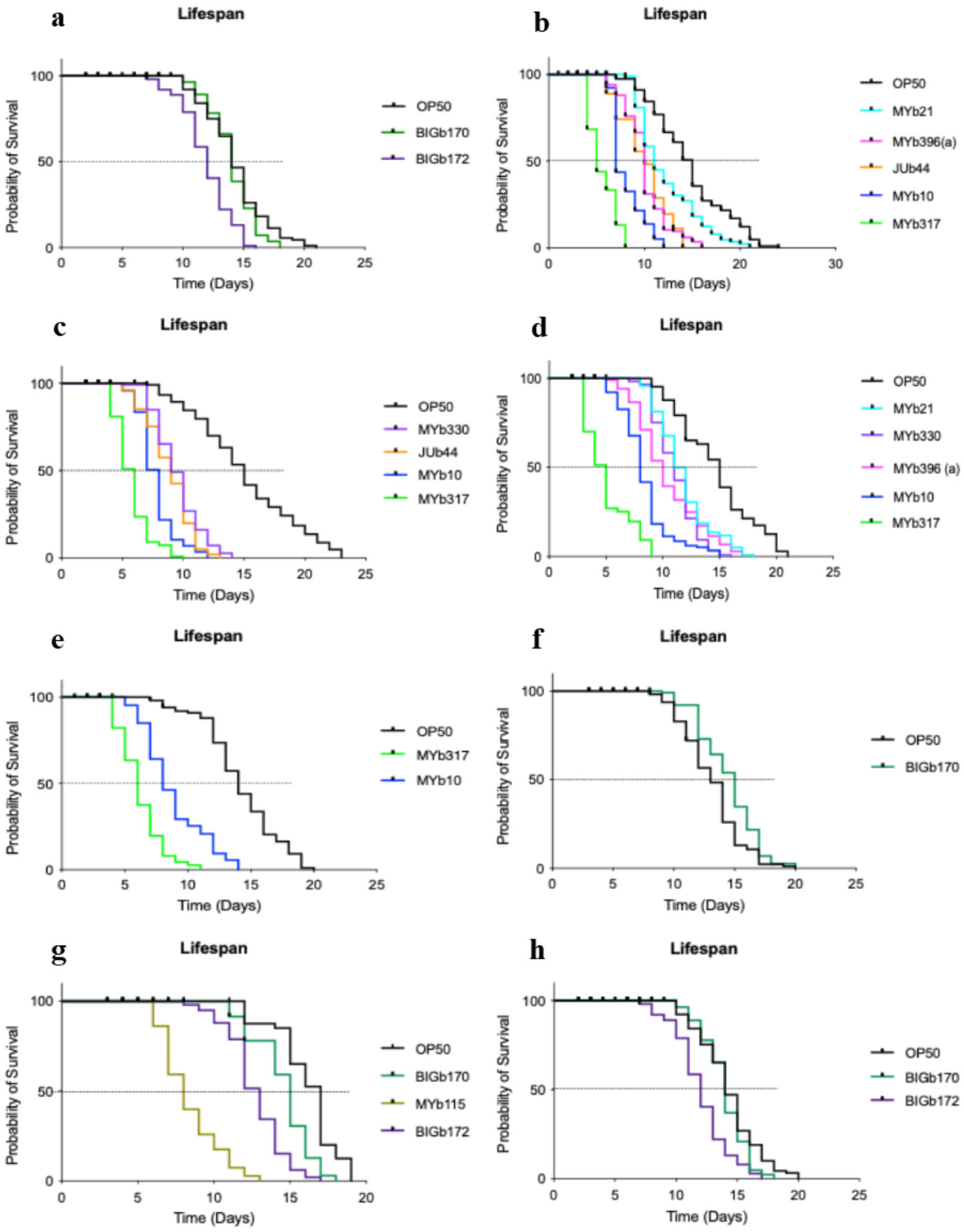
Lifespan experiments using BIGb170, BIGb172, JUb44, MYb10, MYb21, MYb49, MYb71, MYb317, MYb330, MYb396 (a), MYb115, and CEent1 were conducted ***(figure. 44).*** 120 worms were used per condition in each lifespan. The number of lifespan assays was 13 for *E.coli* as it was the control, BIGb170 had 7 repeats, BIGb172, MYb10 and MYb317 had 4 repeats, JUb44, MYb21, MYb49, MYb71, MYb330, MYb396 (a) and MYb115 had 3 repeats, while only one lifespan assay was conducted in CEent 1.

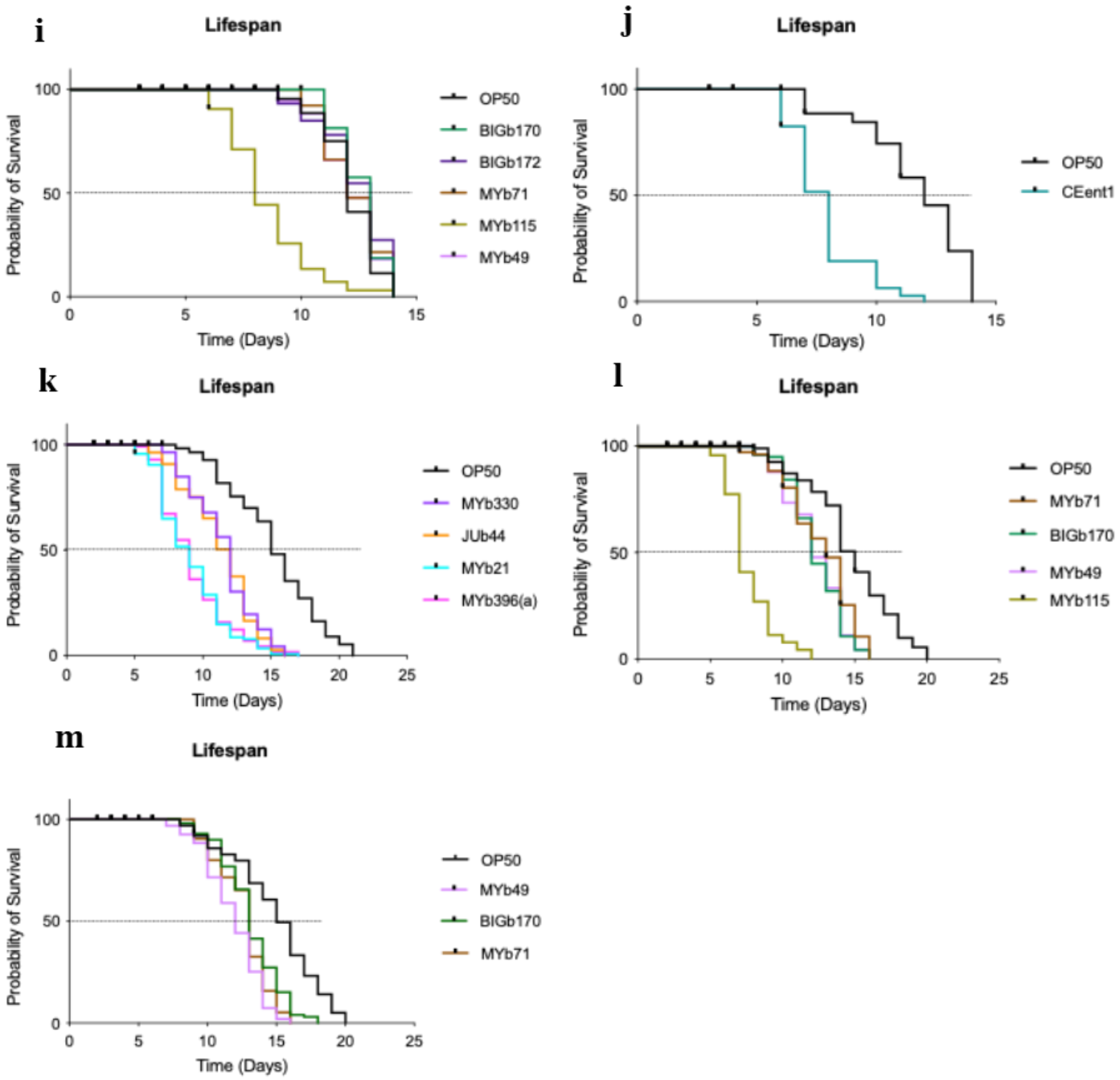
Overall, CeMbio+ bacteria did not increase the lifespan of wildtype worms, with the exception of BIGb170. This suggests that an increase in resistance to heat stress may not necessarily translate into increased lifespan. An exception to this trend was BIGb170, which showed similar results to OP50. In two lifespans, worms survived better on BIGb170, while in others, the survival rate was similar or lower than that with OP50. This suggests that BIGb170 is highly sensitive, and variables such as temperature changes during worm transfer may significantly impact the results.

The median survival of worms on OP50 was Day 15, BIGb170 was Day 13.7, MYb71 was Day 12.7, BIGb172 was Day 12.5, MYb49 was Day 12, MYb330 was Day 10.7, MYb21 was Day 10.5, JUb44 was Day 10.3, MYb396 (a) was Day 9.7, CEent1 was Day 8, MYb10 was Day 7.75, MYb115 was Day 7.7, and MYb317 was Day 5.5.

MYb10, MYb115, and MYb317 almost halved the lifespan of worms when compared to OP50 suggesting that there might be a trade-off between the increase in oxidative or heat stress resistance and lifespan. It could also be that the stress assays were conducted on Day 1 adults, and these bacteria provide benefits at a young age but not in older animals. KM analysis shows significance ($p<0.005$).

After finding out that bacteria which improve heat stress resistance didn't increase lifespan in wildtype worms, I next examined whether they improved lifespan in KP mutants or whether the results were similar to those in wild-type.


a
Lifespan
Probability of Survival
Time (Days)
OP50
BIGb170
BIGb172
b
Lifespan
Probability of Survival
Time (Days)
OP50
MYb21
MYb396(a)
JUb44
MYb10
MYb317
c
Lifespan
Probability of Survival
Time (Days)
OP50
MYb330
JUb44
MYb10
MYb317
d
Lifespan
Probability of Survival
Time (Days)
OP50
MYb21
MYb330
MYb396 (a)
MYb10
MYb317
e
Lifespan
Probability of Survival
Time (Days)
OP50
MYb317
MYb10
f
Lifespan
Probability of Survival
Time (Days)
OP50
BIGb170
g
Lifespan
Probability of Survival
Time (Days)
OP50
BIGb170
MYb115
BIGb172
h
Lifespan
Probability of Survival
Time (Days)
OP50
BIGb170
BIGb172


i
Lifespan
Probability of Survival
Time (Days)
OP50
BIGb170
BIGb172
MYb71
MYb115
MYb49
j
Lifespan
Probability of Survival
Time (Days)
OP50
CEent1
k
Lifespan
Probability of Survival
Time (Days)
OP50
MYb330
JUb44
MYb21
MYb396(a)
l
Lifespan
Probability of Survival
Time (Days)
OP50
MYb71
BIGb170
MYb49
MYb115
m
Lifespan
Probability of Survival
Time (Days)
OP50
MYb49
BIGb170
MYb71

Microbial isolates	**Number of lifespan assays**	**Total number of worms**	**Median lifespan in each assay**	**Average median lifespan**
E.coli OP50 (control)	13	1,560	14,15,15,15,14, 13,17,15,12,12, 15,15,15	14.4
BIGb170 (*Sphingobacterium multivorum)*	7	840	15,14,14,15,13, 12,13	13.7
BIGb172 (*Comamonas piscis)*	4	480	12,12,13,13	12.5
JUb44 (*Chryseobacterium scophthalmum)*	3	360	9,10,12	10.3
MYb10 (*Acinetobacter guillouiae)*	4	480	8,8,7,8	7.75
MYb21 (*Comamonas)*	3	360	11.5,11,9	10.5
MYb49 (*Ochrobactrum)*	3	360	12,12,12	12

MYb71 (*Ochrobactrum vermis)*	3	360	13,12,13	12.7
MYb317 (*Chryseobacterium)*	4	480	6,5,6,5	5.5
MYb330 (*Pseudomonas)*	3	360	9,11,12	10.7
MYb396 (a) (*Comamonas)*	3	360	10,10,9	9.7
MYb115 (*Pseudomonas)*	3	360	8,8,7	7.7
CEent1 (*Enterobacter hormaechei)*	1	120	8	8

Figure 44. Survival curves of wild-type worms on different gut microbiota (BIGb170, BIGb172, JUb44, MYb10, MYb21, MYb49, MYb71, MYb317, MYb330, MYb396 (a), MYb115, and CEent1). 120 worms were used per condition in each lifespan. The table provides the summary of the lifespan experiments. This includes the number of lifespan assays, total number of worms, median lifespan in each assay and average median lifespan.

3.2.7 CeMbio+ strains may impact lifespan in KP mutants

To assess whether gut microbiota impacted the lifespan of KP mutants, lifespan experiments using *kmo-1* mutants and MYb71 bacteria were conducted ***(figure.45).*** *kmo-1* mutants and wildtype worms were exposed to both OP50 and MYb71. 120 worms per condition were used. The results indicate that wildtype worms survived better on OP50 (median survival day 16) compared to MYb71 (median survival day 13). However, *kmo-1* mutants exhibited similar survival rates on both OP50 and MYb71 (median survival day 11). Overall, *kmo-1* mutants have a decreased lifespan compared to wildtype worms. This suggests the potential role of the KP pathway in lifespan regulation through interactions with the gut microbiota. However, this experiment was conducted only once, so additional repeats are necessary to draw a reliable conclusion.

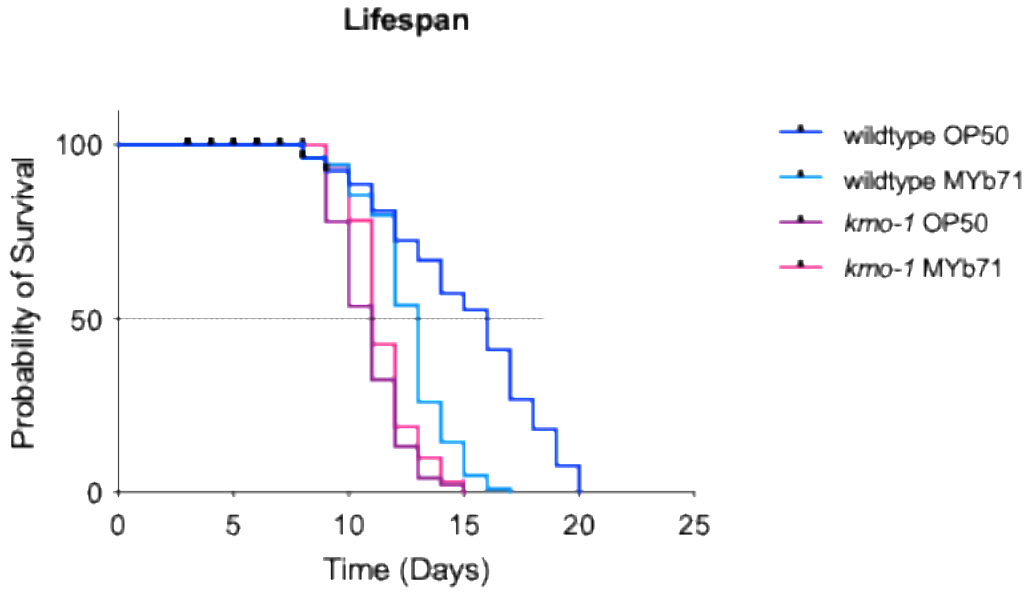


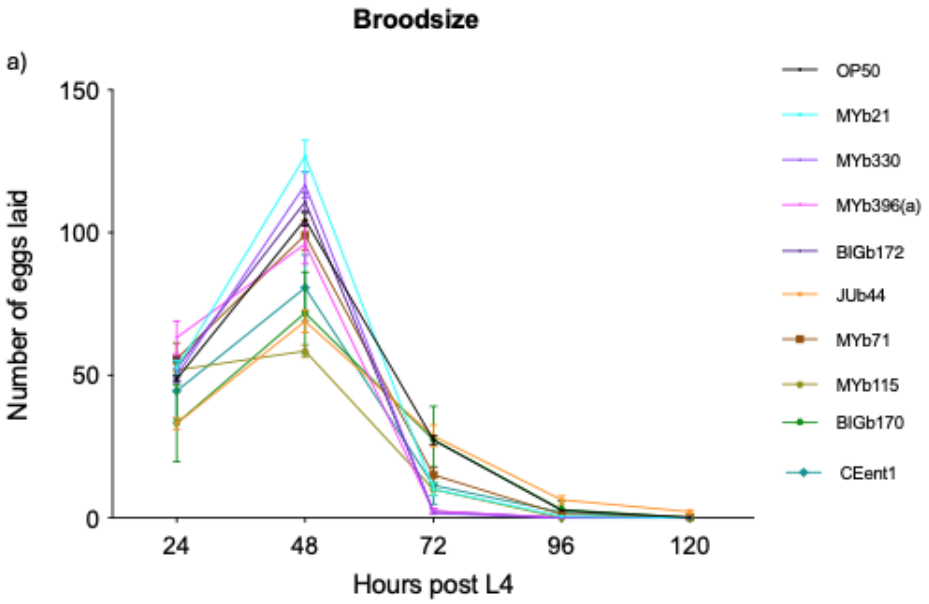
Figure 45. Lifespan of wild-type worms and kmo-1 mutants on MYb71. 120 worms per condition were used.

3.2.8 CeMbio+ strains slightly reduced the worm brood-size

Brood size experiments were conducted to determine if gut microbiota affected the number of eggs laid by worms ***(figure. 46).*** Brood size experiments were conducted in wildtype worms fed on OP50, MYb21, MYb330, MYb396(a), BIGb172, JUb44, MYb71, MYb115, BIGb170 and CEent1 bacteria. Each experiment had 12 worms per condition. The results showed that,

generally, the gut microbes reduced the brood size of worms, with MYb21 having the same effect as OP50. However, the one-way ANOVA followed by Dunnett's multiple comparisons test (OP50 compared to microbial isolates) showed no significance ($p<0.05$). This could be due to low or no repetition. BIGb170 had three repeats. MYb21, MYb330, and MYb71 were repeated twice, while MYb396(a), BIGb172, JUb44, MYb115, and CEent1 had only one repeat. The control OP50 had 6 repeats. Therefore, additional repeats are needed for better data interpretation.

The highest number of eggs was laid 48 hours post-L4 stage, suggesting that the timing of egg-laying was not affected.



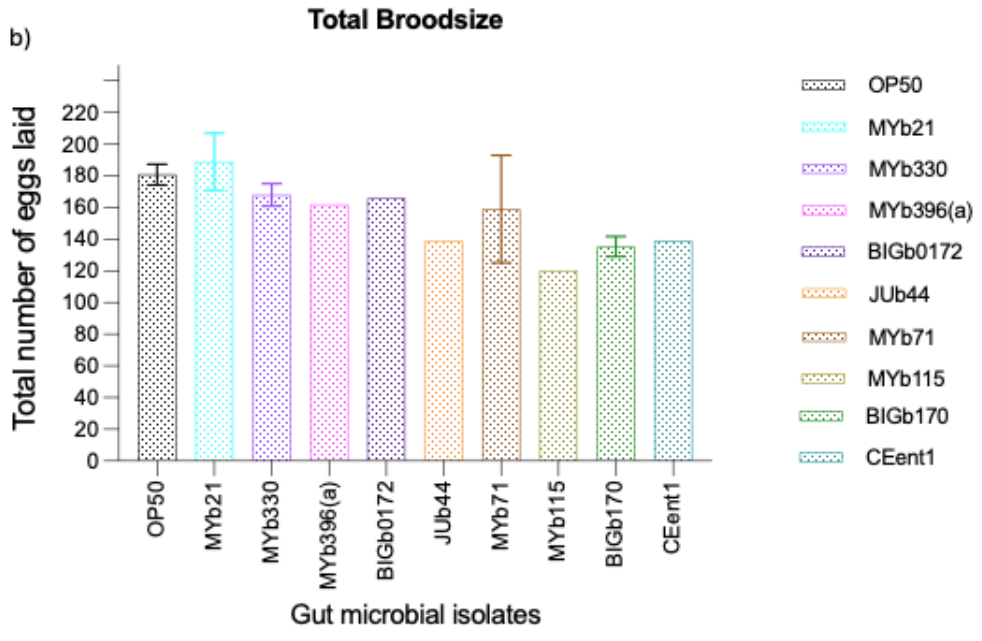


*Figure 46. Brood size of wildtype worms on gut microbial isolates. The highest number of eggs was laid at 48 hours post-L4 stage. One way ANOVA followed by Dunnett's multiple comparisons test was performed using OP50 (CGC/ABA) as the control versus gut microbiota in wild-type worms, statistically significant = * ($p < 0.05$). Error bars represent the standard error of the mean (SEM). Each experiment had 12 worms per condition. No error bars on MYb396 (a), BIGb172, JUB44, MYb115, and CEent1 as the experiment didn't have repeats. OP50 had six repeats, MYb21, MYb330, and MYb71 were repeated twice, and BIGb170 had three repeats.*

3.2.9 Worm movement declines with age

Worm movement was scored in parallel with lifespan experiments ***(figure. 47).*** This included BIGb170, BIGb172, MYb21, JUb44 and MYb396(a). The results show that worms start off with good movement (category A, shown in blue), but over time, their movement becomes slower and less coordinated (category B, shown in pink), eventually declining to the point where they can barely move (category C, also shown in pink). Worms that reach very old age, i.e., those that live longer, remain in this unhealthy state. Worms fed on BIGb170 were faster and better coordinated compared to OP50 suggesting that BIGb170 improves healthspan in worms.

Whilst conducting lifespan and worm movement assays, the impact of the bacterial diet on worm size and colour was evident. However, due to time constraints, imaging could not be performed, which is something that could be explored in the future. Worms raised on MYb10, MYb49, and MYb71 appeared darker, whereas worms fed on MYb317 became lighter. Worms on BIGb170 and MYb330 were thinner and smaller compared to the control, whereas the worms on MYb396(a) and MYb21 were larger and fatter. This could also explain the impact of size on movement as fatter worms exhibited an earlier decline in movement and were more sluggish compared to thinner ones.

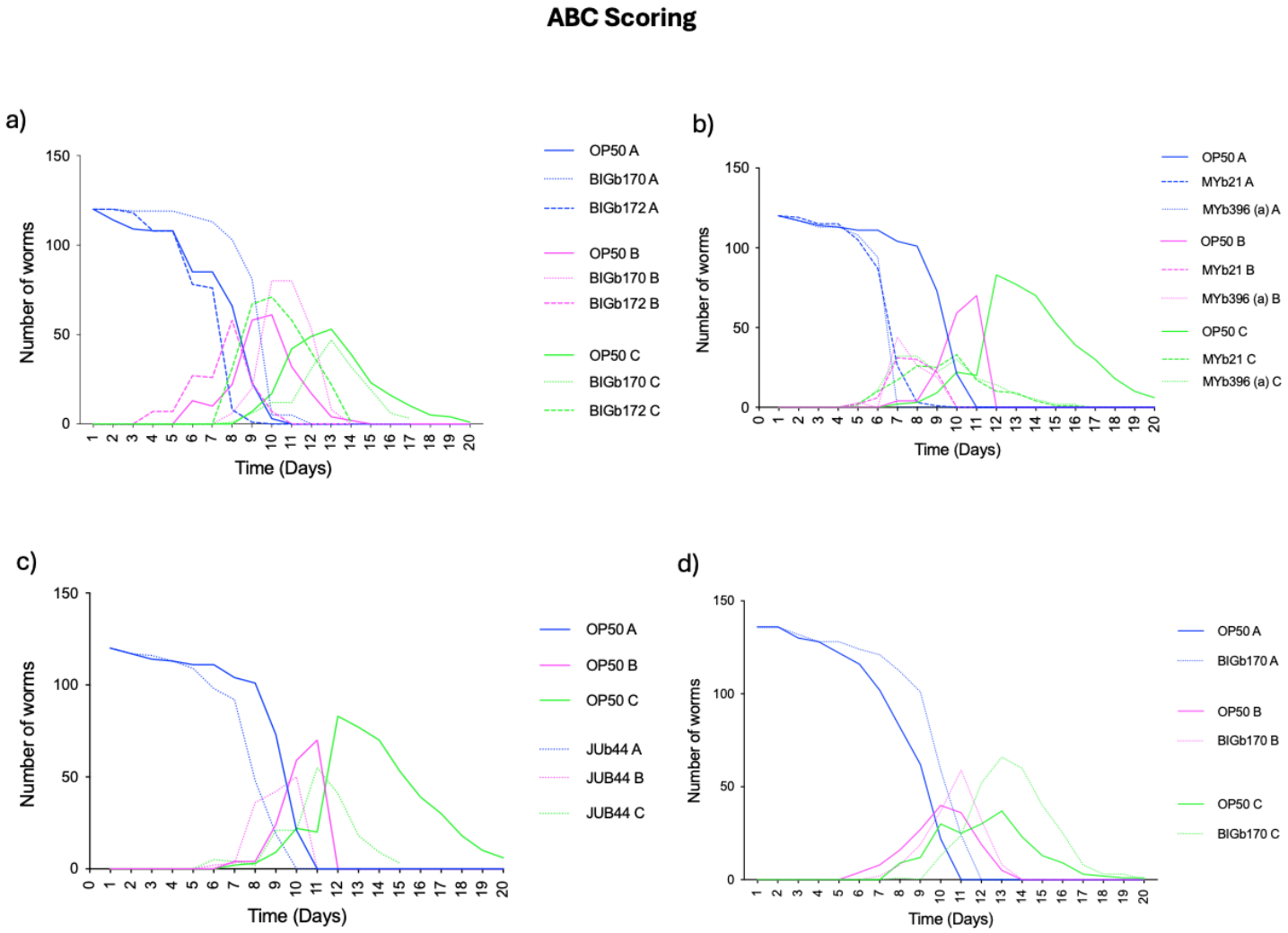


Figure 47. ABC scoring. BIGb170, BIGb172, JUb44, MYb396 (a), and MYb21 with E. coli OP50 as the control, were used to determine the effects of microbial isolates on worm movement. Data from four lifespan experiments in which ABC scoring was conducted in parallel is shown. Worms start off with coordinated and sigmoidal movement (category A, shown in blue), but over time, their movement becomes slower and less coordinated (category B, shown in pink), eventually declining to the point where they can barely move (category C, also shown in pink). a) ABC scoring of OP50 vs BIGb170 and BIGb172 shows that worms stay in category A for the longest when fed on BIGb170. b) Movement starts to decline with age in worms fed on OP50, MYb21 and MYb396 (a). c) Movement starts to decline with age in worms fed on OP50 and JUB44. d) ABC scoring of OP50 vs BIGb170 shows that worms stay in category A for the longest when fed on BIGb170. Each experiment used 120 worms per condition.

3.2.10 Summary

To study the impact of gut microbiota on health, I conducted LFASS heat shock and oxidative stress assays in wild-type, IIS mutants (*daf-2, daf-16;daf-2*) and KP (*kmo-1*) mutants. I found that gut microbial isolates differentially affect oxidative and heat stress resistance in *C. elegans,* and IIS and KP mediate some of the shifts in stress resistance seen with some gut microbiota isolates highlighting the involvement of gut-brain axis host genetic pathways in mediating gut microbiotas effects on *C. elegans* severe stress resistance.

I then compiled a list of bacteria with potential probiotic effects based on their ability to improve resistance to heat or oxidative stress. This included, B. longum 2, BIGb0170, BIGb172, JUb44 , MYb10, MYb21, MYb49, MYb71, MYb186, MYb317, MYb328, MYb330, MYb331, MYb396(a) and conducted lifespan and brood size experiments using these bacteria. I found that although these bacteria improve resistance to stress they don't increase lifespan, suggesting that increased stress resistance does not necessarily translate to a longer lifespan confirming that stress resistance and longevity are physiologically distinct.

I also found that although the gut microbes do not impact the timing of egg-laying as the highest number of eggs was laid 48 hours post-L4 stage, they do generally reduce the brood size of worms. This could be because in nature worms feed on different bacteria whereas I only tested them on individual bacteria.

I also looked at if combination of bacteria can further increase heat stress resistance and found that the combination of BIGb0170, MYb21, and MYb71 increases heat stress resistance the most compared with OP50 alone and other combinations of bacteria. Therefore, making it a potential combination to study for brood size and lifespan assays.

I also had a chance to score some worms in parallel with lifespan experiments and found that BIGb170 improves worm movement. Overall, these finding suggest that the gut microbiota may improve resistance to stress without affecting reproduction too much, which could explain why the worms feed on these bacteria. They may live shorter lives but remain healthier. However, further experiments are needed using a combination of different bacteria to get a fuller picture as individually bacteria do not seem to improve different aspect of health.

3.3 Optimisation of a high-throughput screening pipeline to study microbial impact on *C. elegans* health.

The genome-wide RNAi screen was optimised using the LFASS approach. This involved several steps, such as propagating a large number of *C. elegans* on OP50 bacteria, bleaching them to synchronise their development (Ali et al., 2022) and preparing RNAi bacteria onto which the worms were later placed.

L4 to adult worms were fed dsRNA-producing bacteria for 24 hours, which has been shown to be sufficient for RNAi efficiency of some genes and because knockdown of some genes at earlier larval stages may affect development. For example, RNAi of the transcription factor ELT-2 led to developmental changes at the L1 stage but not at L4, and the RNAi effect was observed when initiated from the L4 stage onward (Zárate-Potes et al., 2020). Worms were maintained at 25 °C, and heat shock was applied after 24–28 hours.

Heat stress was chosen over oxidative stress, as it is a more reliable predictor of longevity. This is because previous results from our lab have shown a strong positive correlation between longevity and resistance to high thermal stress, but not to severe oxidative stress. This may be due to the role of cellular functions that are particularly sensitive to heat stress, such as protein-folding homeostasis (Benedetto et al., 2019).

3.3.1 A bacterial RNAi-driven genome-wide phenotypic screen for new host genetic modulators of heat stress

The RNAi screen library consists of chromosomes X, 1, 2, 3, 4, and 5.

Clones were kept in a freezer at -80°C in sixty 96-well plates. The clones were thawed on LB agar plates, and their growth was recorded. There was some data loss due to several reasons, such as no clone growth and contamination. An example is shown in figure X a). The well in which no clone grew was highlighted in orange.

After the assays, excel files were labelled with gene names. Wells in which no clones grew due to the absence of clones in the library were highlighted in orange ***(figure.48).*** Wells where growth was observed but no clones were present in the library (highlighted in yellow) were suspected of contamination and therefore excluded. Additionally, wells where clones were present but did not initially grow on the plates, yet grew later in Terrific Broth, were also

excluded from the datasets as they were contamination. This approach eliminated false positives and false negatives, but resulted in some data loss. Therefore, the possibility that it might correspond to a potential gene of interest cannot be determined.

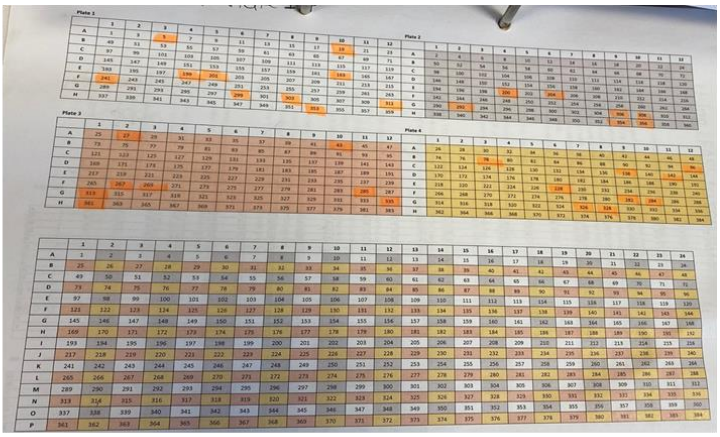
The results generated using MATLAB were also manually checked to ensure that there were no errors during data processing.

Due to individual experimental variations, no threshold value for time of death was used, instead the plate average was used as a control. Then the top 10 (increased the median time of death) and bottom 10 (decreased the median time of death) genes from each 384-well plate were selected and inserted into WormCat for analysis. WormCat can be used online with sequence IDs from the Ahringer RNAi library or downloaded as an R package (Holdorf et al, 2020).

In total, 1,200 genes were analysed using WormCat: 600 genes were selected as potential thermoprotective candidates (those whose RNAi makes worms more sensitive to heat stress), and 600 as potential sensitising candidates (those whose RNAi makes worms more resistant).

WormCat provides enrichment data from the nested annotation list with three categories: 1, 2, and 3, where 1 is the broadest and 3 is the most specific. Some of the genes were unassigned but indicated to be regulated by multiple stresses and for 219 genes there was no information available.

a



d

68.66	K10E9.1		127.93 C41D11.2
78.76	T03F1.1		128.12 W01B11.5
80.77	F23C8.4		128.18 F54A5.2
85.40	F25F1.1		128.24 K09H9.6
87.92	C53H9.2		128.75 T20F5.3
88.98	F53G12.7		128.91 Y44E3B.1
89.90	F28H1.2		128.97 K09H9.5
90.29	C18E3.7		129.64 ZK973.g
90.57	F37F2.3		130.10 Y95B8A_84.f
90.93	F28H1.1	plate average=	132.41 K09H9.7
			111.80

b

02s	20522s	20642s	20762s	20882s	21002s	21122s	21243s	21363s	21483s	21603s	Well positions		Gene names
3 °C	42.3 °C	42.2 °C	42.2 °C	42.2 °C	42.2 °C	42.1 °C	42.1 °C	42.1 °C	42.1 °C	42.1 °C			
3249	3223	3212	3279	3220	3246	3187	3193	3137	3210	3140	A1	68.66	K10E9.1
3769	3805	3730	3830	3764	3786	3693	3729	3670	3684	3644	A2	102.47	F47G6.1
2200	2268	2208	2236	2228	2258	2202	2271	2214	2239	2149	A3		
2207	2272	2206	2177	2187	2219	2173	2187	2162	2194	2131	A4	106.34	F47G6.2
2141	2176	2163	2212	2163	2184	2180	2186	2134	2148	2117	A5	97.84	F56C11.1
2658	2649	2619	2735	2653	2708	2628	2685	2604	2690	2598	A6	106.43	F47G6.3
1960	2054	1971	2019	1936	2016	1955	1948	1921	1956	1882	A7	100.19	F56C11.2
1721	1732	1749	1720	1701	1747	1689	1718	1707	1688	1634	A8	93.51	F47G6.4
2366	2378	2310	2336	2329	2386	2336	2350	2293	2302	2242	A9	104.95	F56C11.3
1552	1580	1547	1618	1572	1514	1555	1553	1534	1536	1519	A10	96.10	M01D7.1
1813	1837	1808	1824	1790	1810	1772	1844	1760	1802	1739	A11	106.31	F56C11.5
1549	1549	1546	1541	1541	1533	1513	1518	1505	1526	1494	A12		
1514	1597	1520	1528	1551	1542	1480	1535	1484	1525	1476	A13	110.32	F56C11.6
1526	1551	1501	1535	1493	1529	1487	1543	1471	1480	1466	A14	106.97	M01D7.3
2601	2584	2647	2624	2580	2617	2563	2589	2527	2572	2526	A15	108.65	F53G12.1
1827	1840	1788	1859	1794	1834	1821	1832	1800	1853	1772	A16		
3267	3306	3188	3270	3140	3213	3148	3221	3085	3103	3112	A17	108.53	F53G12.2
2684	2725	2684	2756	2710	2719	2674	2757	2682	2742	2668	A18	110.70	M01D7.5
1521	1539	1539	1564	1542	1578	1508	1510	1479	1509	1489	A19	94.43	F53G12.3
3349	3445	3370	3414	3318	3437	3317	3406	3331	3367	3294	A20	107.26	M01D7.6
3123	3111	3058	3115	3012	3128	2990	3071	3008	3018	2974	A21	104.05	F53G12.4
2844	2875	2799	2856	2751	2818	2793	2834	2713	2788	2772	A22	102.89	M01D7.7
3074	3097	3016	3073	3039	3051	3004	3029	2931	3040	2877	A23	105.76	F53G12.5
3334	3485	3282	3396	3328	3390	3343	3377	3257	3387	3239	A24	87.92	C53H9.2

c

21122s	21243s	21363s	21483s	21603s	Well positions	Strip meth	Layout		Well positions	
42.1 °C	42.1 °C	42.1 °C	42.1 °C	42.1 °C						
3187	3193	3137	3210	3140	A1	ABA_HS42(	SM1_1	68.66	A1	K10E9.1
3693	3729	3670	3684	3644	A2	ABA_HS42(	SM1_2	102.47	A2	F47G6.1
2202	2271	2214	2239	2149	A3	ABA_HS42(	SM1_3		A3	
2173	2187	2162	2194	2131	A4	ABA_HS42(	SM1_4	106.34	A4	F47G6.2
2180	2186	2134	2148	2117	A5	ABA_HS42(	SM1_5	97.84	A5	F56C11.1
2628	2685	2604	2690	2598	A6	ABA_HS42(	SM1_6	106.43	A6	F47G6.3
1955	1948	1921	1956	1882	A7	ABA_HS42(	SM1_7	100.19	A7	F56C11.2
1689	1718	1707	1688	1634	A8	ABA_HS42(	SM1_8	93.51	A8	F47G6.4
2336	2350	2293	2302	2242	A9	ABA_HS42(	SM1_9	104.95	A9	F56C11.3
1555	1553	1534	1536	1519	A10	ABA_HS42(	SM1_10	96.10	A10	M01D7.1
1772	1844	1760	1802	1739	A11	ABA_HS42(	SM1_11	106.31	A11	F56C11.5
1513	1518	1505	1526	1494	A12	ABA_HS42(	SM1_12		A12	
1480	1535	1484	1525	1476	A13	ABA_HS42(	SM1_13	110.32	A13	F56C11.6
1487	1543	1471	1480	1466	A14	ABA_HS42(	SM1_14	106.97	A14	M01D7.3
2563	2589	2527	2572	2526	A15	ABA_HS42(	SM1_15	108.65	A15	F53G12.1
1821	1832	1800	1853	1772	A16	ABA_HS42(	SM1_16		A16	
3148	3221	3085	3103	3112	A17	ABA_HS42(	SM1_17	108.53	A17	F53G12.2
2674	2757	2682	2742	2668	A18	ABA_HS42(	SM1_18	110.70	A18	M01D7.5
1508	1510	1479	1509	1489	A19	ABA_HS42(	SM1_19	94.43	A19	F53G12.3
3317	3406	3331	3367	3294	A20	ABA_HS42(	SM1_20	107.26	A20	M01D7.6

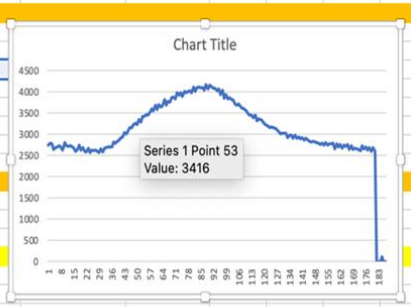


Figure 48. Screen data sorting. a) After thawing the clones from the RNAi library, those that did not grow are recorded and highlighted (shown in orange). b) Excel files are labelled with gene names. Wells in which no clones grew due to the absence of clones in the library are highlighted in orange, while wells where growth was observed but no clones were present are highlighted in yellow and then removed from the dataset. c) Results generated using MATLAB are double checked to ensure that there are no errors. d) The top 10 genes (with increased heat stress resistance, i.e., higher median time of death) and the bottom 10 genes (with decreased time of death) were then selected.

3.3.2 Wormcat analysis provides a detailed view of genes involved in heat stress

Wormcat uses detailed information about *C. elegans* genome to identify coexpressed gene sets and provides a scaled heat map to visually represent gene enrichment. Genes are categorised into different categories and assigned p-values using Fisher's test (Holdorf et al, 2020).

Usually, gene ontology is used to derive information about the biological function of coexpressed genes, categorising each into one of three major classifications: Biological Process (genes involved in a process that an organism is programmed to execute), Molecular Function (describes protein activities), or Cellular Component (identifies the location of activity). One of the limitations of this is that around 30% of *C. elegans* genes are not annotated, excluding these genes from the analysis. Whereas, WormCat organises genes into a concise hierarchy of nested categories, first assigning each gene based on its physiological function, followed by its molecular function or cellular location, thereby providing enrichment information not revealed by gene ontology (Holdorf et al, 2020).

The RNAi screen identified essential genes (those that are not just thermoprotective but generally important genes for general cell functions) such as those involved in signalling, genes regulated by multiple stresses, lipid metabolism, development, mitochondria, proteolysis, and transmembrane transport ***(figure. 49).***

Upon heat shock, several protective proteins (mainly chaperones) are upregulated to prevent protein denaturation and misfolding and exposure to heat stress has been shown to cause shifts in gene expression, affecting genes associated with a wide range of functions, such as cuticle structure, development, stress response, and metabolism. This is to counteract the adverse effects of heat stress. Jovic et al found that during the early phase of heat stress, there is a rapid increase in heat shock proteins. Shortly after, histones and genes associated with nucleosome assembly are upregulated. This allows greater access to the transcription sites of stress-responsive genes, and the incorporation of DNA into nucleosomes may serve as an additional protective mechanism during the stress response. Whereas, genes associated with transcription (*nhr*), metabolic processes, and locomotion are down-regulated (Jovic et al 2017). Therefore, knockdown of genes involved in general cell functions is expected to affect heat stress resistance.

However, most of the results showed no statistical significance. This could be due small gene list or because genes are spread across many functions rather than clustering in a few specific categories. There were statistically significant changes in wormcat category 3 genes (most

specific). This included lipid transmembrane transport, adaptin, unassigned prion domain, transcription, and proteolysis proteosome.

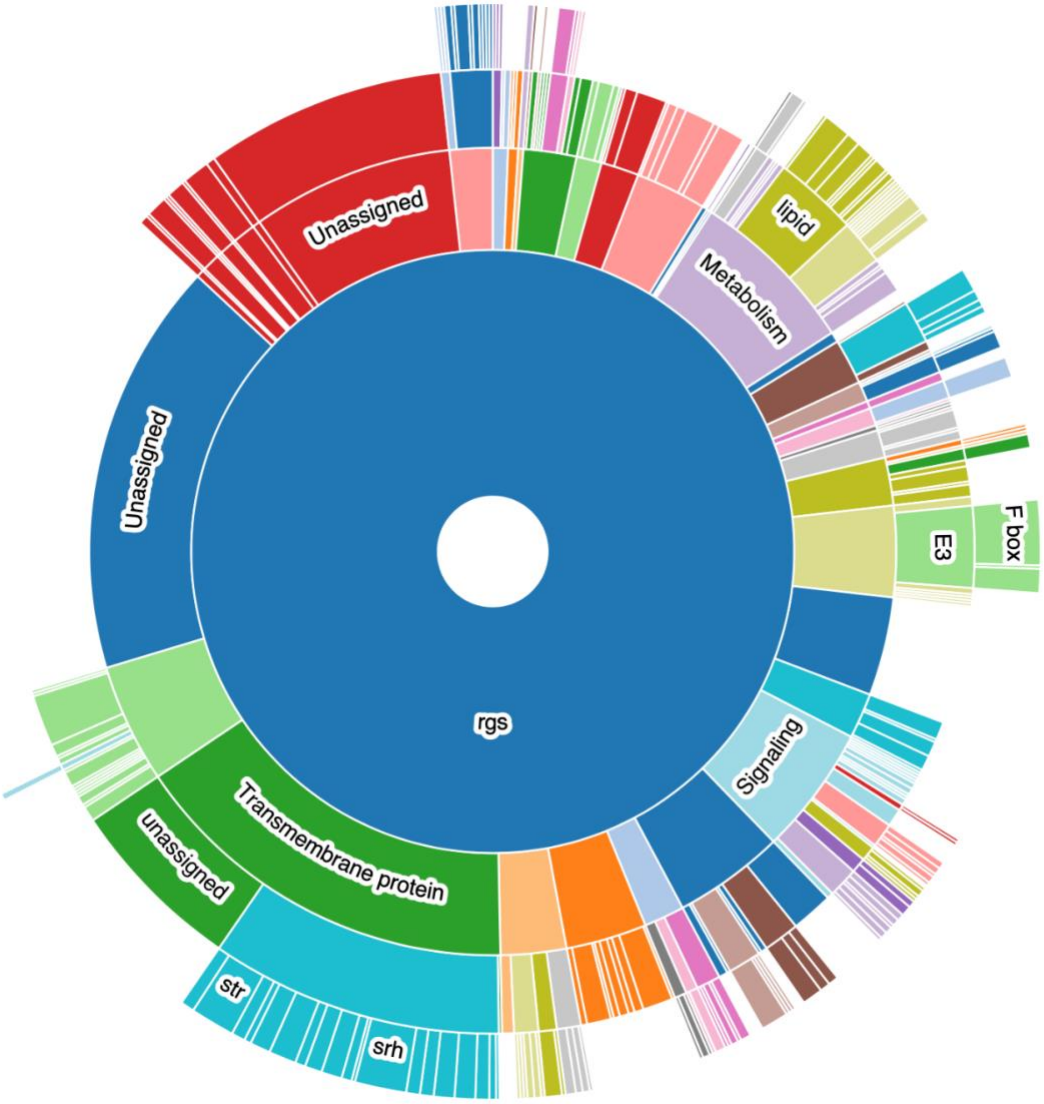
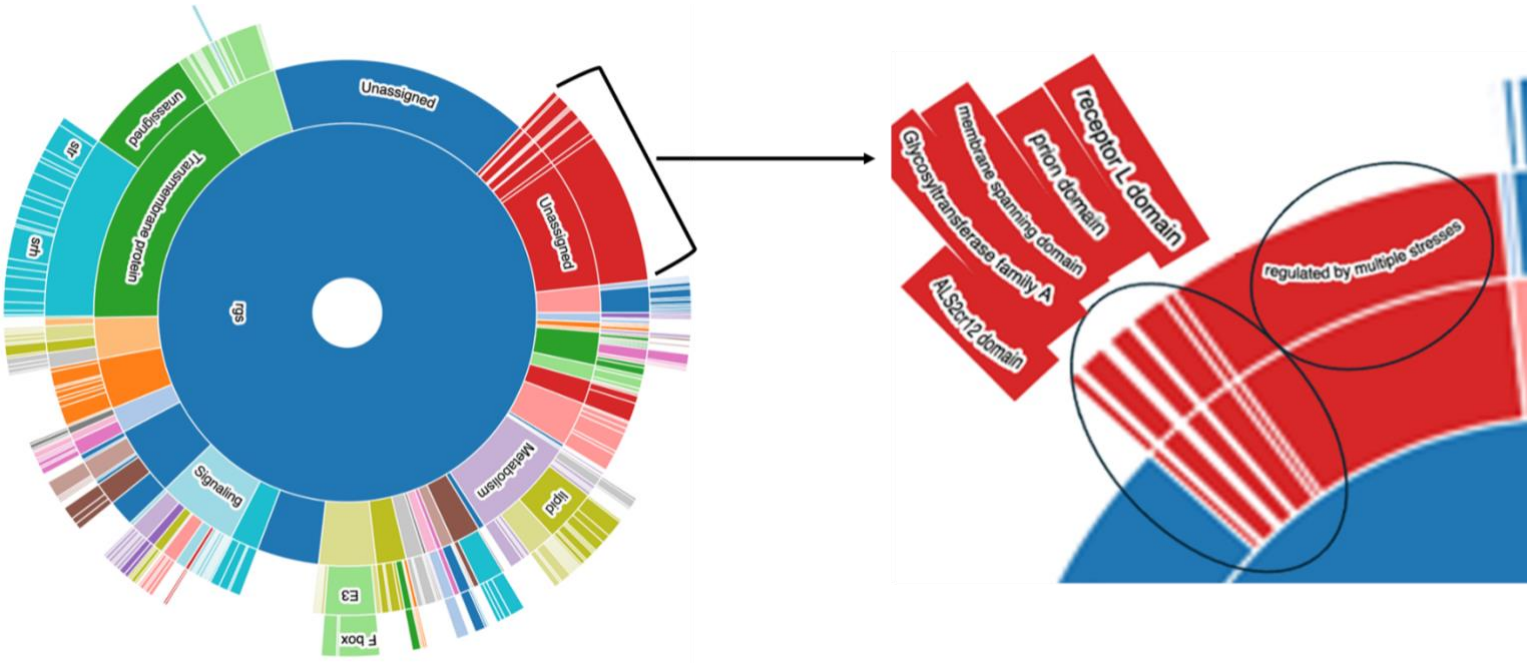


Figure 49. WormCat sunburst showing genes associated with heat stress response in C.elegans. Wormcat organises genes into a hierarchy of nested categories, based on physiological functions, molecular functions and cellular locations, providing enrichment information. The screen has identified essential genes (those that are not just thermoprotective but generally important genes for general cell functions) such as those involved in signalling, genes regulated by multiple stresses, lipid metabolism, development, mitochondria, proteolysis, and transmembrane transport.

Stress associated and various domain genes

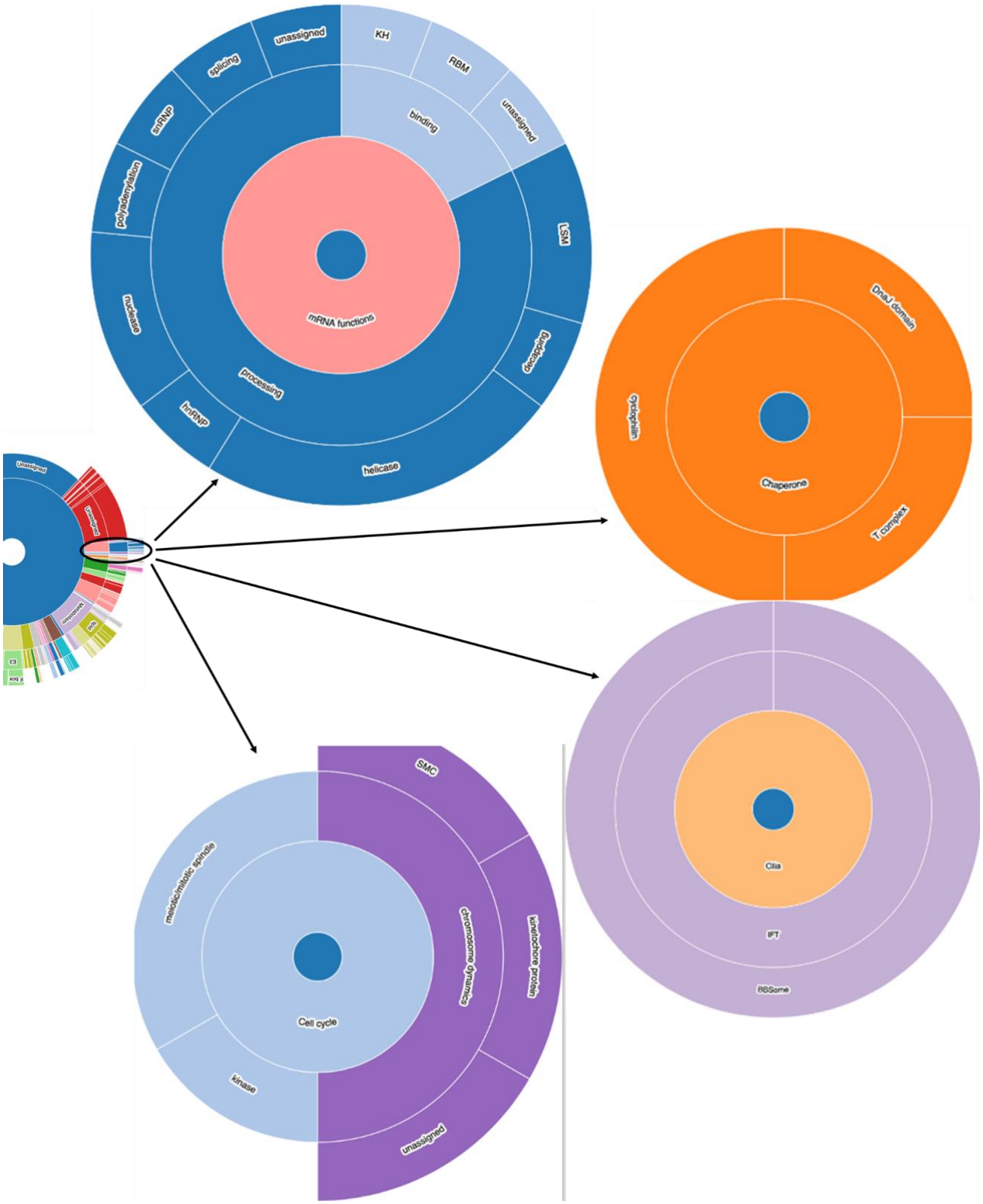
a)



49-a) Zoomed in view of the unassigned section of the Wormcat category 3 genes highlights the presence of stress-associated genes and various domain genes. This includes receptor L domain, prion domain, membrane spanning domain, glycosyltransferase family A and Flagellum Associated Containing Coiled-Coil Domains (ALS2cr12 domain).

Genes associated with mRNA functions, chaperone, cilia and cell cycle

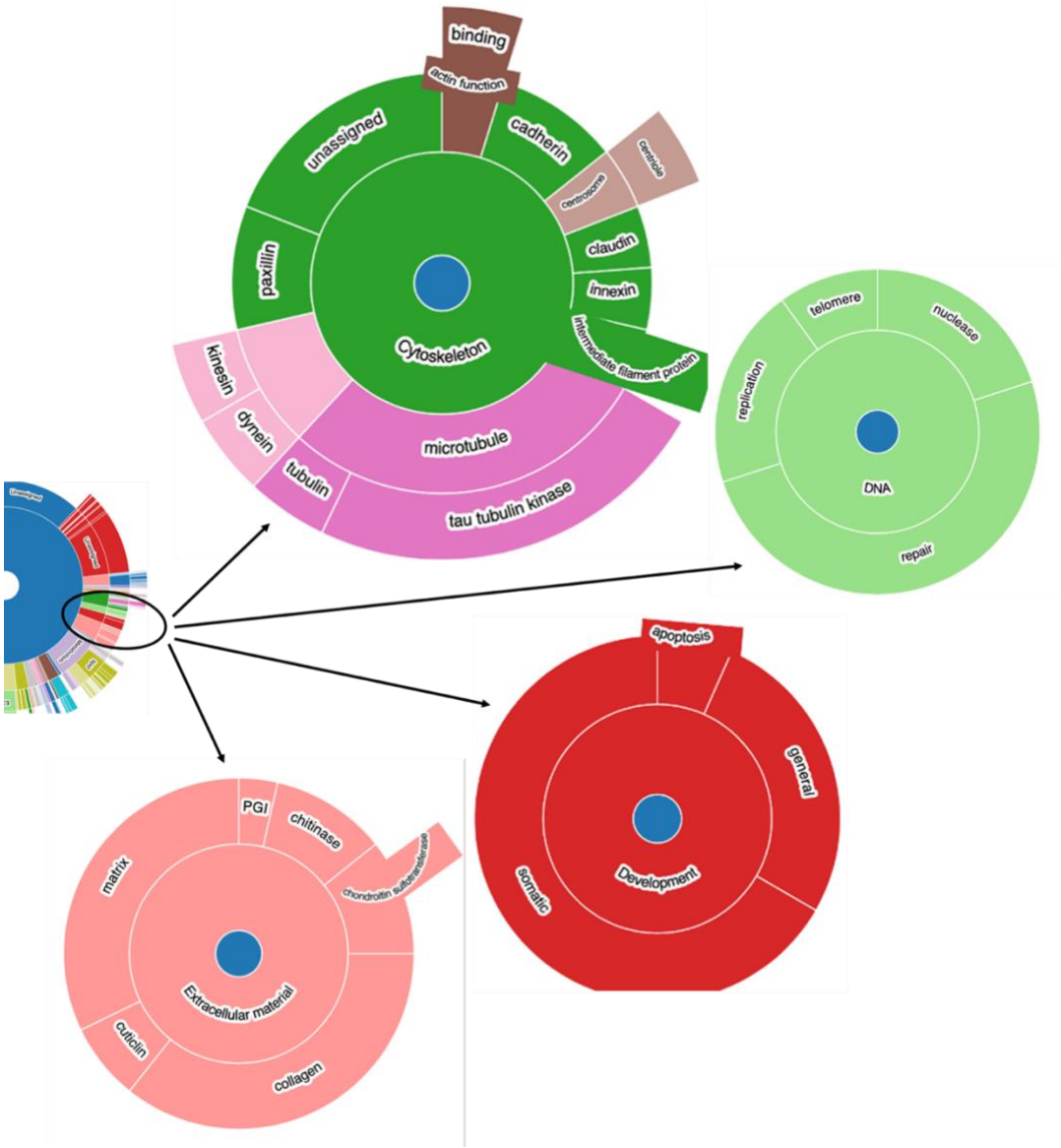
b)



49-b) Sunburst of the zoomed in section shows genes involved in mRNA functions: KH binding, RNA binding motif (RBM), like sm proteins (lsm), Heterogeneous Nuclear Ribonucleoproteins (hnRNP), Small Nuclear Ribonucleoprotein Messenger RNA (snRNP), decapping, helicase, nuclease,

polyadenylation and splicing, molecular chaperones: DnaJ domain (N-terminal J-domain), Tailless complex polypeptide 1 ring complex (T-complex) and Cyclophilins, cell cycle: Structural Maintenance of Chromosomes (SMC), kinetochore protein, kinase and meiotic/mitotic spindle, cilia: Intraflagellar Transport (IFT) and Bardet-Biedl Syndrome proteins complex (BBSome).

c) *Genes associated with cytoskeleton, DNA, development and extracellular*

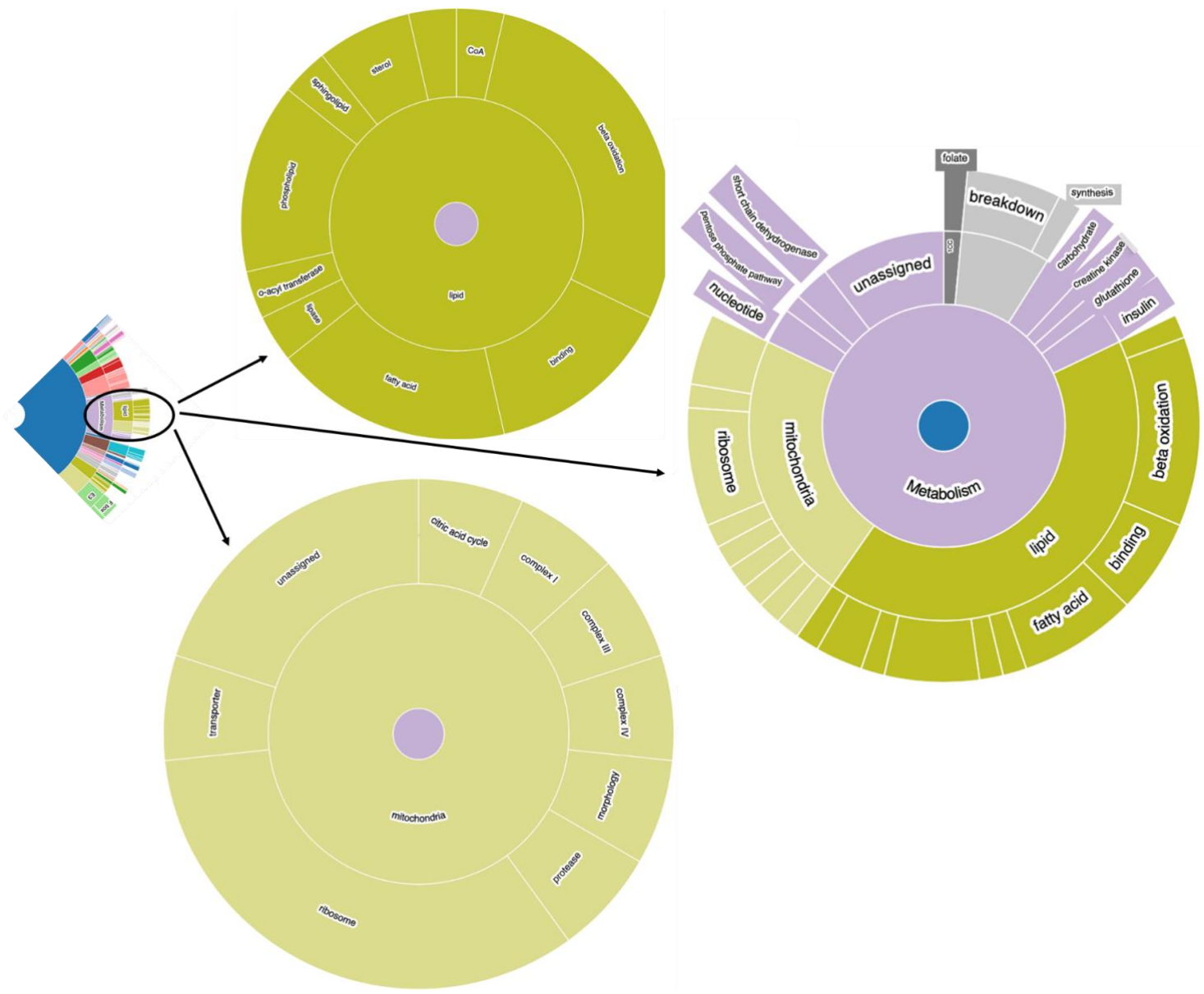


49-c) Sunburst of the zoomed in section shows genes associated with cytoskeleton: paxillin, cadherin, centrosome centriole, actin, claudin, innexin, kinesin and dynein motor proteins, tubulin and tau tubulin kinase microtubule, DNA: replication, repair, telomere and nuclease, development: general, somatic

and apoptosis, and extracellular material: cuticulin, collagen, chitinase, chondroitin sulfotransferase and protein glutamyl phosphotyrosine-1 (PGI).

Genes associated with lipid, metabolism and mitochondria

d)



49-d) Sunburst of the zoomed in section shows genes associated with lipids: beta oxidation, lipid binding, fatty caid, lipase, o-acyl transferase, phospholipid, sphingolipid, sterol, coenzyme A (CoA), metabolism: insulin, glutathione, creatine kinase, carbohydrate, amino acid synthesis and breakdown,

folate, short chain dehydrogenase, pentose phosphate pathway and nucleotide, and mitochondria: citric acid cycle, complex 1, complex 111, complex IV, morphology, protease ribosome and transporter.

e) *Genes associated with protein modification and neuronal function*

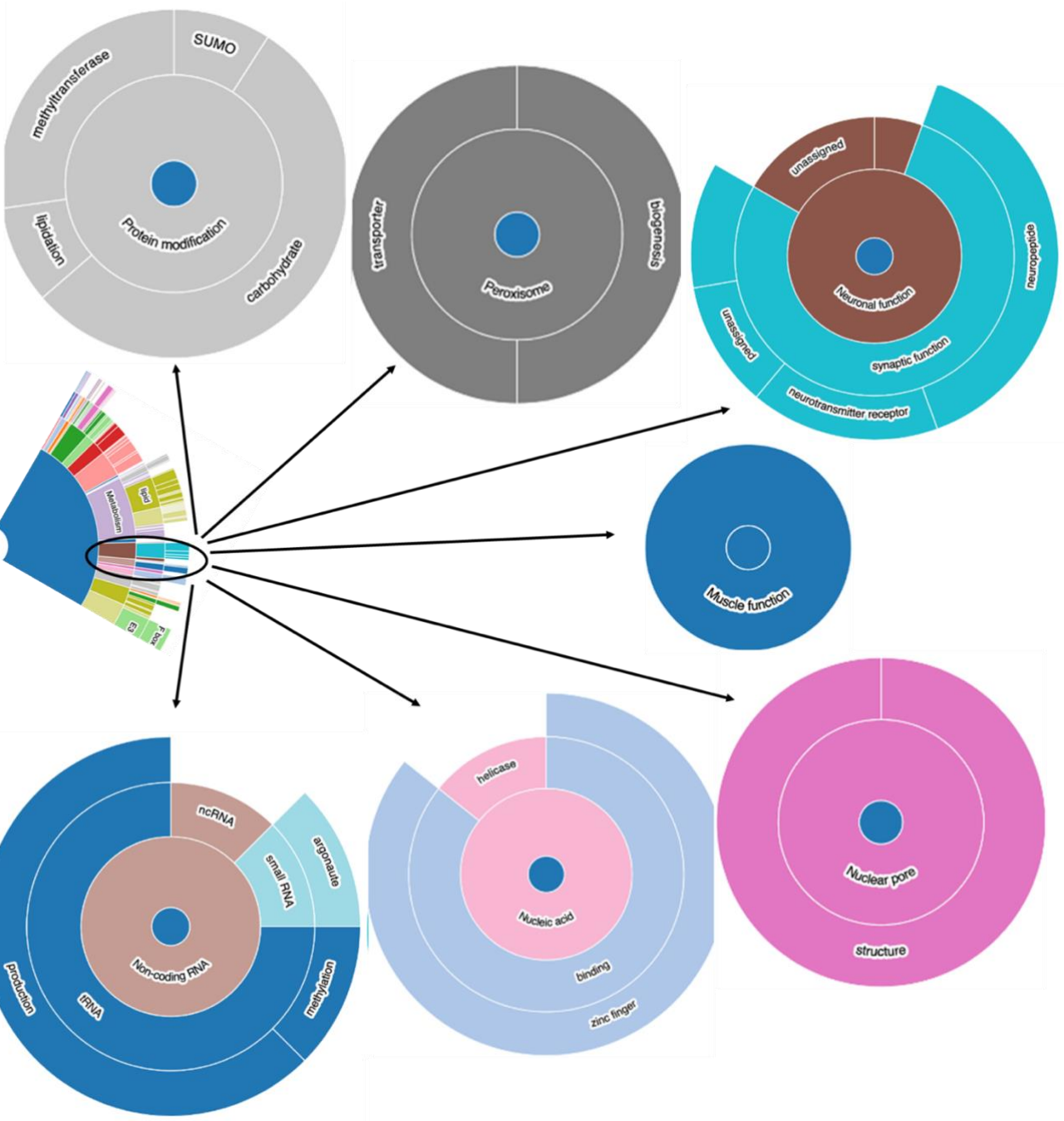


Figure 49-e) Sunburst of the zoomed in section shows genes associated with protein modifications: methyltransferase, Small Ubiquitin-like Modifier (SUMO), methyltransferase, lipidation, carbohydrate; transporter and biogenesis peroxisome, neuronal function: synaptic function,

neuropeptide, neurotransmitter receptor, development and vesicle trafficking; muscle function; nuclear pore structure; nucleic acid: binding, zinc finger and helicase, and non-coding RNA: argonaute small RNA, methylation, tRNA production and non-coding RNA (ncRNA).

f) *Genes associated with proteolysis, proteosome and ribosome*

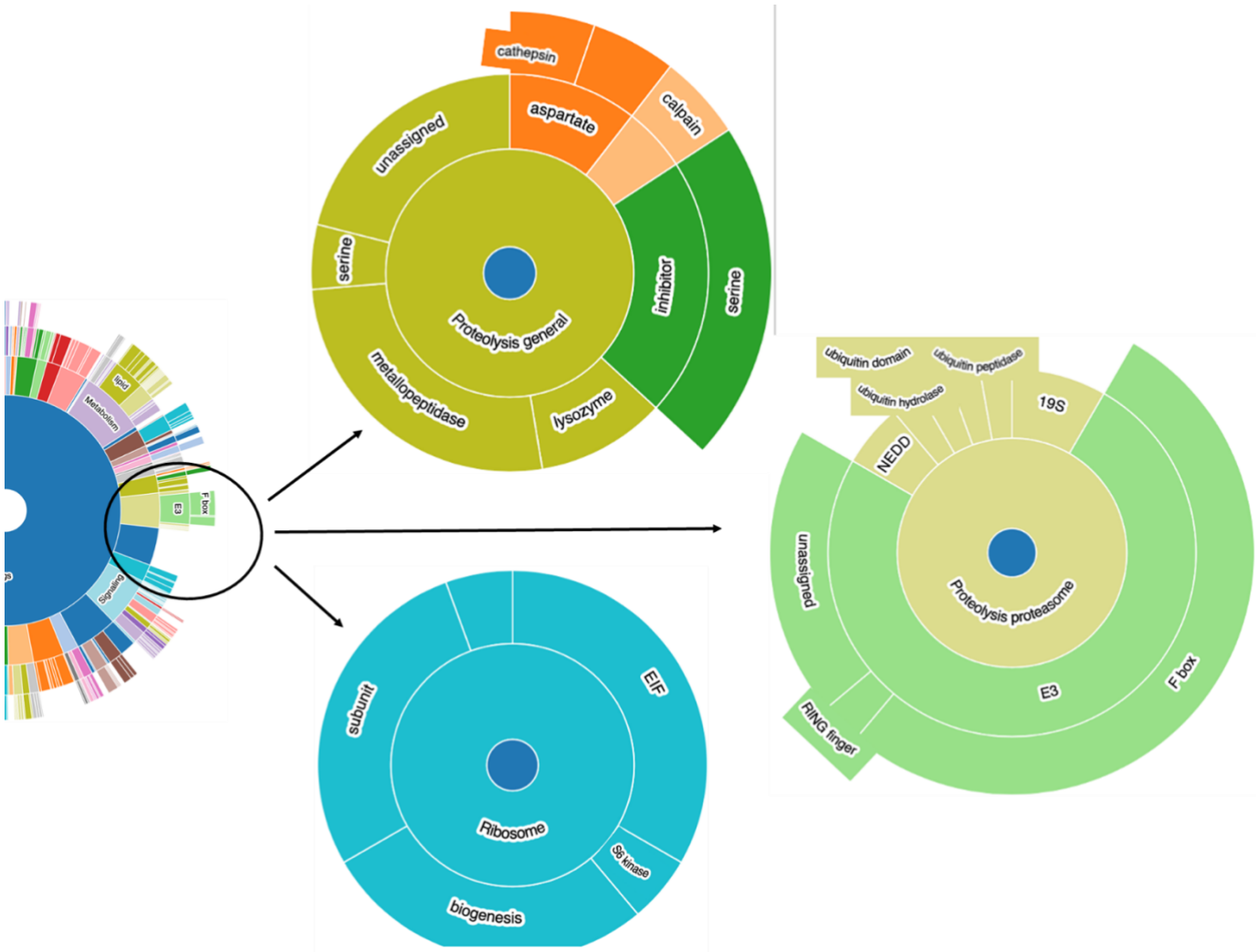


Figure 49-f) Sunburst of the zoomed in section shows genes associated with proteolysis: cathepsin aspartate, calpain cysteine, serine inhibitor, lysozyme, metallopeptidase and serine, proteosome: F box proteins, Really Interesting New Gene (RING) finger E3 (Ubiquitin ligase), NEDD, ubiquitin domain, ubiquitin hydrolase, ubiquitin peptidase and 19S, and ribosome: subunit, biogenesis, S6 kinases (serine/threonine protein kinases) and eukaryotic initiation factor (EIF).

g)

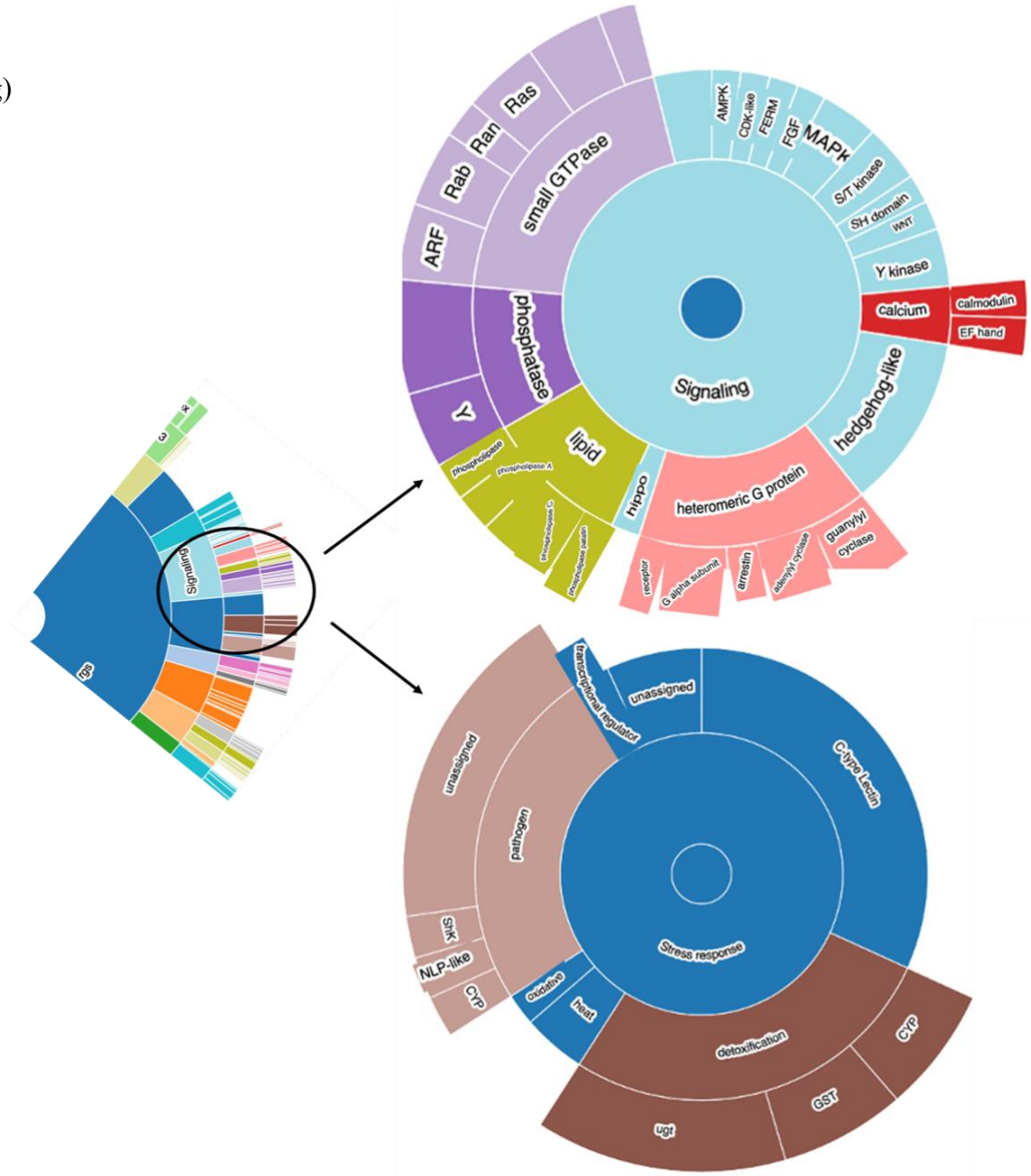


Figure 49-g) Sunburst of the highlighted section shows genes associated with signalling: hedgehog-like, Y kinase, wingless-type MMTV integration site family (WNT) signalling, SH domain, S/T kinase, mitogen activated protein kinase (MAPK), fibroblast growth factor (FGF), (Four, 1-Ezrin-Radixin-Moesin (FERM), cyclin-dependent kinases (CDKs), and AMP-activated protein kinase (AMPK); calmodulin calcium and FF hand (protein motif); ADP-ribosylation factor (ARF), Ras-associated binding (Rab), Ras-related nuclear protein (Ran), Rat Sarcoma (Ras) and Rho/Rac small GTPases;

phosphatase; phospholipase A, C and phospholipase patatin; hippo, and heteromeric G protein: arrestin, adenylyl cyclase, G alpha subunit, receptor and guanylyl cyclase.

h) *Genes involved in trafficking, transcription and transcription factors*

49-h) Sunburst of the highlighted section shows genes associated with trafficking: adaptin endocytosis, retromer and Soluble N-ethylmaleimide-sensitive factor (SNARE) vesicle targeting, attachment protein receptor, Coat Protein Complex I (COP1), endoplasmic reticulum-Golgi intermediate compartment (ERGIC), mannosidase and trafficking protein particle complex, transcription factors: Nuclear Hormone Receptor (NHR), T-box, zinc finger (ZF), basic-helix-loop-helix (bHLH), Basic leucine zipper (bZIP) and forkhead,, and transcription: histone chromatin structure, RNA polymerase 1 and 11, demthylase, deacetylase and SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin modification.

Genes associated with transmembrane proteins

i)

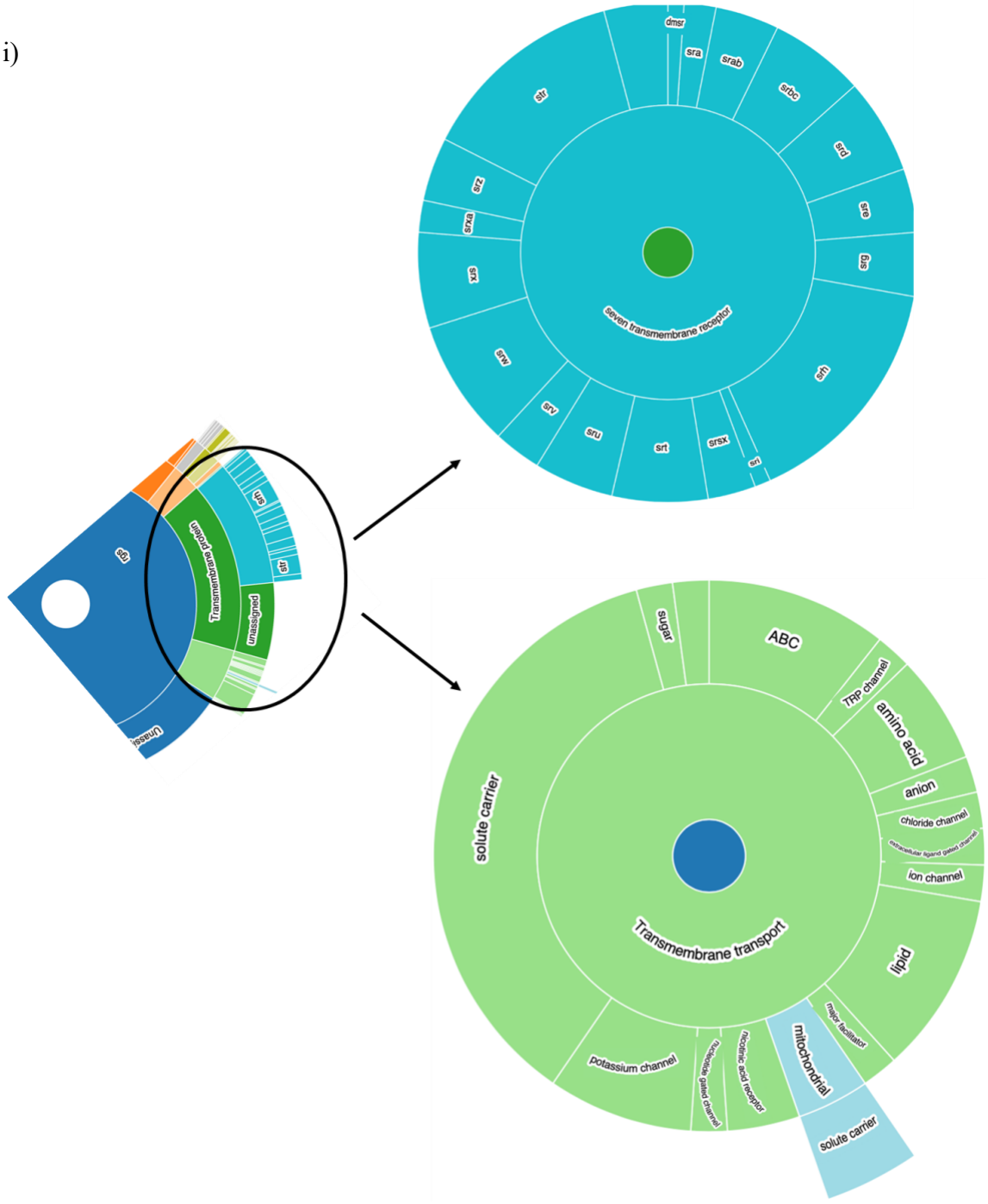


Figure 49-i) Sunburst of the highlighted section shows genes associated with transmembrane proteins: dimehtyl sulfoxide reductase regulator (dmsr), Steroid Receptor RNA Activator (sra), Sterol Regulatory Element Binding Proteins (srab), Sterol Regulatory Element-binding Protein, Class B (srbc), Serpentine receptor class delta (srd), Sterol Regulatory Element (sre), Serpentine receptor class gamma (srg), signal Recognition Particle Receptor protein (sri), Serpentine type 7TM GPCR chemoreceptor (Srsx), sprinter (srt), Serpentine Receptor Uncharacterized (sru), Simian Retrovirus (srv), serpentine type (srw), sulfiredoxin (srx) and Scavenger Receptor Cysteine-rich domain (srxa), and transmembrane transport: solute carrier, sugar, ATP-binding cassette (ABC), trp channel, amino acid, anion,chloride channel, extracellular ligand gated channel, ion channel, lipid, Major Facilitator, mitochondrial solute carrier, nicotine acid receptor, nucleotide gated channel and potassium channel.

3.3.3 RNAi screen identifies known thermoprotective genes

WormBase has a list of 370 genes associated with the heat stress response in *C. elegans* (wormbase.org).

The first step was to look for some of the known key genes involved in heat stress resistance and see if they appeared in the analysis ***(figure.50).***

This included *hsf-1,* which regulates heat stress, and heat shock proteins that are up-regulated during heat stress such as, *hsp-70, hsp-16.1, hsp-16.11, hsp-16.2, hsp-16.41* (Brunquell et al., 2016), small heat shock proteins, *hsp-12.1, hsp-12.2, hsp-12.3* and *hsp-12.6* (Fu et al., 2021); *hsp-90, hsp-110* (Jones et al., 2018), *sip-1* (a heat shock protein found in *C. elegans* embryos) (Fleckenstein et al., 2015); *dnj*-5 *(hsp-40); hif-1* (which encodes the hypoxia-inducible factor-1 and is essential for heat acclimation) (Treinin et al., 2003), whose knockdown is expected to sensitise the worms to heat stress. Also included was *ccar-1*, a negative regulator of the heat-shock response, where knockdown is expected to protect the worms against heat stress (Brunquell et al., 2018).

Surprisingly, the RNAi of *hsf-1* did not seem to sensitise the worms to heat stress suggesting that early adulthood RNAi does not seem to follow the same response as *hsf-1* mutants. The RNAi of *ccar-1* and *hif-1* showed some protection. RNAi of heat shock proteins, *hsp-70, hsp-16.11, hsp-12.2, hsp-12.6, hsp-110, hsp-90,dnj-5,* seemed to sensitise the worms to heat stress, whereas RNAi of heat shock proteins *hsp-16.1, hsp-16.2, hsp-16.41, hsp-12.1, hsp-12.3* seemed to protect worms against heat stress.

Other genes that impact heat stress include *daf-2* and *age-1*, which are involved in the IIS pathway; their knockdown is associated with increased stress resistance (Kenyon et al., 1993; Lithgow et al., 1995). In contrast, *daf-16*, also part of the IIS pathway, is crucial for stress resistance, and its knockdown leads to decreased stress resistance (Lithgow et al., 1995). *daf-2*, *age-1* and *daf-16 RNAi* protected the worms.

I also looked for *sir-2.1.* Sirtuins are a family of NAD^+-dependent deacetylases, and *sir-2.1* is the *C. elegans* ortholog of the mammalian SIRT1. *Sir-2.1* promotes the heat stress response by binding to the heat shock promoter of *hsp-70* enhancing its expression (Westerheide et al., 2009). Increasing *sir-2.1* expression in *C. elegans* has been shown to extend lifespan (Tissenbaum and Guarente., 2001). Knockdown of *sir-2.1* should sensitise the worms to heat stress, which was also observed in the screen.

The *clk-1* gene encodes demethoxyubiquinone monooxygenase, an enzyme involved in mitochondrial respiration. *clk-1* mutants have shown an increase in lifespan, but may have similar survival compared to wildtype during heat stress (Bansal et al., 2015). *clk-1* RNAi sensitised the worms.

Glutathione S-transferases are antioxidant enzyme which suppress ROS formation and protect against oxidative stress (Tao et al., 2017). *RNAi* of *gst-4* and *gst-26* sensitised the worms to heat stress suggesting that they may play a role in protecting the worms against heat and oxidative stress. RNAi knockdown of mitochondrial protein transport gene *tomm-22* and mitochondrial ribosomal protein gene *mrps-5* has shown to increase heat shock resistance (Labbadia et al., 2017). There wasn't much change in *mrps-5* RNAi and *tomm22* knockdown sensitised the worms.

eat-2 mutants have reduced pharyngeal pumping and feeding, leading to an extended lifespan. However, *eat-2* mutants may be more sensitive to heat stress (Bansal et al., 2015). In *eat-20* worms, the pharyngeal pumping rate is reduced by at least 15% compared to the wild type and the worms are starved causing their intestines to appear paler and show reduced autofluorescence (Shibata et al., 2000). For *eat-2* there was data loss due to contamination and RNAi of *eat-20* did not show much effect.

I also looked for autophagy-related genes, as heat shock is known to induce autophagy to reduce protein aggregation (Kumsta et al., 2017). However, *atg-13* and *atg-4.1* RNAi protected worms from heat stress. Whereas, *unc-51 (atg-1)* RNAi sensitised the worms to heat stress.

AAK-2 is an AMP-activated protein kinase involved in regulating lifespan, stress responses, and germ cell cycle arrest during dauer entry. Mutations in *aak-2* may result in hypersensitivity to heat shock (Lee et al., 2008). However, *aak-2* RNAi protected the worms from heat stress.

PEK-1, XBP-1, and IRE-1 are involved in the unfolded protein response in the ER. During ER stress, *xbp-1* is activated by IRE-1, an endoribonuclease that cleaves the mRNA of XBP-1. PEK-1 phosphorylates eIF2α, the alpha subunit of the eukaryotic translation initiation factor 2, which reduces protein synthesis to prevent the accumulation of misfolded proteins in the ER (Richardson et al., 2013). As expected, RNAi knockdown of *pek-1, xbp-1* and *ire-1* sensitised the worms to heat stress. Calreticulin (CRT) is a calcium-binding protein in the ER that is upregulated during stress (Park et al., 2001). *crt-1* RNAi seemed to provide some protection.

pat-10 is another calcium binding protein gene. Upon heat stress HSF-1 upregulates *pat-10* which maintains a functional actin cytoskeleton (Baird et al., 2014). RNAi of *pat-10* sensitised the worms.

The CUL-6 ubiquitin ligase complex decreases HSP-90 levels in the absence of heat shock, thereby improving thermotolerance. This process depends on the lysosomal component VHA-12, which encodes a subunit of the vacuolar-ATPase complex essential for lysosomal function. This regulation is independent of HSF-1, and the enhanced thermotolerance associated with reduced HSP-90 levels appears to be specific to the intestine (Sarmiento et al., 2024). The RNAi of *vha-12* didn't seem to have any effect.

UNC-32 encodes a subunit of the vacuolar-ATPase complex and is important for the function of neurons and muscles. Defects in *unc-32* result in uncoordinated movement (Pujol et al., 2001). *unc-32* RNAi sensitised the worms to heat stress.

SEL-11, a ligase involved in ER function, affects sleep amount in *C. elegans*. Stress can induce a stress-induced sleep state in worms, and mutations in *sel-11* prolong the duration of the quiescent, sleep-like state (Kawano et al., 2023). As expected, *sel-11* RNAi sensitised the worms to heat stress.

UNC-23 is the ortholog of the human Bcl-2-associated athanogene (BAG-2) chaperone, which binds to HSP-70. The UNC-23 protein is expressed in both the body wall muscle and the hypodermis, where it is required for proper attachment of muscle cells to the hypodermis. UNC-23 interacts with the ATPase domain of HSP-70 (HSP-1 in *C. elegans*) to facilitate the release of substrates from the binding cleft of HSP-1. In the absence of UNC-23, HSP-1 cannot efficiently release its substrates in response to mechanical stresses experienced by the hypodermis (Rahmani et al., 2015). RNAi of *unc-23* did not seem to have much of an effect.

Only a few know genes involved in heat stress resistance met the threshold (top 10 and bottom 10 genes with average of the plate = control) and were included in the wormcat analysis.

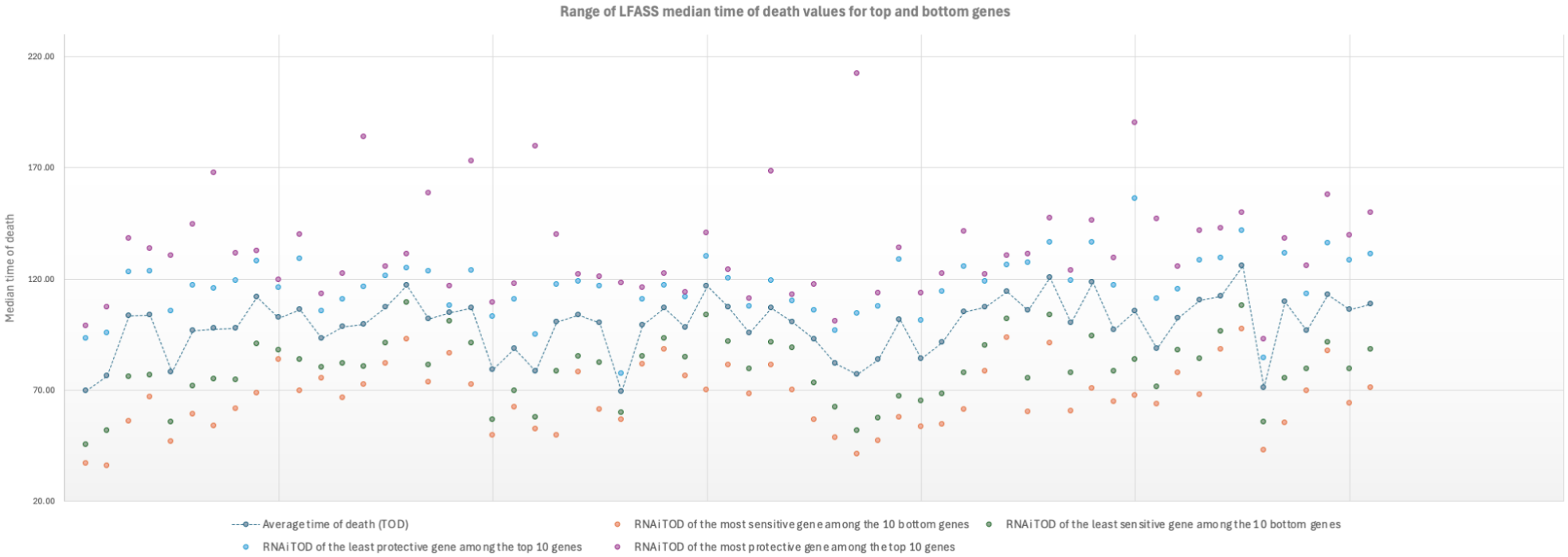
This included F44E5.5 (Hsp70), *sip-1*, *skn-1* and dnj-5 (Hsp40) chaperone.

Knockdown of F44E5.5, *sip-1*, and dnj-5 sensitised the worms to heat stress. Whereas, knockdown of *skn-1* increased the median time of death.

Identification of known thermoprotective genes validates this RNAi screen approach, however the threshold criteria could be less stringent to include more genes in wormcat analysis, for example instead of considering only the top and bottom 10 genes, we could expand the selection to include more genes.

Figure 50 a shows the spread of how different from the average the RNAi top and bottom hits were.

a


Range of LFASS median time of death values for top and bottom genes
Median time of death
220.00
170.00
120.00
70.00
20.00
Average time of death (TOD)
RNAi TOD of the most sensitive gene among the 10 bottom genes
RNAi TOD of the least sensitive gene among the 10 bottom genes
RNAi TOD of the least protective gene among the top 10 genes
RNAi TOD of the most protective gene among the top 10 genes

b

Gene	RNAi expected to sensitise or protect	outcome	Average time of death (TOD) of the plate	RNAi TOD	RNAi TOD of the most sensitive gene among the 10 bottom genes	RNAi TOD of the least sensitive gene among the 10 bottom genes	RNAi TOD of the least protective gene among the top 10 genes	RNAi TOD of the most protective gene among the top 10 genes	included in wormcat
hsf-1	sensitise	no effect	*99.6*	100.69	72.33	80.47	116.44	183.87	no
hsp70	sensitise	sensitised	100.16	80.34	61.11	82.24	116.84	121.01	yes
hsp-16.1	sensitise	protection	88.65	99.5	63.76	71.56	111.26	146.86	no
hsp-16.11	sensitise	sensitised	88.65	74.4	63.76	71.56	111.26	146.86	no
hsp-16.2	sensitise	protection	120.47	134.66	91.15	103.71	136.53	147.32	no
hsp-16.41	sensitise	protection	120.47	131.35	91.15	103.71	136.53	147.32	no
hsp-12.1	sensitise	protection	98.38	107.15	66.6	81.81	110.89	122.24	no
hsp-12.2	sensitise	sensitised slightly	107.4	102.2	50.08	91.74	120.12	124.07	no
hsp-12.3	sensitise	mild protection	84.16	86.3	53.42	65.15	101.33	113.59	no
hsp-12.6	sensitise	sensitised	84.16	73.4	53.42	65.15	101.33	113.59	no
hsp-110	sensitise	sensitised slightly	107.39	102.58	56.08	91.74	120.12	124.07	no
hsp-90	sensitise	no effect	112.8	111.42	87.6	91.52	136.14	157.79	no
sip-1	sensitise	sensitised	95.65	70.87	68.36	79.42	107.63	111.25	yes
dnj-5	sensitise	sensitised	78.48	53.76	52.34	57.76	95.13	179.98	yes
hif-1	sensitise	protection	96.75	106.62	69.62	79.48	113.19	125.76	no
ccar-1	protect	protection	107.44	114.87	78.31	90.1	119.03	121.91	no
daf-2	protect	mild protection	*100.59*	104.81	70.14	88.92	110.17	112.73	no
age-1	protect	protection	103.84	110.7	78.09	85.06	118.82	122.09	no
daf-16	sensitise	protection	98.38	105	66.6	81.81	110.89	122.24	no
sir-2.1	sensitise	sensitised	91.3	76.48	54.51	68.37	114.3	122.32	no
clk-1	sensitise	sensitised	98.25	87.94	76.31	84.9	111.79	114.12	no
gst-4	sensitise	sensitised	91.3	77.62	54.51	68.37	114.3	122.32	no
gst-26	sensitise	sensitised	69.39	61.85	56.59	59.94	77.43	118.27	no
mrps-5	protect	no effect	110.48	111.74	67.78	84.23	128.47	141.76	no
tomm-22	protect	sensitised	106.97	98.73	72.58	90.96	123.95	172.91	no
skn-1	sensitise	protection	83.71	110.72	47.25	57.32	107.75	113.6	yes
eat-20	sensitise	no effect	97.62	97.7	53.78	74.98	115.6	167.74	no
atg-13	sensitise	mild protection	107.39	111.68	56.08	91.74	120.12	124.07	no
atg-4.1	sensitise	protection	99.6	110.6	72.33	80.47	116.44	183.87	no
unc-51	sensitise	sensitised slighlty	112.8	107.75	87.6	91.52	136.14	157.79	no
aak-2	sensitise	mild protection	97.62	110.88	53.78	74.93	115.6	167.74	no
pek-1	sensitise	sensitised	78.22	64.32	46.8	55.53	105.29	130.24	no
xbp-1	sensitise	sensitised slightly	98.25	92.48	76.31	84.9	111.79	114.12	no
ire-1	sensitise	sensitised slightly	100.56	98.35	49.73	78.43	117.59	139.97	no
crt-1	sensitise	protection	96.95	105.58	64.85	78.39	117.14	129.58	no
pat-10	sensitise	sensitised	102.54	97.96	83.69	87.91	115.99	119.43	no
VHA-12	sensitise	no effect	76.49	77.56	36.1	51.73	95.83	107.11	no
unc-32	sensitise	sensitised	107.39	98.33	56.08	91.74	120.12	124.07	no
sel-11	sensitise	sensitised	112.8	105.3	87.6	91.52	136.14	157.79	no
unc-23	sensitise	no effect	88.65	85.35	63.76	71.56	111.26	146.86	no

c

d

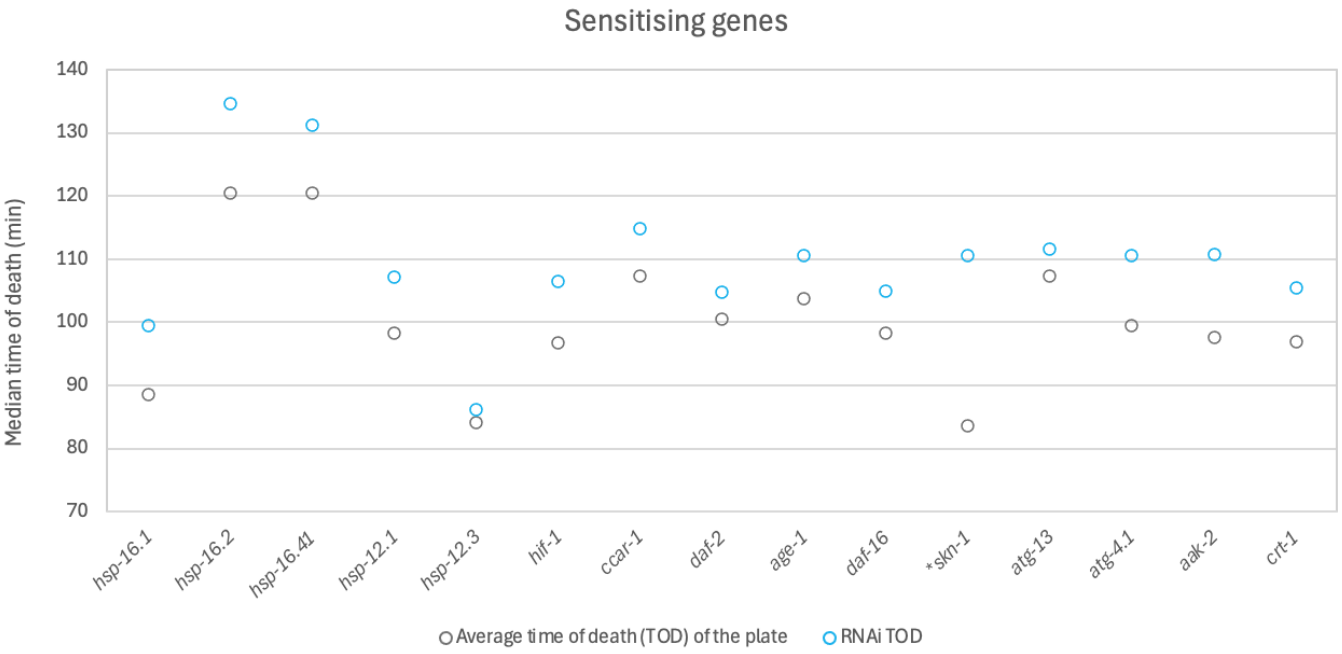


*Figure 50. A) Visual representation of the screen. This shows the average time of death of worms on a plate, as well as the range of death times for the selected top and bottom genes. B) This shows the RNAi-induced time of death for known key genes involved in heat stress resistance, whether RNAi of these genes should protect or sensitise the worms, whether the outcome matched expectations, and whether they met the threshold for inclusion in the WormCat analysis. C) Thermoprotective genes (RNAi of which sensitised the worms to heat stress) and thermosensitive genes (RNAi of which protected the worms to heat stress). *= included in the wormcat analysis.*

3.3.4 RNAi screen identifies new thermoprotective genes

RNAi identified some potential thermoprotective genes, the knockdown of which decreased the median time of death in worms, indicating that these genes are important for thermoprotection.

This included several unassigned genes regulated by multiple stresses (B0218.7, *moa-2*, C10G11.6, C11E4.7, F13A7.7, F42C5.3, F44G4.5, KO7A1.4, K10G4.3, M01H9.4, Y59C2A.3, zip-12), unassigned transmembrane proteins (B0207.2, *igeg-1,* F37C4.4, F53C3.6, W09G12.9, ZC190.8) unassigned genes (*hpo-27,* F13E9.9, F53H4.2, K08B5.2, R10D12.8, Y75B8A.25), pseudogenes (fbxa-187, F55C5.2) and neuropeptide *nlp-4,* which are indicated to be affected by several genes including *hsf-1.*

Other genes which could be potentially studied further to determine their role in heat stress could be genes involved in other stress responses, such as the c-type lectin genes involved in immune stress response, such as *clec-212, clec-20, T25C12.3, clec-134, clec-130, clec-37, clec-261, clec-94* and *clec-96*, those involved in detoxification stress, *ugt-1, ugt-60,* F10D2.8, *gst-25, ugt-10, gst-23, cyp-37A1, cyp-14A5* and pathogen stress response, *ilys-2,* F20G2.5, *hpo-31.*

On the other hand, RNAi knockdown of genes which improved resistance to heat stress and therefore can be explored further to see if their knockdown provides other advantages, include, C-type lectins; *clec-178, clec-34, clec-121, clec-112* and *clec-91*, genes involved in pathogen stress response, C14C6.5, C39H7.4, *irg-3, fipr-13, hrg-3, bre-5, hpo-21*, those involved in detoxification stress, *cyp-34A5, cyp-33E1, ugt-40, ugt-34,* T07E3.3 and *sod-2* involved in oxidative stress.

Unassigned genes regulated by multiple stresses and indicated to be affected by *hsf-1* include, B0496.1, F10D11.3, bath-29, F42C5.3, F45D3.3, M03B6.1, W03D8.5, W09G12.9, Y59C2A.3, unassigned transmembrane proteins, C32C4.3, T14B4.5, Y71G12B.3, unassigned genes, B0353.1, *pals-9,* K09C4.10, M57.1, R10D12.8, Y102E9.5, ZK131.11, proteolysis proteasome *fbxa-125, fbxa-95* and C54G4.9 involved in development.

3.3.5 Summary

I conducted an RNAi screen to identify novel *C. elegans* genes involved in severe heat resistance and possibly healthy ageing.

I looked for known thermoprotective genes, such as *hsf-1* and found that the RNAi of *hsf-1* did not seem to sensitise the worms to heat stress suggesting that early adulthood RNAi does not seem to follow the same response as *hsf-1* mutants or RNAi needs to be done at L1 stage to see the effect. RNAi of heat shock proteins, *hsp-70, hsp-16.11, hsp-12.2, hsp-12.6, hsp-110, hsp-90, dnj-5,* seemed to sensitise the worms to heat stress, whereas RNAi of heat shock proteins *hsp-16.1, hsp-16.2, hsp-16.41, hsp-12.1, hsp-12.3* seemed to protect worms against heat stress, suggesting that the knock down of some heat shock proteins may be beneficial as it may up-regulate other heat shock proteins.

I had an inclusion criteria for wormcat analysis to include only the top 10 and bottom 10 genes with the average of the plate as control (based on the findings in result chapter 1). In total, 1,200 genes (600 thermoprotective and 600 thermosensitive) were analysed using WormCat.

I found genes involved in general cell functions, such as those involved in signalling (MAPK, WNT, hedgehog like), genes regulated by multiple stresses (heat, pathogens, oxidative), lipid metabolism, development, mitochondria, proteolysis, and transmembrane transport, suggesting that heat stress response is affected by various interlinked pathways.

Only a few know genes involved in heat stress resistance met the threshold and were included in the wormcat analysis. This included F44E5.5 (Hsp70), *sip-1 (1* (HSP exclusively expressed in oocytes and embryos), *skn-1* and dnj-5 (Hsp40) chaperone. Knockdown of F44E5.5, *sip-1* and dnj-5 sensitised the worms to heat stress which was expected.

Identification of known thermoprotective genes validates this RNAi screen approach, however the threshold criteria could be less stringent to include more genes in wormcat analysis, for example instead of considering only the top and bottom 10 genes, we could expand the selection to include more genes. I also identified some new thermoprotective candidate genes, such as *clec-20, F20G2.5, pals-30* (immunity) and *R08F11.1, fat-7* (fat metabolism).

4. Discussion

The ability to maintain homeostasis decreases with age, which means that response to physiological perturbations, such as heat and oxidative stress can be used as a determinant of health (Bansal et al., 2015).

The LFASS assay relies on recording the time of death based on the measurement of intestinal blue death fluorescence, which means delay in death readout due to impairment of organismal death pathway could potentially be misinterpreted as increased stress resistance. My results have shown that modulation of organismal death pathway genes does not dramatically affect heat and oxidative stress outcomes in the LFASS assay.

The death fluorescence readouts are impacted by the bacterial species on which the worms feed. However, despite the difference in amplitudes, death waves are well-defined due to significant death peaks and therefore we can determine the time of death accurately. This means that it is possible to precisely pinpoint times of death and compare them across bacterial strains. The differences in death fluorescence amplitude across different microbes suggest different anthranilate levels and accordingly different KP activities.

Gut microbial isolates differentially affect oxidative and heat stress resistance in *C. elegans* and the IIS and KP mediate some of the shifts in stress resistance seen on gut microbiota isolates. I have also performed an RNAi screen to identify genes that may be involved in promoting heat stress resistance.

4.1 LFASS provides sensitive, unbiased and high-throughput results

Impairment of the organismal death pathway could affect death-associated fluorescence and consequently delay the timing of death, which might be misinterpreted as increased stress resistance. My results showed that the most mutants involved in the organismal death pathway were not critically different from the wild-type in their response to heat and oxidative stresses suggesting that impairment of the organismal death pathway does not critically impact the timing of death in LFASS assay.

IIS is a lifespan-regulating genetic pathway in worms and therefore *daf-2* (long-lived, stress-resistant strain) was used as a positive control and as expected *daf-2* mutants were more

resistant to both stresses on both days compared to the wild-type and the death pathway mutants.

Genes involved in the organismal death pathway, *itr-1*, *unc-25*, and *aex-2* showed some differences in median time of death compared with the wildtype, whereas *eat-2*, *unc-17*, and *egl-8* behaved similarly to the wild type. None of the strains, except for *unc-25* mutants on day 1 of the heat shock assay, showed an increased time of death (by five minutes) compared to *daf-2*. This was not observed in day 2 heat shock animals or in the oxidative stress assay.

We expect some delay in the timing of death in our assay, as organismal death processes that lead to death fluorescence can significantly influence the screening results. This also helps define a threshold to consider when selecting top and bottom candidate genes in the RNAi screen.

The inositol 1,4,5-trisphosphate receptor (InsP3R) functions as the calcium release channel in the ER. In *C. elegans*, a single gene, *itr-1*, encodes the receptor, in contrast to mammals, that have three genes (*ITPR1*, *ITPR2*, and *ITPR3*) encoding three isoforms (Allman, E., Thyagarajan, B. and Nehrke, K., 2012). There was a slight but significant increase in median time of death in heat and oxidative stress assays in *itr-1*(sa73) worms on day 1, but not on day 2. In *itr-1* mutants, we know that slower calcium leakage leads to delayed death fluorescence, resulting in a later fluorescence peak. Stress resistance itself is not affected, as the worm will die once the calcium release signal is triggered. However, the delayed appearance of death fluorescence can give the false impression that the worms are more stress-resistant.

The *unc-25* gene encodes for the biosynthetic enzyme glutamic acid decarboxylase (GAD), which synthesises GABA from glutamate in the cytoplasm of the neuron. 26 out of 302 neurones in *C. elegans* contain Gamma-Aminobutyric acid (GABA) neurotransmitter (Schuske, K et al., 2004). GABA signalling is involved in triggering the death fluorescence and unpublished data from our lab has shown that it delays organismal death readout. This could explain the increased median time of death observed in *unc-25* mutants in both day 1 and day 2 heat stress assays.

In *C. elegans*, LITE-1 is a seven-transmembrane gustatory receptor that mediates UV light avoidance. Worms respond to short wavelengths of light, with their photosensory neurons inducing negative phototaxis to UV light through calcium ions surge into muscle cells causing contraction. This serves as a protective mechanism against lethal doses of sunlight-derived UV radiation (Gong, J et al., 2016). *Lite-1(xu7)* worms exhibited increased median time of death to heat stress on Day 2 compared to Day 1, whereas reduced timing to oxidative stress on Day

1. Oxidative stress resistance significantly reduced in day 1 *daf-16;daf-2* and day 2 *aex-2* worms.

aex-2 mutants are characterized by defective anterior body wall muscle contraction and expulsion. *aex-2* encodes a G-protein-coupled receptor that activates a signalling pathway in the nervous system to regulate defecation. The defecation cycle in *C. elegans* involves three muscle contractions occurring every 45 seconds. The cycle begins with a posterior body wall muscle contraction, followed by an anterior body wall muscle contraction 2–3 seconds later. One second after that, enteric muscles contract, leading to the release of intestinal contents (Mahoney, T.R et al., 2008). *aex-2* is likely required for the full calcium release in the gut that leads to the DF. It is understandable that it can lead to a delay in TOD measured by DF.

eat-2 worms, which have a longer lifespan compared to the wild type due to their reduced pharyngeal pumping and, consequently, decreased food intake (Bansal et al., 2015), exhibited similar timing of death to oxidative and heat stress as the wild type. The pharynx has two pumping rates: it pumps once per second in the absence of food, and in the presence of food, the pumping rate increases to four pumps per second. This pumping rate is controlled by excitatory MC motoneurons. EAT-2 is a nicotinic acetylcholine receptor subunit that functions postsynaptically in the pharyngeal muscle. Stimulation of MC neurons opens the EAT-2 channel, allowing current to flow in, which triggers the opening of voltage-activated calcium channels and subsequent muscle contraction (McKay, J.P et al., 2004).

unc-17 mutants are smaller, uncoordinated, and slow-growing. In *C. elegans*, the *unc-17* gene encodes the vesicular acetylcholine transporter (VAChT). Coordinated muscle movement requires the release of acetylcholine from motor neurons. In motor neurons, choline acetyltransferase synthesizes acetylcholine, which is then transported into synaptic vesicles by VAChT. Acetylcholinesterase then hydrolyzes acetylcholine into choline and acetyl-CoA (Mathews, E.A et al., 2012). The egl-8/PLCβ (phospholipase Cβ) mutation has also been shown to increase lifespan, possibly through mechanisms involving daf-16. *egl-8* is also involved in calcium release (Mack, H.I et al., 2022).

Perturbing the organismal death pathway should not critically, but might occasionally, confound stress assay analyses based on LFASS. The results presented here give us an idea of the extent to which time of death may be affected, and help define the appropriate time threshold for identifying hits in our stress resistance screens. Inter- and intra-experimental variability is higher in t-BHP assays compared to heat stress assays, suggesting that heat stress assays are more reliable. Moreover, heat stress resistance assays are better predictors of

longevity phenotypes in LFASS, making them a valuable tool provided potential confounding factors are taken into account.

4.2 Gut microbiota influences *C.elegans* heat tolerance, oxidative stress response and health, and modulates IIS and KP pathways

4.2.1 Gut microbiota influences response to stress in IIS dependent or independent manner

Worms found in the environment harbour a variety of microorganisms in their guts, unlike laboratory worms, which are typically fed a single type of bacterium and undergo regular hypochlorite treatment (Dirksen et al., 2016). Thus, considering the natural microbiome of worms provides a more realistic and unbiased understanding of *C. elegans* biology (Dirksen et al., 2020).

Therefore, I investigated the impact of gut microbiota on the stress response in wild-type worms. Since the IIS pathway is known to play a role in stress resistance and longevity, I explored whether the effects of the gut microbiota on stress resistance are mediated through the IIS pathway by using insulin receptor mutants, *daf-2* (long-lived) and *daf-16;daf-2* (short-lived) strains.

For instance, Stuhr and Curran have shown that bacteria promoting increased lifespan also affect IIS genes by downregulating *daf-2* and *age-1* while upregulating DAF-16 target genes. This finding suggests that microbiota can regulate lifespan through the IIS pathway (Stuhr, N.L. and Curran, S.P., 2020).

I exposed IIS mutants *daf-2* and *daf16;daf-2* to *C.elegans* natural bacteria (CeMbio+ microbes) before exposing them to heat and oxidative stress. My results showed that gut microbial isolates can differentially impact stress resistance in wild-type worms, for example MYb021, critically improved resistance to heat stress, whereas JUb066 (a), MYb011, MYb025, MYb264, MYb375, MYb398, MYb416, MYb526 (a) and MYb535 (b) sensitised wild-type worms to heat stress ($p = <0.05$).

I also found that some gut bacteria influenced stress response in IIS dependent manner (changes in wild-type worms but not in *daf-2* mutants) such as BIGb170, BIGb172 and MYb49 improved resistance to heat stress, in an IIS dependent manner, whereas *B. longum* 2, JUb44, MYb21, MYb71, MYb317, MYb330 and MYb396 (a) led to an IIS independent increase to heat stress. On the other hand, BIGb0393, CEent1, JUb66, MSPm1, MYb11, MYb58, MYb69

(b), MYb121, MYb174, MYb176, MYb177, MYb264, MYb375, MYb388 (a), MYb398, MYb416 (a), MYb526 (a) and MYb535 (b) led to an IlS independent decrease in heat stress. In addition, some bacteria such as MYb10, MYb69 (a), MYb191, MYb371, MYb396 (b) and MYb526 (b) compensated the *daf-16* in *daf-2* background, (higher or same median time of death in *daf16;daf2* than *daf-2* worms), whilst MYb115, MYb158, MYb328 and MYb536 suppressed *daf-2* effects. Bacteria reducing the protective effect of DAF-2 mutation during stress suggests the potential pathogenicity of some gut bacteria. This can be due to stress itself as it can cause commensal bacteria to become pathogenic (Stevens et al., 2021).

On the other hand, *B. longum* 2, JUb44, MYb10 and MYb21 improved resistance to oxidative stress in an IIS dependent manner, whereas MYb186, MYb328, and MYb331 improved oxidative stress resistance in an IIS independent manner. However, JUb134, MYb25 (b), and MYb382 led to an IIS independent reduction in oxidative stress resistance.

Interestingly, JUb44 and MYb21 caused an IIS dependent increase to oxidative stress resistance, but an IlS independent increase to heat stress, suggesting that the same bacteria may be impacting different stresses through different molecular pathways indicating that a single gut bacterium species can influence different stress response pathways in opposite directions. This is consistent with the idea that a metabolic shift can favour one molecular pathway over another due to energy trade-offs, potentially leading to a beneficial effect on one hand and a detrimental one on the other.

4.2.2 What is the relationship between kynurenine pathway and stress resistance?

After examining the impact of the IIS pathway on stress resistance, I explored the relationship between microbiota and the kynurenine pathway using wild-type and KP *kmo-1* mutant worms, as previous work from our lab and the literature has identified the KP as an important gut-brain axis pathway that mediates the impact of gut microbes on host health.

The results showed that KP is strongly involved in stress resistance. However, its inhibition had opposite effects on worm resistance to different stresses. Hence, *kmo-1* mutants generally resisted severe oxidative stress better than wild-type worms but were more susceptible to heat stress. This supports the observation that *kmo-1* mutants are more sensitive to heat stress, consistent with their reported shorter lifespan. It also aligns with findings from the LFASS paper, which suggest that oxidative stress resistance does not generally correlate well with longevity, whereas heat stress resistance does.

Interestingly, the increased resistance of *kmo-1* mutants to oxidative stress was also recently reported in the context of paraquat-induced stress (Choi et al., 2021). Choi et al demonstrated that *kmo-1* expression is essential for normal worm longevity, as knockdown of *kmo-1* decreased life-span, despite conferring increased resistance to paraquat-induced oxidative stress.

But, why would kmo-1 mutants be more resistant to t-BHP and paraquat-induced oxidative stress? One reason could be that as KP produces a number of compounds with oxidative potential, *kmo-1* mutants could be producing less endogenous oxidising compounds, or accumulate more antioxidant compounds. To verify this, endogenous oxidised biomolecules can be tested (using fluorogenic probes) in *kmo-1* versus wildtype worms, for example, ROS molecules can oxidise the amino acid residues of proteins to generate protein carbonyls (PC) content that can be used as a marker for identifying oxidative modification of proteins. PC can be measured using the 2,4-dinitrophenylhydrazine (DNPH) method. Lipid peroxidation results in the end product, Malondialdehyde, that can be measured using thiobarbituric acid reactive substances (Katerji et al., 2019).

Another reason for the increased resistance to oxidative stress in *kmo-1* mutants could be that inhibiting the downstream effects of KMO increases the levels of KP metabolites, which mediate antioxidant effects. For example, Giorgini et al conducted a study in mice with no KMO activity and showed that these mutants have elevated levels of other KP metabolites: kynurenine, kynurenic acid, and anthranilic acid (Giorgini et el., 2013). Anthranilic acid causes antioxidant effects in several ways such as by forming complexes with copper ions to inactivate hydroxyl radicals inducing anti-inflammatory, downregulating ROS formation through iron complexes and by inhibiting the respiratory chain complexes I–III from interfering with the mitochondrial function. Kynurenine and kynurenine acid can scavenge ROS, hydrogen peroxide and superoxide reducing oxidative damage (Mor et al., 2021).

To further test this possibility, the resistance to stress of other KP mutants, such as *kynu-1* and *tdo-2,* which block the pathway at different levels could be tested and compared to *kmo-1* mutants, to see if the accumulation of specific KP compounds correlate with resistance to oxidative stress. Wild-type could alternatively be supplemented with compounds accumulating in *kmo-1* mutants, and their resistance to severe oxidative stress could be compared to a non-supplemented control group. However, some of the mutants of the pathway, such as *kynu-1* don't fluorescence well because they produce less anthranilic acid (Komura et al., 2021). This means that LFASS assays may not be appropriate for them and instead, a more time-consuming and lower throughput traditional oxidative stress assays would need to be performed.

Another explanation for the heightened resistance of *kmo-1* mutants to oxidative stress could be due to differences in feeding behaviour caused by pharyngeal pumping rate which might differ between different strains. Additionally, some microbes could also potentially alter the rate of pharyngeal pumping affecting the amount of food being eaten by the worm. Therefore, some worms might be feeding less than others (Stuhr, N.L. and Curran, S.P., 2020).

These could lead to either *kmo-1* mutants consuming less t-BHP or paraquat in oxidative stress assays, or upregulating starvation-associated stress defence mechanisms such as autophagy. Pharyngeal pumping rate can be measured by counting grinder movements using a stereomicroscope (Raizen et al., 2018) and could be compared between wild type and *kmo-1* mutants. On the other hand, disrupting Tryptophan catabolism by mutations in the KP might impact the production of serotonin and dopamine, which are also produced from Tryptophan metabolism and modulate worm feeding behaviours.

SKN-1 transcription factor mediates oxidative stress response in *C. elegans* by inducing the expression of detoxifying genes which encode enzymes that synthesize glutathione and scavenge free radicals (An at al., 2005).The antioxidant defence in *kmo-1* vs *wild-type* worms could be studied using fluorescent stress reporters such as, SKN-1:GFP. Transparency of C.elegans allows GFP fluorescence to be assessed in vivo non-invasively (Braeckman et al., 2016).

Because the gut microbiota can modulate the KP (Kennedy et al., 2017), we can use fluorescent microscopy to test and compare differences in bacterial colonisation in wild-type and *kmo-1* mutants to see if certain bacteria are more invasive in *kmo-1* mutants. During gut infections KP compounds are produced (Martin J PhD, benedetto lab, unpublished) which may be antioxidants. Worms tend to produce antioxidant compounds during other innate immune responses too, for example, during *Pseudomonas aeruginosa* infection, the PMK-1 p38 MAPK pathway in *C. elegans* is activated and upregulates a range of antimicrobial genes, including C-type lectins, ShK-like toxins, and CUB-like domain-containing proteins. These effectors contribute to pathogen defence by targeting microbial structures or interfering with pathogen physiology (Troemel et al., 2006). Thus one possibility is that exposure to new bacteria or bacteria that the worm detects as potentially pathogenic could elicit a KP response with antioxidant effects, raising worm resistance to oxidative stress.

4.2.3 Specific combinations of gut microbes can further increase C. elegans heat resistance

Five promising microbial isolates BIGb170, JUb19, CEent1, MYb21, and MYb71, with *E. coli* OP50 as the control, were used to determine the effects of the combination of microbial isolates on heat stress resistance. This provides a better understanding of bacterial community effects and how gut microbiotas naturally regulate host heat stress resistance, as there are multiple bacteria present in the animal gut.

These experiments were conducted in parallel to the RNAi screen and therefore the same worm strain (NL2099) was used for convenience, but it is important to note that it introduced a confounding factor, with NL2099 being a *rrf-3* (hypersensitive to RNAi) mutant. For example, BIGb170, which previously showed increased heat stress resistance in wild-type and insulin signalling mutants, appeared to make NL299 worms more susceptible to heat stress.

When compared to the control, CEent1, MYb21, and MYb71 showed improved heat stress resistance, while JUb19 was similar to the control. The combination of CEent1, MYb21, and MYb71 further enhanced heat stress resistance, suggesting that these bacteria, both individually and in combination, are beneficial to worms. Further enhancement in combination could suggest parallel or additive mechanisms of action. Bacteria inside the gut interact with each other and form symbiotic relationships such as Mutualism, an interaction in which both bacterial strains benefit, and commensalism, in which one bacterial strain gains an advantage, while the other strain is unaffected. Bacteria can also utilise the same resources in different ways based on their genetic differences, allowing them to avoid competition with each other in a process known as Specialisation (Kern, L et al., 2021).

On the other hand, all combinations of BIGb170 and JUb19 showed higher median times compared to their individual results, suggesting that in the presence of bacteria promoting heat stress resistance, these strains do not diminish those effects. Instead, the observed outcomes are primarily driven by bacteria that enhance stress resistance. This could be a result of competition elimination, such as predation, where one strain feeds on another. Bacteria compete with each other by sharing resources and limiting access to competing resources in a process known as exploitation competition and can also directly compete with other strains by releasing toxins or modifying molecules harming neighbouring strains, in a process known as interference competition. (Kern, L et al., 2021).

Therefore the results could be explained by interbacterial relationships, whereby if one bacterium eliminates the competition then the effects might be due to that bacterium alone.
The BCJ (BIGb70,CEent1,JUb19) combination and the two-strain combinations (B+C, B+J, C+J) are not particularly effective, especially when compared to BCJ combined with other bacterial mixes. While looking at combinations of two or three among B, C, or J might suggest that the metabolites produced by these strains are not beneficial for promoting stress resistance, it could also indicate that the strains do not interact well with each other—possibly due to adverse microbe-microbe interactions. MYb21 and MYb71 appear to work best as a simple two-strain combination. They may complement each other metabolically to enhance worm health, or they may each individually (and together) induce a stress-protective response. However, since the combination of the two does not outperform MYb21 alone, it suggests they might act through a similar mechanism, especially given that both have been shown to increase heat stress resistance in an insulin-independent manner.
The combined effects of all the bacterial strains were like the control, potentially indicating a balance of different strain effects. This is worth investigating as it does not seem to be down to the influence of one specific microbe and it would be interesting to know about the fitness of the animals in these conditions through brood size, movement and lifespan experiments.

4.2.4 Bacterial-Mediated Stress Resistance in the Host: A Trade-Off with Lifespan?

After discovering that some bacteria may increase resistance to heat and oxidative stress, possibly through the modulation of molecular pathways, I investigated whether they also extend the lifespan of wild-type worms or shorten it. I conducted lifespan experiments in wildtype worms by feeding them one of the following bacteria: BIGb170, BIGb172, JUb44, MYb10, MYb21, MYb49, MYb71, MYb317, MYb330, MYb396 (a), MYb115, or CEent1, which had improved resistance to heat or oxidative stress in worms.
I found that microbial isolates may improve stress resistance but do not extend lifespan compared to OP50, suggesting that increased stress resistance does not necessarily translate to a longer lifespan. This confirms that stress resistance and longevity are physiologically distinct. MYb10, MYb115, and MYb317 nearly halved the lifespan of worms compared to OP50, suggesting a potential trade-off between increased oxidative or heat stress resistance and

lifespan, and indicating that these bacteria may have pathogenic potential. This is consistent with the studies examining CeMbio impact on worm lifespan and health, for example, Kissoyan, K.A.B et al showed that *Pseudomonas lurida* MYb11 and *P. fluorescens* MYb115 protect the worm against pathogens such as *Bacillus thuringiensis (Bt),* but there seems to be a trade-off between protective effects and longevity. Their results showed that MYb11 protects *C. elegans* against *Bacillus thuringiensis* by producing antimicrobial compounds massetolide E that inhibits the pathogen's growth (Kissoyan, K.A.B at al., 2022). Whereas, MYb115 does not directly inhibit the growth of *Bacillus thuringiensis*, but may provide protection by altering host lipids, which in turn reduces the epithelial barrier dysfunction caused by *Bacillus thuringiensis* (Singh, A. and Luallen, R.J., 2024). Despite providing protection against pathogens, MYb115 did not increase worm lifespan, and MYb11 reduced lifespan, suggesting a trade-off between pathogen protection and lifespan. Therefore, these results align somewhat with my observations and suggest that, although some of the CeMbio+ bacteria offer benefits against certain types of stress, they may also reduce lifespan and have pathogenic potential.

Another possibility for the increased stress resistance but decreased lifespan is that the stress assays were conducted on Day 1 adults, meaning these bacteria may provide benefits at a young age but not in older animals. To test this, exposing older worms to stress could be a useful approach.

In two lifespan experiments, worms survived better on BIGb170, while in others, the survival rate was similar or lower than that with OP50. This suggests that BIGb170 is highly sensitive, and environmental variables such as slight temperature changes during worm transfer may significantly impact the results.

Additionally, paraformaldehyde (PFA) can be used to kill bacteria, thereby minimizing the confounding effects of bacterial metabolism. Beydoun et al. demonstrated that a 0.05% PFA treatment for 1 hour was sufficient to kill *E. coli*, allowing its use as an inactive food source for worms. PFA permeabilizes cells without destroying their internal structures (Beydoun et al., 2021), suggesting this approach could be applied to BIGb170 or other environmental bacteria. Our lab is currently working with heat-killed BIGb170. So far, the results indicate that killed BIGb170 also improves resistance to heat stress in wild-type worms, suggesting that the effect does not require metabolically active bacteria.

After discovering that most bacteria shorten lifespan in wildtype worms the next logical step was to conduct lifespan experiments in KP and IIS mutants as I already knew that some bacteria increase resistance to heat and oxidative stress through the modulation of KP and IIS.

However, due to time constraints, I was only able to conduct one lifespan assay with wild-type worms and *kmo-1* mutants on MYb71.

My results showed that, although *kmo-1* mutants have a decreased lifespan compared to wild-type worms, they exhibited similar survival rates on both OP50 and MYb71 (median survival day 11). In contrast, wild-type worms survived longer on OP50 (median survival day 16) than on MYb71 (median survival day 13), suggesting that MYb71 may be beneficial for *kmo-1* mutants. This points to a possible role of the KP pathway in lifespan regulation through interactions with the gut microbiota.

4.2.5 Studying the impact of the C. elegans natural gut microbiota on worm health

Various studies have found a correlation between an increase in lifespan and healthspan. As a result, lifespan has been studied extensively and studies have focused on increasing lifespan. However, contrary to this, some studies have demonstrated that this might not always be the case and therefore healthspan and lifespan should potentially be separated when studying ageing. For example, Bansal et al studied healthspan by measuring multiple physiological parameters, such as movement on solid and liquid media, heat and oxidative stress resistance with age in wild-type and long-lived *C. elegans* mutants of IIS (*daf-2*), DR (*eat-2*), protein translation (initiation factor-2) and mitochondrial signalling (*clk-1*) longevity paradigms, and observed that long-lived mutants had an extended period of frailty. This showed that some long-lived mutants stay unhealthy for longer and therefore an increase in lifespan may not translate into improved healthspan, suggesting that healthspan requires further examination (Bansal et al., 2015).

The parameters for studying healthspan in *C. elegans* include pharyngeal pumping rate (feeding), movement and the ability to maintain homeostasis, which decline with age (Bansal et al., 2015). This means I can potentially test these other parameters as well as stress resistance to better understand healthspan in *C. elegans.*

The pharynx is a neuromuscular organ that contracts and relaxes to ingest food in *C. elegans*. Pharyngeal pumping reaches its peak rate of around 300 pumps per minute in day 2 adults, then gradually declines with age and typically ceases by day 12. The rate of pumping can be studied to determine health. However, in some mutants, reduced pharyngeal pumping and consequently reduced food intake may lead to calorie restriction and an increase in lifespan.

For example, *eat-2* mutants, which exhibit slow pumping, have a longer lifespan compared to wild-type animals (Bansal et al., 2015).

Additionally, some bacteria could have fewer calories or are too difficult to digest suggesting the role of CR as a possible cause for the increased longevity. To see if this is the case, I could conduct a size comparison as dietary restriction decreases body weight and fertility in *C. elegans*. If there are no differences in body and brood size, then the increase in longevity could be considered due to dietary restriction independent manner.

Worm sinusoidal movement becomes slower and less coordinated with age due to the degeneration, loss and quality of the skeletal muscle. In worms, movement can be studied on solid and liquid media, for example, by observing the distance travelled over a certain period on solid media and counting the number of body bends per minute of a worm to measure the level of thrashing (Bansal et al., 2015). This sinusoidal movement can be counted as waves per minute using a dissecting microscope or defined into three categories, rhythmic sinusoidal movement (Class A), uncoordinated and inactive (Class B) and unable to progress but move head and tail spontaneously when prodded (Class C). Sensory perception also declines with age which can be measured by chemotaxis towards food (Collins et al., 2007).

4.2.6 In lab conditions, CeMbio+ microbes reduce brood size

I conducted brood size experiments to determine if gut microbiota affected the number of eggs laid by worms. The results showed that, generally, the gut microbes reduced the brood size of worms, with MYb21 having the same effect as OP50. However, the one-way ANOVA (OP50 compared to microbial isolates) followed by Dunnett's multiple comparisons test showed no significance. This could be due to low or no repetition. Therefore, additional repeats are needed for better data interpretation.

The highest number of eggs was laid 48 hours post-L4 stage, suggesting that the timing of egg-laying was not affected. This suggests that the gut microbiota may improve resistance to stress without affecting reproduction too much, which could explain why the worms feed on these bacteria. They may live shorter lives but remain healthier.

Another consideration is that the CeMbio+ conditions tested are not representative of what happens in the wild. I tested the bacteria individually, whereas in nature, worms feed on a variety of bacteria. Therefore, a combination of bacteria may increase brood size. Based on my

results, a combination of MYb21 and MYb330 could be worth investigating as individually their brood size was similar to on OP50.

There is a possibility that laboratory *C. elegans* have undergone genetic drift across generations to adapt to the *E. coli* OP50 diet, leading to a preference for OP50 over wild microbial isolates in laboratory settings. To test this hypothesis, several recent wild isolates can be examined to determine if the same trend is observed. Alternatively, the oldest available N2 stock from CGC or Bristol) can be compared to recent stocks.

Lastly, I have only examined self-reproduction, whereas males are more common in wild isolates, and male-hermaphrodite reproduction is likely much more frequent in the wild than in the laboratory. In this context, natural bacterial isolates may provide additional benefits as brood size after mating does not follow the same pattern as brood size from self-reproducing hermaphrodites. (Wegewitz et al., 2008).

4.2.7 Longer-living worms spend a significant portion of their later life in poor health

My results showed that some gut microbiota may improve stress resistance, however they may lead to a reduction in brood size and shorten lifespan. Therefore, I conducted an ABC worm movement assay, as movement can serve as an indicator of health. I conducted worm movement scoring in parallel with some of the lifespan experiments. This included, OP50, BIGb170, BIGb172, MYb21, JUb44 and MYb396(a).

The results showed that worms initially exhibit good movement (category A; sigmoidal and fast), but over time, their movement becomes slower and less coordinated (category B), eventually declining to the point where they can barely move and only respond when prodded with the worm pick (category C).

Worms that reached very old age, i.e., those that lived longer, remained in this unhealthy (category C) state. This suggests a similarity between human and worm ageing. Just as longer-living humans often spend a significant proportion of their lives in poor health with multiple comorbidities, worms also remain in an unhealthy state as they age. This illustrates that longevity does not necessarily translate into healthspan, and the goal should be healthy ageing rather than simply increasing lifespan.

Compared to OP50, worms fed on BIGb170 were faster and better coordinated, suggesting that BIGb170 improves movement in worms.

Although the ABC assay is effective for observing worm movement, it is subject to human error and observation bias. A way to overcome this bias is usually to score blindly (without bacteria labelling), however since CeMbio+ bacteria are easily identifiable through their colour this was not feasible. Therefore, other assays, such as the swimming assay, can be used to track worm movement by counting bends per minute under the microscope. Additionally, the pharyngeal pumping assay can assess worm movement and, subsequently, health parameters. In the future, these assays can be conducted in parallel to determine if they produce similar results.

4.2.8 Imaging with gut microbial isolates can provide further insight into worm behaviour

I observed phenotypic changes in worms with different bacterial diets while conducting lifespan and movement assays. For instance, compared to OP50, worms fed on MYb21 and MYb396(a) appeared significantly larger and fatter, while those on BIGb170 appeared smaller and skinnier. The reason for these size differences could be that some bacteria lead to an increase in fat storage, whereas others provide fewer calories.

There is a possibility that the size of worms had an impact on their movement, for example, worms fed on BIGb170 were skinnier and smaller, which may explain why they were faster, whereas worms fed on MYb396(a) were fatter and therefore more sluggish, showing an earlier decline in movement.

On the other hand, a decline in movement with age may result in some worms being farther from the bacterial source and not receiving adequate nutrients.

Other noticeable observations included worm food preferences. For example, MYb330 is a very wet bacterium, which caused worms to move toward the edges of the plates while trying to escape. This led to some worms becoming desiccated and resulted in data loss. In contrast, worms seemed to remain within the bacterial lawn of MYb10 exhibiting a darker appearance due to feeding on dark coloured bacteria. MYb10 reduced worm lifespan significantly and it could be that MYb10 is only beneficial in certain amounts and an excess results in toxicity. As worms prefer to stay in MYb10 bacterial lawn, over time with bacterial buildup, this leads to pathogenicity.

The impact of these phenotypic changes on worm behaviour could also be investigated through imaging experiments. However, due to time constraints, imaging could not be conducted.

4.2.9 Research in C. elegans provides valuable insights into the mechanisms of probiotics

4.2.9.1 Probiotics can mediate beneficial effects through the IIS pathway

In the context of human health, probiotics are defined as living bacteria which if administered in adequate amounts can suppress pathogen proliferation to rebalance the altered microbiome to provide beneficial effects to the host (Markowiak, P. and Śliżewska, K., 2017). These beneficial effects exceed nutritional effects (Wieërs et al., 2020).

The most common human probiotics belong to *Lactobacillus, Bifidobacterium, Lactococus, Streptococcus* and *Enterococcus* species of bacteria, which may provide beneficial effects, such as lipid-reducing activities and the maintenance of glucose homeostasis (Goyache et al., 2024). The beneficial effects of probiotics are strain-specific which means that strains from the same species can produce different effects (Roselli et al., 2019).

However, probiotics mechanisms of action are not yet fully understood and therefore, research in *C. elegans* provides valuable insights into these mechanisms (Goyache et al., 2024). For example, *Clostridium butyricum* (gram-positive) is a marketed probiotic in humans, Kato et al studied the influence of *Clostridium butyricum* MIYAIRI 588 (CBM 588) on *C. elegans* lifespan and stress resistance. The results showed that the wild-type worms grown on CBM 588 lived longer and had improved locomotion than those that were cultivated on *E.coli* OP50. Additionally, worms fed on (CBM 588) and later infected with pathogenic bacteria (*Salmonella enterica* or *Staphylococcus* aureus) lived longer compared to worms fed on OP50. The transcriptional profiling using RNA sequencing showed the involvement of the IIS pathway as DAF-16-dependent genes (dod-22, dod-23, dod-24) were downregulated by CBM 588. Therefore, *daf-2, daf-16* and *skn-1* were studied, the results showed that CBM 588 did not promote longevity in individual mutations in *daf-2, daf-16* and *skn-1* (mammalian ortholog Nrf2), suggesting that both DAF-16 and SKN-1 may be required for the CBM 588-mediated lifespan extension. Another possibility is that longevity is mediated through SKN-1 activation via the p38 MAPK pathway (Kato et al., 2018). This suggests that some probiotics may be mediating beneficial effects through the IIS pathways. I have identified that BIGb170, BIGb172 and MYb49 increase resistance to heat stress and JUb44, MYb10 and MYb21 increase oxidative stress resistance in an IIS dependent manner.

4.2.9.2 Probiotics can mediate beneficial effects through the KP pathway

The KP is modulated by ageing, resulting in an increase in kynurenine, kynurenic acid and quinolinic acid and a decrease in tryptophan concentration in serum and cerebrospinal fluid (Sorgdrager et al., 2019; Wu et al., 2021). These increased levels of kynurenines can produce neurotoxic effects, thus a decrease in kynurenines can be beneficial. Therefore, some probiotics have shown to provide beneficial effects through the modulation of the KP, for example, Rudzki et al showed that Probiotic *Lactobacillus Plantarum 299v* which is a natural habitat of human gut microbiota and is widely distributed in the GI tract improves cognitive functions in patients with depression by decreasing kynurenine concentration (Rudzki., 2018). A study by Van der Goot et al. showed that in *C.elagans* tryptophan 2,3-dioxygenase (*tdo-2*), the first enzyme in the KP pathway of tryptophan degradation, functions as a metabolic switch regulating age-related protein homeostasis and lifespan. *tdo-2* expression naturally increases with age and contributes to age-associated α-synuclein toxicity. In wild-type worms, depletion of *tdo-2* suppressed the accumulation of aggregation-prone proteins, including amyloid-β and worms fed increasing amounts of L-tryptophan also showed reduced α-synuclein toxicity. The depletion of *tdo-2* leads to elevated tryptophan levels, which may influence other signalling molecules involved in regulating proteotoxicity, as tryptophan itself does not appear to act directly on α-synuclein aggregation (Van Der Goot et al., 2012).

Similarly, Strasser et al studied the impact of a probiotic supplement on tryptophan metabolism and incidence of upper respiratory tract infections in athletes after aerobic exercise for three months. Tryptophan levels decreased in placebo group compared to the concentrations measured before the intervention, but remained unchanged in the probiotic group (Strasser et al., 2016). This suggests that bacteria can provide beneficial effects by increasing tryptophan levels. Therefore, it can be studied if CeMbio+ bacteria can increase tryptophan levels in mutants of the KP which have decreased levels of tryptophan.

4.2.9.3 Mechanisms of resistance to heat stress in probiotics

Heat stress increases intestinal barrier permeability and also affects microbiota metabolites which impact host signalling pathways altering the function of the microbiota-gut-brain axis. Bacteria can produce neurotransmitters in the host, for example, *Lactobacillus spp.* and *Bifidobacterium spp.* generate GABA, *Bacillus spp.* produces dopamine, and *Lactobacillus spp.* generates acetylcholine. Gut microbiota also convert amino acid metabolites to regulate intestinal epithelial cell homeostasis. Therefore, organisms have developed protective mechanisms to deal with heat stress to maintain homeostasis, such as upregulating the immune response. However, the changes in the levels of pro-inflammatory cytokines can negatively affect the brain function by causing neuroinflammation. Heat stress has been shown to promote opportunistic pathogens and diminish some probiotics during heat stress. In some animal studies, providing animals with probiotics before exposing them to heat stress has been shown to increase stress resistance response, for example, oral administration of *Bacillus subtilis* strain prevented the complications associated with heat stress in rats, characterised by increased thickness of the intestine and bacterial translocation (Wen et al., 2021).

Bacteria response to heat stress by secreting heat shock proteins, for example, upon heat stress (30 to 42 °C) *E. coli* strain Nissle 1917, synthesis more that 20 HSPCs. *Bacilli* strains are usually tolerant to environmental stresses, such as heat, and *Bacillus coagulans* and *Bacillus licheniformis* strains can pass through the GI tract easily and therefore are used as probiotics. *B. subtilis* heat response consists of 5 classes of heat shock inducible genes and also related to lipid function. The fatty acid composition changes (increase in unsaturated fatty acids) during heat stress to maintain membrane integrity as heat stress increases the fluidity of the bacterial membrane (Mansilla et al.; 2004).

My results have shown that BIGb170 (*sphingobacterium)* improves worm resistance to heat shock suggesting the protective role of sphingolipid metabolism during heat stress. It could be that the bacteria provides worms with sphingolipids, which protects worms during heat stress by contributing to maintaining membrane integrity. Sphingolipids are lipid components of cell membranes that are involved in the formation of lipid raft domains, which contain receptors and signalling proteins involved in a variety of responses. Sphingolipid metabolism influences development and lifespan in *C. elegans*. Sphingolipids are synthesized by the enzyme serine palmitoyl-transferase (SPT), which catalyses the production of 3-dihydrosphinganine from serine and palmitoyl-CoA. The 3-dihydrosphinganine undergoes further modifications to

produce sphingosine, ceramide, and sphingomyelin. In response to the activation of cell surface receptors for cytokines and growth factors, sphingomyelinases cleave sphingomyelin, generating sphingosine-1-phosphate and gangliosides (Cutler, R.G et al., 2014).
Together, these findings suggest that some bacteria are better adapted to cope with heat stress by producing protective metabolites or altering membrane composition. Since the molecular mechanisms underlying resistance to heat stress in probiotics are not fully understood, research using *C. elegans* provides valuable insights into these mechanisms. Therefore, probiotics such as those that modulate lipid metabolism could potentially be administered to humans to help maintain membrane integrity during heat shock.

4.2.10 Differential effects of natural microbial isolates on C. elegans

Based on my combined results from heat and oxidative stress, brood size, lifespan, and worm movement, I have selected a few targets of beneficial bacteria for further study: MYb21, MYb71, MYb330, BIGb170, and MYb396(a). These bacteria improved resistance to stress without negatively impacting reproduction ***(table. 8).*** Although they slightly decreased lifespan, they did not cause an acceleration in movement deterioration, suggesting that while worms may be living slightly shorter lives than those fed on OP50, they are still leading healthier lives.
On the other hand, MYb317 and MYb10 showed potential to be pathogenic. The reason for this is unclear; it could be that they exert beneficial effects during the earlier days of life but later become pathogenic. Another possibility is that they are only beneficial at lower doses, and over time, their accumulation reaches toxic levels. Additionally, MYb10 may provide protection against pathogenic bacteria, and in the presence of other CeMbio+ bacteria, it may not reduce lifespan. Conducting lifespan experiments with bacterial combinations could provide more insight into this. If MYb317 and MYb10 reduce lifespan even in the presence of other bacteria, they would likely be pathogenic. However, if other bacterial strains mitigate their negative effects due to competition, the levels of bacteria may remain optimal for host health.
MYb317 is a species of *Chryseobacterium*. Recently, Page et al. demonstrated that *Chryseobacterium* strains JUb129 and JUb275, isolated from *C. briggsae*, are pathogenic to both *C. briggsae* and *C. elegans*, killing the worms within three hours. Interestingly, the worms were not repelled by the bacteria but were, in fact, attracted to the bacterial lawn and

remained there. The bacteria-initiated digestion of the worms from the inside, eventually degrading the external cuticle. Their results also showed that JUb44 did not kill the worms (Page et al., 2019).

My results have shown that although MYb317 did not kill the worms instantly, it significantly shortened their lifespan, suggesting it is somewhat pathogenic. This indicates that while some species of *Chryseobacterium* are pathogenic to worms, the worms are nonetheless attracted to them. However, since these lifespan experiments were conducted on a single bacterium, the next step would be to perform experiments with bacterial combinations to account for bacterial-bacterial interactions on the host.

Table 8. Beneficial and pathogenic bacteria

Bacteria	**Improved resistance to heat stress**	**Improved resistance to oxidative stress**	**Median Lifespan (OP50 Control= Day 15)**	**Brood size**	**Movement**
BIGb0170 *Sphingobacterium multivorum*	✓		Slightly reduced (13.7)	reduced	Better – more worms with A movement for a longer duration compared to the control
BIGb172 *Comamonas piscis*	✓		Slightly reduced (12.5)	slightly reduced	-
JUb44 *Chryseobacterium scophthalmum*	✓	✓	Reduced (10.3)	reduced	Show a similar age-related decline in movement as OP50
MYb10 *Acinetobacter guillouiae*		✓	Halved (7.75)	-	-
MYb21 *Comamonas*	✓	✓	Reduced (10.5)	same	Show a similar age-related decline in movement as OP50

MYb49 *Ochrobactrum*	✓		Slightly reduced (12)	-	-
MYb71 *Ochrobactrum vermis*	✓		Slightly reduced (12.7)	same	-
MYb317 *Chryseobacterium*	✓		Pathogenic (5.5)	-	-
MYb330 *Pseudomonas*	✓		Reduced (10.7)	Slightly reduced	-
MYb396(a) *Comamonas*	✓		Reduced (9.7)	Slightly reduced	Show a similar age-related decline in movement as OP50

4.2.11 Why do some bacteria act the same way?

I have highlighted the bacteria which act similarly to observe if these bacteria are phylogenetically related (Dirksen et al., 2020). I have highlighted in yellow a few bacteria that may be potential targets for probiotics, as they increase stress resistance without significantly reducing lifespan or negatively impacting brood size. Additionally, I have highlighted in red the bacteria that can be pathogenic ***(figure.51).***

I observed that bacteria from the same family affected stress response differently, for example, *Chryseobacterium,* bacteria MYb317 increased resistance to heat stress, but MYb25 decreased stress resistance. Similarly, *Comamonas* bacteria MYb21 and MYb396 (a) increased stress resistance whereas MYb69 (a) decreased stress resistance. This suggests that the effects of the bacteria are strain specific.

On the other hand, most of the bacteria that decreased resistance, such as CEenT1, BIGb393 and JUb66, belonged to *Enterobacter* species. An age-related increase in *Enterobacteriaceae* has been observed in *C. elegans*, and this shift is associated with a decline in immunity and heightened vulnerability to infections (Choi et al., 2024).

Bacteria from the same family can behave differently due to genomic variations, as unique genes can lead to new enzymatic functions. For example, some *E. coli* strains contain virulence factors, while others are commensals. Even strains with very similar genetic sequences can function differently; for instance, *E. coli* CFT073 (pathogenic) and *E. coli* Nissle 1917 (probiotic) share 99.98% sequence similarity (Liao, H., Ji, Y. and Sun, Y., 2023).

Metabolic differences between bacterial strains could also explain these effects. One well-known factor is the production of vitamin B12. *E. coli* OP50 and HT115 differ in their levels of carbohydrates, fatty acids, and vitamin B12, with OP50 being slightly deficient in vitamin B12 compared to HT115. *C. elegans* acquires vitamin B12 from its bacterial food source, and this vitamin is essential for its development and growth. A diet of bacteria severely deficient in vitamin B12 can lead to chronic B12 deficiency in the worms, resulting in growth defects, infertility, and reduced lifespan. Bacterial strains that provide higher levels of vitamin B12 may help protect worms against toxic agents such as thiol-reducing compounds, which disrupt protein disulfide bonds. For example, in the presence of the thiol-reducing agent dithiothreitol (DTT), wild-type worms fed OP50 arrested development at the larval stage, while worms fed HT115 at the same DTT concentration developed into fertile adults (Winter et al., 2022).

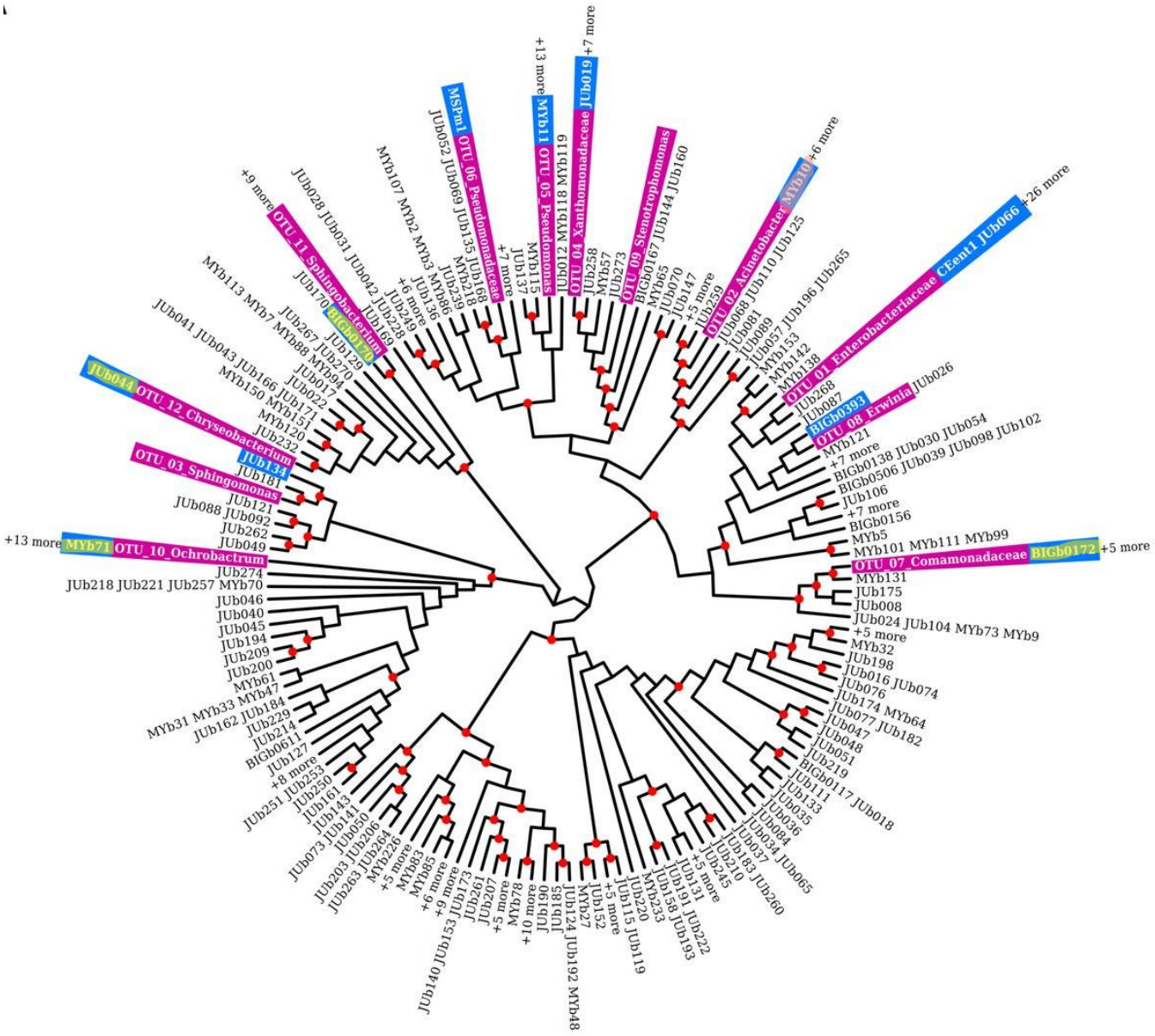


Figure 51. CeMbio strains. Bacteria that increase resistance to stress are highlighted in yellow and bacteria that decrease resistance to stress are highlighted in red (Dirksen et al., 2020).

4.3 RNAi screen identifies essential genes

C. elegans was the first metazoan organism to have its genome completely sequenced. After the discovery of RNAi, multiple RNAi libraries were developed for genome-wide knockdown screens (Holdorf et al., 2020).

The final part of my research was to identify genes that are involved in heat stress resistance. To achieve this, I conducted an RNAi screen by exposing fully developed adult worms to dsRNA-producing bacteria, as earlier (larval stages) knockdown of some genes may affect worm development. The RNAi screen identified essential genes (those that are not just thermoprotective but generally important genes for general cell functions) such as those involved in signalling (MAPK, WNT, hedgehog like), genes regulated by multiple stresses (heat, pathogens, oxidative), lipid metabolism, development, mitochondria, proteolysis, and transmembrane transport, suggesting that heat stress response is affected by various interlinked pathways.

Several studies have examined changes related to heat stress in humans.

In humans, heat stroke (uncontrolled hyperthermia above 40.1°C) induces a heat shock response through the activation of heat shock proteins, chaperones, and co-chaperone genes. Abdulmalek et al. examined genome-wide transcriptomic changes in patients who experienced heat stroke and compared them with those who did not in the same high-temperature environment. The genes with the highest upregulation were those involved in protecting proteins from misfolding and aggregation, such as IRE1 and PERK, which detect misfolded proteins in the ER, and HSPD1, HSPE1, and HSPA9, which are involved in mitochondrial protein homeostasis. Other differentially expressed genes include those involved in immunity, energy metabolism, oxidative stress, and DNA repair. On the other hand, genes associated with energy production, including oxidative phosphorylation and ATP synthesis, were inhibited (Abdulmalek, N et al., 2023). This suggests that during a heat shock focus is on repair mechanisms, such as protein damage control and the organisms' ability to produce energy declines.

4.3.1 RNAi screen identifies known thermoprotective genes

I first looked for key genes known to impact heat stress resistance, such as heat shock proteins, to see whether they appeared in the WormCat analysis. Only a few of these known heat stress-related genes showed up in the results. This suggests that my inclusion criteria may have been too stringent. Nonetheless, the genes that did meet the threshold are likely to have a strong influence on the heat stress response. This included F44E5.5, *sip-1*, *skn-1* and *dnj-5* chaperone. Knockdown of F44E5.5, *sip-1*, and *dnj-5* sensitised the worms to heat stress which was expected. Whereas, knockdown of *skn-1* increased the median time of death. SKN-1 mediates the antioxidant response through the MAPK pathway by promoting the expression of genes involved in detoxification and stress resistance. The role of SKN-1 is well established in oxidative stress resistance. A study conducted by Frankino et al. showed that activation of SKN-1 increased resistance to oxidative stress but decreased survival under heat stress (Frankino et al., 2022). It could be that RNAi of *skn-1* increases oxidative stress, which results in the upregulation of the mitochondrial unfolded protein response, ultimately protecting the worms against heat stress.

The RNAi knockdown of *sip-1* sensitised the worms to heat stress. *sip-1* is the only HSP exclusively expressed in oocytes and embryos. *sip-1* is not found in larvae but is present in aged adults after their reproductive phase, indicating that the protein is not degraded during ageing. Sip-1 is essential for heat shock survival in reproducing adults and embryos, as knock out of *sip-1* reduces the lifespan of wild-type worms from 21 days to 14 days. After heat shock (37 °C for 1.5 hours), only 12% of *sip-1* mutants survived, compared to a 72% survival rate in wild-type worms (Fleckenstein, T et al., 2015).

I then looked at the RNAi screen results of heat shock proteins. The RNAi of *hsf-1* did not seem to sensitise day 1 adult worms to heat stress. It could be that despite RNAi there is resilience from the system and early adulthood RNAi does not seem to follow the same response as *hsf-1* mutants or it could be that RNAi needs to be done at L1 stage to see the effect. It could also be that on Day 1, the adult worms are in good health, with all their stress pathways functioning well. Therefore, knocking down one stress pathway may upregulate other stress pathways. For example, Kovács et al. showed that day 1 adult *hsf-1* RNAi worms tolerated heat stress (35°C for 6 h) significantly better than control worms, however, this effect was rapidly abolished as the animals grew older. This could be due to the upregulation of compensatory effects mediated by other stress response pathways such as innate immunity and

the unfolded protein response related to ER stress (Kovács et al., 2024). I could expose day 2 *hsf-1* RNAi adult worms to heat shock and compare them with day 1 animals to see if I observe similar results.

On the other hand, the RNAi of *ccar-1* which is involved in the negative regulation of heat shock response showed some protection. CCAR-1 inhibits SIR-2.1, which is a deacetylase that removes acetyl groups from HSF-1. Deacetylation of HSF-1 reduces its DNA-binding ability and limits the activation of stress response genes. Therefore, *ccar-1* RNAi relieves this inhibition, leading to increased HSF-1 activity. As a result, *hsp-70* levels increase, which enhances heat stress resistance (Brunquell et al., 2018).

RNAi of heat shock proteins, *hsp-70, sip-1, hsp-16.11, hsp-12.2, hsp-12.6, hsp-110, hsp-90, dnj-5,* seemed to sensitise the worms to heat stress, whereas RNAi of heat shock proteins *hsp-16.1, hsp-16.2, hsp-16.41, hsp-12.1, hsp-12.3* seemed to protect worms against heat stress. It could be that knock down of some heat shock proteins may be beneficial as it may up-regulate other heat shock proteins. For example, depletion of Hsp90 (RNAi at L1) has shown to upregulate several heat shock proteins, such as HSP-16.2, HSP-16.1, and HSP-16.48, as well as other co-chaperones of Hsp90, such as STI-1 and UNC-45 leading to the induction of the HSR in *C. elegans* body wall muscle and intestinal cells. The RNAi knockdown of Hsp90 has also shown an upregulation of some of the genes involved in the innate immune response, such as Y41C4A.11 and Y94H6A.10 (Eckl et al., 2017) which could be protecting the worms.

hif-1 RNAi improved resistance to heat stress in worms. It has been proposed that at high temperatures *hif-1*activates DAF-16 which promote stress resistance (Leiser et al., 2011).

Autophagy-related genes are upregulated in response to heat stress. *unc-51 (atg-1)* RNAi sensitised the worms to heat stress, but *atg-13, atg-4.1* RNAi provided slight protection to worms from heat stress. It could be that although autophagy is activated during heat stress, prolonged exposure to elevated temperatures causes it to become dysfunctional.

Overall, I found known thermoprotective genes that protect Day 1 adult worms from heat stress, validating this RNAi approach. However, the screening criteria could be slightly adjusted to include more genes in the WormCat analysis.

4.3.2 Innate immune response and heat shock response may be interlinked

The analysis from wormcat results highlighted genes involved in immunity that may be involved in heat stress response. For example, one of the genes that flagged up was the *C. elegans* gene F20G2.5, which is involved in the defence response to gram-negative bacteria and the innate immune response. RNAi knockdown of F20G2.5 sensitised the worms to heat stress suggesting that this is a thermoprotective gene.

Some previous studies have suggested that the intracellular pathogen response (IPR) and the heat shock response may share common mechanisms. IPR is regulated by the *pals-22* (repressor) and *pals-25* (positive regulator) genes. The loss of *pals-22* has been shown to increase thermotolerance and resistance against pathogens (Huang, Y et al., 2021). My results have shown the involvement of several *pals* genes: RNAi of *pals-9* and *pals-30* protected the worms from heat stress, while RNAi of *pals-28* sensitised them, which can be looked at in the context of heat stress.

Exposure to heat stress has also been shown to improve resistance to pathogens. For example, worms exposed to heat shock at 35 °C either 2 hours before or 2 hours after infection with Orsay virus (the only known natural virus that affects *C. elegans*) and given a 1-hour recovery period at 20 °C, showed a lower viral load compared to control (Huang, Y et al., 2021).

C. elegans possess a diverse family of C-type lectin-like domain (CTLD) proteins, encoded by 283 *clec* genes, which are involved in immunity. *Clec* genes are upregulated in response to pathogen exposure (Pees, B et al., 2021). My results showed the involvement of *clec* genes in heat stress. RNAi knockdown of *clec-20*, *clec-37, clec-94, clec-96, clec-130, clec-134, clec-212* and *clec-261* sensitised worms to heat stress, suggesting their thermoprotective role during heat shock.

Together these results suggest a link between innate immune response and heat stress resistance.

4.3.3 Heat stress affects lipid metabolism

Other than its thermoprotective role, HSF-1 plays another important function in lipid homeostasis. It affects lipid balance through the metazoan-specific nuclear hormone receptor NHR-49 (Watterson, A et al., 2022). *C. elegans* has more than 280 nuclear hormone receptors, which is considerably more than humans, who have only 48 (Vohanka, J et al., 2010).

In the presence of intracellular lipids, the post-translational modification of the small G protein RAB-11.1 results in the sequestration of NHR-49 into endocytic vesicles in the cytosol. The depletion of intracellular lipids reduces the de novo synthesis of lipid sensors, preventing the vesicular sequestration of NHR-49. As a result, NHR-49 is translocated to the nucleus.

Once inside the nucleus, NHR-49 activates the *rab-11.2* gene, which regulates endosome recycling and the transcription of β-oxidation genes. This process increases nutrient intake and restores lipid homeostasis (Watterson, A et al., 2022).

RNAi knockdown of *hsf-1* has been shown to reduce triglyceride levels and decrease intestinal lipid droplets in *C. elegans*, possibly due to the upregulation of β-oxidation genes. In contrast, overexpression of *hsf-1* in all tissues results in increased lipid accumulation, suggesting that *hsf-1* plays a role in lipid storage regulation in *C. elegans* (Watterson, A et al., 2022).

Elevated temperature can increase cell membrane fluidity which can compromise cellular integrity. To cope with this cells suppress fatty acid desaturation, leading to a reduced ratio of unsaturated fatty acids which preserves membrane fluidity. This is mediated by FAT enzymes (Zhou et al., 2012). My results have shown that the RNAi of *fat-7* (ortholog of human stearoyl-CoA desaturase) sensitises the worms to heat stress, highlighting the protective role of it during heat stress.

RNAi knockdown of sphingolipid R08F11.1 (ortholog of human glucosylceramidase beta 2) also sensitised the worms to heat stress. This shows the protective role of sphingolipid metabolism during heat stress and could explain why BIGb170 (*sphingobacterium)* improved worm resistance to heat shock. It could be that the bacteria has an impact on sphingolipids, which protects worms during heat stress by contributing to maintaining membrane integrity.

Studies have shown an age-related accumulation of certain lipids. For example, in mice, during normal ageing, there is a progressive increase in the amounts of specific types of sphingomyelin and ceramide in the brain. Similarly, some species of sphingomyelins and ceramides accumulate in abnormally large amounts in Alzheimer's disease. Cutler, R.G., et al. found that inhibiting sphingolipid production increased the lifespan of *C. elegans*. Inhibition of SPT

slowed the development rate and extended the egg-laying period. Conversely, dietary supplementation with palmitoyl-CoA accelerated the development rate, suggesting that sphingolipid production facilitates development (Cutler, R.G et al., 2014).

4.3.4 Mitochondrial function affects heat shock response

My results have identified multiple mitochondrial genes whose RNAi sensitised worms to heat stress, such as *mpc-2*(mitochondrial pyruvate carrier), *mrpl-40* (mitochondrial ribosomal protein L40), and *dars-2* (aspartyl-tRNA synthetase 2) as well as genes whose RNAi improved heat stress resistance, such as *sod-2* (involved in superoxide dismutase activity) and *cox-5A* (an ortholog of human *COX5A*, cytochrome c oxidase subunit 5A), to name a few.

Previous studies have shown that intestine-specific knockdown of *sod*-2 increases resistance to heat stress while decreasing resistance to oxidative stress (Liontis et al., 2014).

Reduced mitochondrial activity is linked with increased lifespan in *C. elegans.* Mitochondria may play a role in restoring heat stress resistance, as mild disruptions to the electron transport chain can influence HSF-1 activity by suppressing the programmed repression of the HSR in early adulthood. In *C. elegans*, there is a significant decline in HSR between day 1 and day 2 of adulthood (Labbadia, J et al., 2017).

Labbadia et al. conducted an RNAi screen to identify genes whose knockdown maintained heat stress resistance in day 2 worms. Day 1 and day 2 worms were exposed to a 33°C heat shock for 6 hours. At day 1, approximately 80% of the animals moved normally 48 hours after the heat shock, whereas on day 2, only 20% retained normal movement (Labbadia, J et al., 2017). The study found that knockdown of F29C4.2 (a cytochrome c oxidase subunit) fully restored stress resistance and increased the expression of mitochondrial unfolded protein response genes, *hsp-6* and *hsp-60*. Although *F29C4.2* RNAi did not induce the basal expression of HSR genes, upon heat shock, the levels of HSP-16 protein were elevated in *F29C4.2* RNAi-treated worms (Labbadia, J et al., 2017).

RNAi targeting subunits of mitochondrial complexes I, III, and IV induced *hsp-6* expression in adult worms. Furthermore, long-lived worms with loss-of-function mutations in mitochondrial genes survived heat shock better than short-lived mutants. These worms also showed increased levels of *hsp-70*, *F44E5.4*, and *hsp-16.11* mRNA, as well as elevated levels of HSP-16 protein compared to wild-type (Labbadia, J et al., 2017). This suggests that RNAi

knockdown of mitochondrial genes may protect worms against heat stress. I have identified *sod-2* and *cox-5A* as genes whose knockdown increases heat stress resistance in worms.

4.3.5 Transmembrane receptor proteins are involved in the induction of heat stress

Seven-transmembrane receptor proteins, also referred to as G-protein-coupled receptors (GPCRs), are expressed on various cell types and play roles in numerous physiological processes. *C. elegans* possess many transmembrane receptor proteins that are involved in chemo sensation, lipid homeostasis, and immunity (Pu, L et al., 2023). The exact number of GPCRs is unknown but predicted to be more than 1300 (Maman, M et al., 2013).

In *C. elegans*, thermosensory neurons sense elevated temperatures and initiate the heat stress response through various, as yet unknown, neuronal receptors. Maman et al. have demonstrated that both chemosensory and thermosensory neurons are involved in the induction of the heat shock response (Maman, M et al., 2013).

Their results showed that GPCR thermal receptor 1 (*gtr-1*), expressed in chemosensory neurons, does not play a role in heat sensing, as (L1) worms treated with *gtr-1* RNAi moved away from both hot and cold regions. However, knockdown of *gtr-1* impaired the induction of the heat stress response downstream of both DAF-16 and HSF-1, indicating that *gtr-1* is specifically required for the induction of the heat stress response. Fluorescent microscopy revealed that *gtr-1* is expressed in neurons of the head ganglia, the ventral cord, and the tail but not in the AFD thermosensory neurons (Maman, M et al., 2013).

Knockdown of *gtr-1* sensitised wild-type and *daf-2* worms to heat stress, suggesting that *gtr-1* is involved in the enhanced stress resistance of *daf-2* worms. However, it did not impact lifespan, indicating that lifespan and stress resistance are separable traits (Maman, M et al., 2013).

The screen has identified several GPCRs, RNAi knockdown of which sensitised the worms to heat stress suggesting that they could be involved in regulating heat shock response. This includes, *str-205* (enriched in the muscle cell), *srh-283, srh-250* and *str-4*.

4.3.6 Uridine diphosphate-glycosyltransferases (UGTs) play a role in heat stress resistance

Glycosylation is the modification of lipids and proteins through the addition of glycans. Glycans are synthesized in the Golgi apparatus and the endoplasmic reticulum, which contain distinct glycosyltransferases responsible for adding or removing sugars to/from the growing oligosaccharide chain (Kellokumpu, S et al., 2016).

Uridine diphosphate-glycosyltransferases (UGTs) are a family of enzymes involved in the second phase of xenobiotic metabolism. C.elegans possess approximately 250 glycosyl-transferases, with the UGT family comprising around 67 genes (Asif, M.Z et al., 2023).

UGTs play a role in immunity and are upregulated during pathogenic exposure. For example, UGTs detoxify 1-HP (1-Hydroxyphenazine), a toxin produced by the pathogenic bacterium *Pseudomonas aeruginosa*, by adding glucose molecules to it. Knockout mutants of *ugt-23* and *ugt-49* are more sensitive to 1-HP compared to the wild type. (Asif, M.Z et al., 2024). My results have shown that knockdown of *ugt-34* and *ugt-40* sensitises worms to heat stress, suggesting that these UGTs play an important role in the heat stress response, possibly by detoxifying byproducts generated during stress.

4.3.7 The nervous system plays an important role in sensing and responding to heat stress

Neuropeptides are signalling molecules that bind to GPCRs to mediate a diverse set of neuroendocrine signalling and extra synaptic communication, such as feeding, sleep, and learning. ALA neurons release neuropeptides, such as NLP-8, NLP-14, and FLP-24, that induce sleep-like behaviour upon heat stress, including quiescence of feeding, locomotion, and defecation (Watteyne, J et al., 2024). My results have shown that the RNAi of neuropeptide-like proteins *nlp-10* and *nlp-32* sensitises the worms to heat stress, suggesting that these neuropeptides could be involved in responding to heat stress.

The cytosolic stress response in the nervous system may be coordinated by glial cells rather than neurons. *C. elegans* have 56 glial cells, which provide neurotransmitters to neurons, participate in synapses, and support neuronal development. Four of the cephalic sheath (CEPsh) glial cells resemble mammalian astrocytes, and the overexpression of *hsf-1* in CEPsh glia has been shown to increase stress resistance not only in wild types but also in *unc-25*

mutants (GABA), *eat-4* (glutamate), *cat-2* (dopamine), and *unc-17* (acetylcholine) mutants (Gildea, H.K et al., 2022). It is possible that a number of neurotransmitters signal the glial HSR or a novel cargo may be responsible for this signalling, Moreover, *hsf-1* overexpression in CEPsh glial cells can also increase resistance to pathogens, suggesting that this pathway is also linked to immunity (Gildea, H.K et al., 2022). Knockdown of several unassigned genes, such as F53F4.13, F53H4.2 (expressed in the amphid and the phasmid sheath cell), and *pals-28* (enriched in dopaminergic neurons and the cephalic sheath cell) sensitised the worms to heat stress.

4.3.8 The mRNA pathway is involved in heat stress response

The microRNA (miRNA) pathway is involved in the heat shock response and may also play a role in recovery after heat shock. Several specific miRNAs have been shown to be up- or downregulated during a heat shock response. For example, deletion of *miR-71* or *miR-246* has been shown to decrease survival during heat shock (Pagliuso, D.C et al., 2021).

The Argonaute-like gene (ALG-1) in *C. elegans* binds to most miRNAs. ALG-1 and miR-85 may be involved in the regulation of HSP-70. *alg-1* mutant worms exposed to heat shock exhibit reduced survival. During a normal heat shock response, HSP-70 mRNA is upregulated and returns to basal levels after 24 hours. However, in *alg-1* mutants, hsp-70 mRNA levels remain elevated compared to the control, suggesting that ALG-1 is required for the downregulation of hsp-70 (Pagliuso, D.C et al., 2021).

Using an miRNA target prediction tool, it was shown that miR-85 has two binding sites in the 3'UTR of hsp-70, suggesting that miR-85 may be involved in its regulation.

In worms lacking miR-85, several stress-responsive genes, including *hsp-70*, were highly overexpressed. Deletion in the hsp-70 3'UTR removes both miR-85 target sites, and worms lacking miR-85 or its target sites overexpress *hsp-70* under normal temperature (20 °C). During heat shock, wild-type and mutant worms exhibit similar *hsp-70* levels. However, during recovery after heat shock, mutants lose the ability to rapidly downregulate *hsp-70* expression, which normally declines after heat shock. This inadequate response to downregulate *hsp-70* levels post-heat shock also reduces worm survival (Pagliuso, D.C et al., 2021).

It is unclear why an excess of *hsp-70* is detrimental to survival, but possible explanations include disruption of protein homeostasis or cellular toxicity resulting from interactions with lipids, leading to aberrant membrane interactions.

On the other hand, the *hsp-70* overexpression observed in *miR-85* mutants did not result in developmental abnormalities, suggesting that *miR-85* is not involved in development but is required for recovery post-heat shock (Pagliuso, D.C et al., 2021).

My results showed that miR-85 RNAi protected the worms from heat stress, possibly by up-regulating hsp-70 during heat shock.

5 Future work

I have identified several beneficial bacteria. This includes MYb71 (*Ochrobactrum vermis)*, MYb330 (*Pseudomonas)*, BIGb170 (*Sphingobacterium multivorum)*, and MYb396(a), MYb21 (*Comamonas)*, as they improved stress resistance without negatively impacting reproduction. Although they slightly decrease lifespan, they do not accelerate movement deterioration, suggesting that while worms may live slightly shorter lives than those fed on OP50, they still lead healthier lives.

However, I found MYb317 and MYb10 to be pathogenic. These bacteria improve stress resistance but halve worm lifespan. The exact reason for this is unclear, but it could be due to several reasons. For example, it could be that they exert beneficial effects during the earlier days of life but later become pathogenic, or they may only be beneficial at lower doses and become toxic as their accumulation increases over time.

On the other hand, the combination of CEent1, MYb21, and MYb71 enhanced heat stress resistance, both individually and together. Logically, the next step will be to use a combination of these bacteria in lifespan and brood size experiments. I have observed that some gut microbes can differentially affect oxidative and heat stress resistance in *C. elegans* through the modulation of IIS and KP pathways. It would be interesting to see the impact of combination of CEent1, MYb21, and MYb71 on KP and IIS mutants' broodsize and lifespan.

After conducting an RNAi screen, I identified some thermoprotective (F44E5.5, *sip-1*, and *dnj-5* chaperone) and thermosensitising gene *skn-1*, already known to be involved in heat stress response. I also found new thermoprotective candidates genes. This includes genes involved in immunity: *clec-20*, *clec-37, clec-94, clec-96, clec-130, clec-134, clec-212* and *clec-261,* F20G2.5, *pals-28,* fat metabolism: sphingolipid R08F11.1 and *fat-7,* mitochondrial function: *mpc-2*(mitochondrial pyruvate carrier), *mrpl-40* (mitochondrial ribosomal protein L40), and *dars-2* (aspartyl-tRNA synthetase 2), GCPRs: *str-205* (enriched in the muscle cell), *srh-283, srh-250* and *str-4,* UGTs: *ugt-34* and *ugt-40*, neuronal function: *acr-14.* I also found sensitising genes, knockdown of which may be beneficial for heat stress. This includes *sod-2*, *cox-5A* and miR-85.

As this was just an initial screening, the next logical step would be to repeat and replicate the experiment with the identified genes to ensure the accuracy of the results. In Addition, RNAi screen needs to be completed by ordering the Vidal RNAi library clones for the genes that are missing in the Ahringer library. Then the genes will be used in conjunction with the beneficial

microbial isolates to determine whether they produce a synergistic response. Possible combination of RNAi can be used followed by gut microbiota composition changes to investigate whether the stress protective mechanisms involved are specific to an *E. coli* diet, and whether they act via the gut-brain axis. This could involve a combined target RNAi (hits from the screen) and gut microbiota cocktails. Then it would be followed by brood size, lifespan, healthspan assays and pathology analyses to identify gene-gut microbiota interactions that maximise healthspan and study mechanistically how they achieve it.

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Abbreviations:

ABC- ATP-binding cassette
ALS2cr12 domain- glycosyltransferase family A and Flagellum Associated Containing Coiled-Coil Domains
AMPK- AMP-activated protein kinase
ARF- ADP-ribosylation factor
ATG- Autophagy related genes
BBSome- Bardet-Biedl Syndrome proteins complex
bHLH- basic-helix-loop-helix
bZIP- Basic leucine zipper
Co A- coenzyme A
COPI- Coat Protein Complex I
CR- calorie restriction
CPR- C-reactive protein
$CaCl_2$ - Calcium chloride
$CaCl_2.2H_2O$ - Calcium chloride dihydrate
CDK- cyclin-dependent kinases
CMA- Chaperone mediated autophagy
Dmsr- dimehtyl sulfoxide reductase regulator
DnaJ domain- N-terminal J-domain
DNMTs- DNA methyltransferases

EIF- Eukaryotic initiation factor

ER- Endoplasmic reticulum

ERGIC- Endoplasmic reticulum-Golgi intermediate compartment

EV- Extracellular vesicles

FGF- fibroblast growth factor

FERM- Four, 1-Ezrin-Radixin-Moesin

hnRNP - Heterogeneous Nuclear Ribonucleoproteins

Hsps- Heat shock proteins

IPTG - Isopropyl β-d-1-thiogalactopyranoside

IFT- Intraflagellar Transport

IIS- Insulin like/IGFR signalling

KP- Kynurenine pathway

KH_2PO_4 - Monopotassium phosphate

K_2HPO_4 - Dipotassium phosphate

Lsm- like sm proteins

LB - Lysogeny Broth

LFASS- Label-Free Automated Survival Scoring

$MgSO_4$ - Magnesium sulphate

$MgSO_4.7H_2O$ - Magnesium sulphate heptahydrate

mTOR- mammalian target of rapamycin

miRNA- MicroRNAs

ncRNA- non-coding RNA

NaCl - sodium chloride

$Na_2KPO_4.2H_20$ - Sodium phosphate dibasic dihydrate

NaOH - Sodium Hydroxide

NaClO - Sodium Hypochlorite

NHR- Nuclear Hormone Receptor

PGI- Protein glutamyl phosphotyrosine-1

UPS- ubiquitin-proteasome system

RBM- RNA binding motif

Rab- Ras-associated binding

Ran- Ras-related nuclear protein

Ras- Rat Sarcoma

RING- Really Interesting New Gene

SIRTs- sirtuins

snRNP- Small Nuclear Ribonucleoprotein Messenger RNA

SNARE- Soluble N-ethylmaleimide-sensitive factor

Sra- Steroid Receptor RNA Activator

Sre- Sterol Regulatory Element

Srab- Sterol Regulatory Element Binding Proteins

Srd- Serpentine receptor class delta

Srg- serpentine receptor class gamma

Sri- signal Recognition Particle Receptor protein

Srsx - Serpentine type 7TM GPCR chemoreceptor

Srt- sprinter

Sru- Serpentine Receptor Uncharacterized

SUMO- Small Ubiquitin-like Modifier

Srv- Simian Retrovirus

Srw- serpentine type

Srx -sulfiredoxin

Srxa- Scavenger Receptor Cysteine-rich domain

SWI/SNF - SWItch/Sucrose Non-Fermentable

T-complex- Tailless complex polypeptide 1 ring complex

ZF- Zinc finger