Investigating how microbiota affects amyloid-beta toxicity in *C. elegans* models.

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Master of Research: Microbiology

Bioscience

Word count: 17290

05/09/2024

Abstract

Alzheimer's disease is a progressive neurodegenerative condition and affects millions of people globally causing symptoms such as memory loss, cognitive decline, and loss of motor skills. The development of Alzheimer's disease is linked to the deposition of amyloid beta plaques in the brain, however no effective prevention or cure exists yet. Emerging evidence suggests that microbiota may influence neurodegeneration, highlighting the potential of microbiota-based interventions, such as probiotics, in mitigating amyloid beta toxicity. However, the mechanisms contributing to this relationship are still poorly understood.

This study aimed to investigate the protective effects of specific microbiota against amyloid beta toxicity using *C. elegans* as a model system for the development of Alzheimer's disease therapeutics. Previous unpublished work by the Ezcurra laboratory showed *Stenotrophomonas terrae* (St1) and MYb57 protects against amyloid beta toxicity in GMC101 *C. elegans.* This study found these findings were generalisable across other strains such as *C. elegans* CL2006 and CL4276.

To ensure there are no detrimental effects on *C. elegans* health by microbiota, development assays were conducted on worm length and L4 development speed when fed on different microbiota. The results showed some microbiota slightly reduced worm size and delayed L4 development, but these effects were minor and unlikely to confound aging or reproductive studies. Additionally, ~400 St1 transposon insertion mutants were generated to identify genes involved in microbial protection against Aβ toxicity, but no specific gene was isolated.

Supplementation assays using vitamin B12 and NAC were performed due to previous studies suggesting they protect against amyloid beta toxicity [1]. However, this study was unable to replicate those findings.

A *C. elegans* strain (unc-54p::Aβ-1-42::unc-54 3'-UTR + mtl-2p::GFP; drp-1(tm1108) IV) was created to assess whether mitochondrial fission (using *drp-1*) is involved in the protection given by St1 and MYb57 against amyloid beta. Whilst initial results suggest *drp-1* is not the pathway responsible, further validation is needed due to limited replicates.

Overall, this research provides insight into the relationship between microbiota and neurodegeneration and highlights the potential of microbiota-based interventions, such as probiotics, in preventing amyloid beta toxicity. Further research of microbiota-host interactions is crucial for developing novel therapeutic strategies for Alzheimer’s disease.

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Chapter 1 Introduction:

1.1 The Microbiota

The microbiota can be defined as an ecological community made of commensal, symbiotic and pathogenic microorganism within an environment or bodily space [2].

These communities are constructed of a variety of organisms such as bacterium, archaea, viruses, parasites, and fungi [3]. These are known to inhabit a handful of locations within and on the human body. Some of these locations include the respiratory tract, oral cavity, genital organs, and the gut [4]. Microbiota have been identified to have an integral role in humans. They have been correlated to major operations in the body such as immune system response, synthesisation of vitamins and amino acids as well as being linked to the breaking down of potential toxic food and neurological function [5].

Research has revealed correlations between the composition of the microbiota and various health conditions, suggesting that shifts in microbiota communities could

have a role in influencing health and disease. Microbiologists became intrigue with understanding more about the different species found in specific locations and how these may function in a way that affect health. As a result of this The National Institutes of Health Human Microbiome Project was launched [6]. The project conducted metagenomic sequencing of gut microbiotas from 124 European individuals from both healthy and inflammatory bowel disease patients to create a microbial gene catalogue [6]. They used comparative analysis between patients and controls, taxonomic profiling, and functional profiling to understand the diversity and functional capabilities of the gut microbiota in health and disease [6]. This project has been running since 2007 and has now completed its secondary stage [6]. It has been significant in discovering and identifying interactions between microbiotas in humans and the related health outcomes observed [6]. For example, during the secondary stage they examined how these host-microbiota interactions can influence inflammatory bowel disease (IBD), prediabetes stressors and the effects on pregnancy and preterm birth [6]. The project found that in IBD patients' beneficial bacterium such as *Faecalibacterium prausnitzii* is decreased whereas species such as *Escherichia coli* are increased, making inflammation worse [6]. They found that *Lactobacillus* is crucial for a healthy vaginal microbiota during pregnancy and that an overgrowth of *Gardnerella vaginalis* can increase the risk of preterm birth [6].

1.1.1 The Fundamental Human Microbiota:

Since each person's microbiota is unique, it is impossible to define a single "healthy" microbiota. Most variations in microbiota do not negatively affect health. These variations can be proportional differences of prevalence of the same microorganism

species and/or different microorganisms found between individuals. A multitude of components can cause these differences; however, diet is thought to be a main contributor [7]. Researchers used 16S rRNA gene sequencing to analyse stool samples from ten participants before, during and after they switched to either an animal-based diet or plant-based diet for five days [7]. They found that there was an increase in bile-tolerant bacteria such as *Alistipes*, *Bilophila*, and *Bacteroides* in the animal-based diet group [7]. Whereas the plant-based diet group had an increase in carbohydrate-fermenting bacteria such as *Roseburia*, *Eubacterium rectale*, and *Ruminococcus bromiil* [7]. As a result, researchers have been working on a fundamental core microbiota based on commonalities between individual that is thought to be linked to normal health in humans or certain health issues. The dominant phyla in the human microbiota linked to health quality were identified as *Bacteroidetes* and *Firmicutes* [8]. Whilst others *Proteobacteria* and *Actinobacteria* were identified, these were in lower abundance [8].

1.1.2 The gut microbiota and health:

The gut microbiota has been identified to interact with a multitude of different health issues as seen in Figure 1. It has been linked to symptom development of those with obesity. One study found that the gut microbiota plays a significant role in regulating fat storage [9]. Germ-free mice had significantly less body fat compared to conventionally raised mice with a normal gut microbiota, despite consuming more food [9]. This suggests that gut microbiota affect host genes involved in energy storage and metabolism, including upregulation of genes related to lipid storage and insulin sensitivity [9]. Using 16S rRNA gene sequencing and metagenomic analysis

in mice model organisms, it was discovered those with obesity had an increase in *Firmicutes* and a decrease in *Bacteroidetes* in the gut microbiota [10].

The potential use of prebiotics in obese children to lower body weight, serum levels of interleukin six, and fat deposition by increasing *Bifidobacterium spp*. and decreasing *Bacteroides vulgatus* prominence was identified [11]. The gut microbiota has been identified as a risk factor for sepsis [12]. Reduced bacterial taxa due to gut microbiota disruption has been shown to allow for colonization by pathogens and alter immune response, increasing sepsis likelihood [12]. Furthermore, the gut microbiota has been linked to colon cancer, rheumatoid arthritis and Alzheimer's disease illustrating how influential the gut microbiota to health [13].

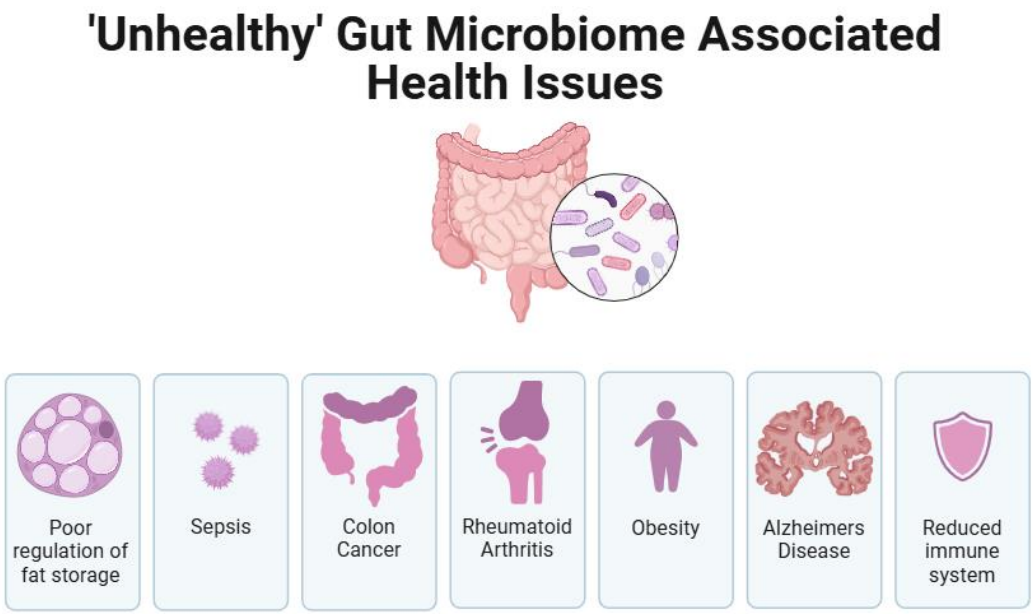

Figure 1: Overview of health issues an 'unhealthy' gut microbiota is associated with. Created using BioRender [14].

1.1.3 The aging gut microbiota

During a human's lifespan, the composition of the gut microbiota changes with microbial diversity becoming more reduced in later life [15]. Pathogenic and opportunistic microbiota has been seen to increase within the gut as aging occurs. A study identified age related changes within the gut microbiota such as the increase of facultative anaerobes and changes of prominence of domain species [16]. Research has shown a decrease in prominence of *Bifidobacteria* and *Lactobacillus* in comparison to younger individuals [17]. The study also found healthy elderly individuals had increased *Bacteroides* diversity and decreased *Bifidobacterial* diversity [17]. This research highlighted the clear change in gut bacteria composition due to aging which could be associated with age-related diseases.

1.1.4 The microbiota as a drug target

Due to the broad volume of genes, the pharmaceutical industry has suggested that the human microbiota could give rise to millions of microbial drug targets [18]. Understanding the gut microbiota could lead to more specific and effective methods of treatments. To develop these microbiota-based therapies the causal mechanisms between the microbe and host needs to be established [19]. However, it is difficult to locate these specific targets that trigger the mode of action due to the extensive number of different microbiota strains [20]. Whilst there is deeper understanding of the microbiota due to projects such as The National Institutes of Health Human Microbiome Project, there is still much to be understood about how these genes, pathways, and compound cause effects [6]. Researching this is crucial as it can

enable for the development of drugs that are more specific and traceable such as probiotics and prebiotics.

1.2 Probiotics and Prebiotics

Prebiotic and probiotics could be used to influence the gut microbiota and create a more beneficial bacterial community for hosts [21].

Prebiotics are non-digestible compounds which that support and promote the growth of desired “healthier” bacterial species improving host health [21]. Prebiotics consist of nutrients such as Oligosaccharide carbohydrates which promotes *Bifidobacterium* as well as *Firmicutes* and *Enterobacteria* [22]. Furthermore, prebiotics contribute to the production of short-chain fatty acids such as lactic acid, butyric acid, and propionic acid which support gut health and metabolism [22].

Probiotics are living organisms such as bacteria (e.g. *Lactobacillus, Bifidobacterium*, and *Streptococcus*) and fungi (e.g. certain *Saccharomyces sp.*) that when in adequate volume can promote gut microbial balance and homeostasis in a way the beneficially affects the host [23] [24]. Probiotics can be found in a variety of forms such as tablets, or in food products such as fermented vegetables and natural yogurts. Whilst these products claim to strengthen the immune system, help digestion, and reduce inflammation, they still pose some risks. For example, probiotics could be detrimental to those with immunodeficiency disorders and therefore should be administered with caution [25]. It is crucial to note the properties of different probiotic species can vary and can be strain specific so the effects cannot be generalised to other species [25].

Recent studies have indicated that probiotics and prebiotics may influence gut health as well as neurological functions through the gut-brain axis. Gut microbiota has been seen to play a crucial role in the regulation of inflammation, metabolic pathways, and immune response, all of which have been linked to neurodegenerative diseases [26]. The imbalance of gut microbial composition, known as dysbiosis, has been linked to the development of increased systemic inflammation and oxidative stress [27]. Both factors are known contributors to Alzheimer's disease [27]. The use of probiotic supplementation has been associated with improvements in cognitive function in Alzheimer's disease patients through the restoration of gut microbiota balance and by reducing neuroinflammatory markers [28]. Probiotic supplementation may influence brain function through the gut-brain axis, with evidence supporting their role in reducing inflammation and oxidative stress which are two key contributors to neurodegenerative diseases such as Alzheimer's disease. By understanding these interactions, new therapeutics for microbiota-targeted strategies for Alzheimer's disease prevention and management can be developed. Whilst microbiota influences several physiological processes, emerging evidence suggests they have a role in neurodegenerative diseases, including Alzheimer's disease.

1.3 Alzheimer's disease:

Alzheimer's disease is a degenerative neurological condition that affects approximately 416 million globally and is an escalating health issue [29]. Alzheimer's disease causes multiple symptoms including memory loss, cognitive decline, and loss of motor skills [30]. This encapsulates those with Alzheimer's disease dementia, prodromal Alzheimer's disease, and preclinical Alzheimer's disease [29]. Due to the

high prevalence of people this disorder affects, there has been a multitude of research conducted into the development and cause of Alzheimer's disease.

Alzheimer's is associated with the accumulation and deposition of amyloid beta into plaques [31]. A secondary factor known as tau was identified to form neurofibrillary tangles in brain cells through deposition [32]. Research using quantitative neuroimaging techniques indicated Alzheimer's disease development originates in the cerebral regions and then expands further into the brain before finally reaching the cerebellum triggering the loss of synapse and neurons [32]. The plaques are neurotoxic and disrupt the communication between neurons and thus interfere with normal cellular functions [33]. As these neurons die, the brain experiences atrophy in the hippocampus and the cortex which are responsible for memory and cognitive function [34].

Through understanding of underlying mechanisms of Alzheimer's disease development of effective therapeutic treatments can be developed. The role of amyloid beta, a key protein associated with Alzheimer's disease progression, has been widely studied. The accumulation of amyloid beta is considered a hallmark of Alzheimer's disease and contributes to synaptic dysfunction, neurotoxicity, and neuronal death. Amyloid beta processing within the brain can significantly impact how the disease develops and progresses [35].

1.3.1 Amyloid beta

The amyloid precursor protein (APP) is a transmembrane protein that regulates synaptic formation, cell growth, and neurite outgrowth [36]. APP can be processed

through two primary pathways known as the non-amyloidogenic or an amyloidogenic pathway as seen in Figure 2 [36].

The non-amyloidogenic pathway causes the APP to be cleaved by α-secretase within the amyloid-beta region, preventing the formation of amyloid beta peptides [36]. This produces a soluble fragment called sAPPα, and a C-terminal fragment called CTFα [36]. These are considered to be non-toxic and potentially neuroprotective [36]. It is believed that this pathway prevents amyloid beta accumulation making it a protective element against Alzheimer's disease [35].

In contrast, the amyloidogenic pathway cleaves APP using β-secretase (BACE1) producing a different C-terminal fragment known as CTFβ, and a soluble fragment known as sAPPβ [36]. The CTFβ fragment is further cleaved by γ-secretase generating amyloid beta peptides, which are primarily the amyloid beta 40 and amyloid beta 42 variety [35]. Amyloid beta 40 and amyloid beta 42 are prone to aggregation which is more hydrophobic and neurotoxic [35]. The accumulation of amyloid beta 42 can form amyloid plaques which have been associated with Alzheimer's disease [35]. Amyloid beta plaques can contribute to neuroinflammation, synaptic dysfunction and neuronal death [35]. These have been associated with the cognitive decline that is observed in Alzheimer's disease patients [35]. This means the balance between APP processing by the non-amyloidogenic pathway and amyloidogenic pathway is crucial for the pathogenesis of Alzheimer's and can determine the risk of plaque formation and disease progression.

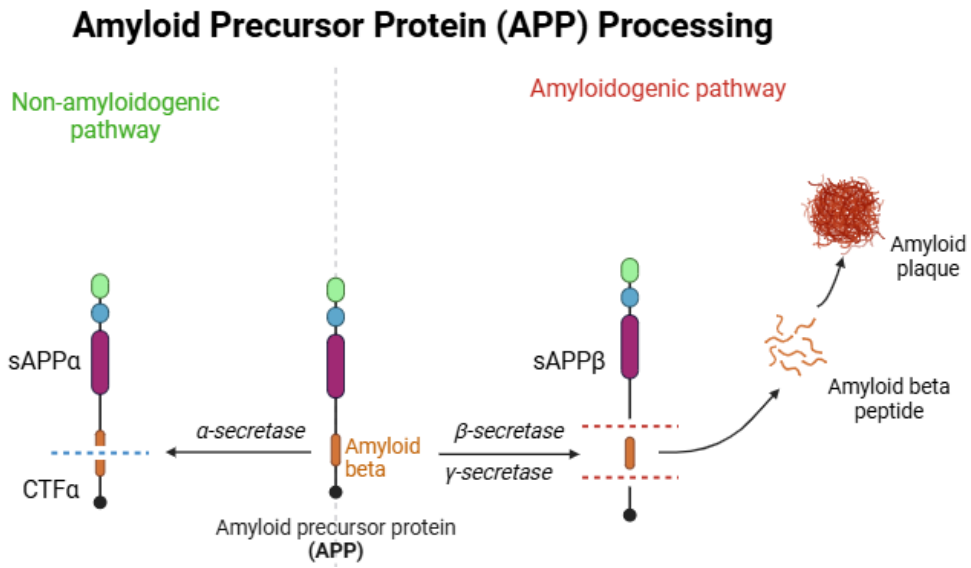

Figure 2: Amyloid precursor protein (APP) undergoing two primary processing sequences (the non-amyloidogenic pathway and the amyloidogenic pathway). Created using BioRender [14].

1.3.2 The Gut Microbiota and Alzheimer's Disease

Research has been conducted linking the gut microbiota and Alzheimer's disease development [37]. An overview of findings can be seen in Table 1. One study was completed by analysing the bacteria present in the gut microbiota of those with normal cognitive function with amyloid beta deposition (pre-clinical Alzheimer's) and those without amyloid deposition [38]. This study did find a discrepancy between the genera of bacteria found in those with the amyloid beta deposition and those without. It was determined that five of the genera found were decreased, and four of the genera were increased between those with amyloid beta deposits vs those without amyloid deposits [38]. *Megamonas*, *Serratia*, *Leptotrichia*, and *Clostridium* (family *Clostridiaceae*) were increased and *CF231*, *Victivallis*, *Enterococcus*, *Mitsuokella*, and *Clostridium* (family *Erysipelotrichaceae*) were decreased in those with amyloid beta deposition [38]. The genera's that were increased in the amyloid beta group

have been associated with the development of Alzheimer's disease. For example, *Leptotrichia* has been linked to Alzheimer's disease through its proinflammatory nature in the saliva microbiota which may trigger the same or similar pathway or response through the intestinal tract [39]. A genus that they identified as decreased is *Enterococcus* [38]. This could be significant since *Enterococcus* species have been linked to anti-inflammatory properties [40]. Therefore, the reduced levels of *Enterococcus* may result in the amyloid beta deposition being increased.

Further research has been conducted reinforcing the concept that the gut microbiota has a link to Alzheimer's development. This study analysed stool samples using bacterial 16S rRNA gene sequencing to determine any microbiota differentiation between those participants with an Alzheimer disease diagnosis and those without [41]. It was found that those who had Alzheimer's disease had decreased diversity and microbial richness in the gut microbiota in comparison to those without Alzheimer's disease and that this correlated with the CSF biomarkers of Alzheimer's disease pathology [41]. Additionally, it was found that those who had Alzheimer's disease had decreased levels of *Bifidobacterium* and *Firmicutes* and increased *Bacteroidetes* [41]. The reduced levels of *Bifidobacterium* in Alzheimer's disease participants may result in pro-inflammatory reactions which may lead to the development of Alzheimer's disease. *Bifidobacterium* has since been used as probiotics since it has the potential to delay this development [42].

Furthermore, a study was completed using probiotics containing Lactobacillus acidophilus, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* [43]. These have been seen to have anti-inflammatory properties [44]. All

participants had Alzheimer's disease and half of these participants were given these probiotics and half were given a control of milk [43]. The mini-mental state examination score was recorded before and after on all participants as well as blood samples to assess the related markers [43]. After 12 weeks intervention, it was found that those who had consumed the probiotics had significant improvements in their mini-mental state examination score [43]. This supports the idea that the gut microbiota plays an important part in developing Alzheimer's disease and using probiotics to help replenish loss of diversity in the gut microbiota could be key to slowing/preventing Alzheimer's disease development.

The bacteria located in the gut microbiota will each activate certain metabolic pathways and produce different metabolites. The presence and absence of these pathways and metabolites is a potential cause for the development of the amyloid beta plaques. Understanding how each pathway and metabolite influences the human body could lead to understanding prevention/treatments for Alzheimer's disease.

There are a multitude of factors and triggers for this accumulation of amyloid beta that have been identified. Some of these are mutations in genes and neurological changes. However, recent investigations have suggested a link between the gut microbiota and the development of this disease. As individuals age, their gut microbiota undergoes transformations influenced by environmental factors, including lifestyle choices, diet, and can be affected by genetic predispositions [45].

Table 1: Summary of gut microbiota findings from numerous studies associated with Alzheimer's disease and cognitive function.

Bacterial Species	**Findings in study**	**Reference**
Leptotrichia	Increased prevalence pre-clinical Alzheimer's disease patients	[38]
Megamonas	Increased prevalence pre-clinical Alzheimer's disease patients	[38]
Serratia	Increased prevalence pre-clinical Alzheimer's disease patients	[38]
Clostridium (family *Clostridiaceae*)	Increased prevalence pre-clinical Alzheimer's disease patients	[38]
Victivallis	Decreased prevalence pre-clinical Alzheimer's disease patients	[38]
Enterococcus	Decreased prevalence pre-clinical Alzheimer's disease patients	[38]
Mitsuokella	Decreased prevalence pre-clinical Alzheimer's disease patients	[38]
Clostridium (family *Erysipelotrichaceae*)	Decreased prevalence pre-clinical Alzheimer's disease patients	[38]
Bifidobacterium	Decreased prevalence in those with Alzheimer's disease	[41]
Firmicutes	Decreased prevalence in those with Alzheimer's disease	[41]
Bacteroidetes	Increased prevalence in those with Alzheimer's disease	[41]
Lactobacillus acidophilus	Probiotic using this significantly improvements mini-mental state examination score after 12 weeks	[44]
Lactobacillus casei	Probiotic using this significantly improvements mini-mental state examination score after 12 weeks	[44]
Lactobacillus fermentum	Probiotic using this significantly improvements mini-mental state examination score after 12 weeks	[44]

1.4 *C. elegans* as a model organism:

Model organisms can provide a powerful experimental opportunity when investigating biological effects of different bacterial species in vivo [46]. Traditional mammalian models can be time consuming, expensive and are biologically complex

in early research when used for investigating the complexity of the microbiota [20]. A common model organism utilised across biological disciplines is the nematode *Caenorhabditis elegans* [47]. Sydney Brenner first identified *C. elegans* as a potential biological model for molecular biology in 1963 [47].

C. elegans are a type of transparent nematode located in soil with diverse bacterial communities [49]. *C. elegans* make effective models for biological research due to their cellular complexity and the conservation of disease pathways [50]. They are advantageous to use due to their short lifespan, easy laboratory cultivation and small size [51]. Since *C. elegans* are self-fertilising, one worm can populate a whole plate with genetically identical progeny [52]. The *C. elegans* genetic code has been well researched, with homologs found for around 60-80% of human protein coding genes [53]. Scientists are easily able to investigate how drugs and compounds may affect humans using this model as they have multiple organs and biochemical pathways without the ethical restrictions of using mammalian models [20].

Through genetic manipulation and due to the nematode being transparent, microbe colonisation and behavioural changes can be monitored easily through a microscope [54]. The *C. elegans* model has been used to examine microbiomics effects on a wide range of disease including Parkinson disease, Huntington's disease, and Muscular Dystrophy [50]. *C. elegans* disease models can be generated through mutant knock out or RNAi knock down of human disease gene homologous to investigate desired phenotypes [55]. They can also be generated by inducing disease-related phenotypes through human gene expression [55]. *C. elegans*

therefore are a powerful model organism in the potential of developing future drug studies to change patient health.

1.4.1 *C. elegans* anatomy and lifespan:

Newly hatched larvae are approximately 0.25mm and will grow to approximately 1mm long [54]. *C. elegans* primarily exist as hermaphrodites (XX) with the probability of males (XO) existing at 0.1% and developing due to a spontaneous loss of an X chromosome [56]. *C. elegans* hermaphrodites are self-fertilising and produce homozygous genetically identical progeny [51]. The *C. elegans* reproductive system is composed of the somatic gonad, their germ line, and the vulva from which eggs are laid [57]. The oocytes are fertilised by sperm located in the spermatheca creating diploid zygotes which are stored in the uterus before being laid [57]. Approximately 300 progenies can be produced by hermaphrodites in the first three/four days of adulthood [57]. However, if a hermaphrodite mates with a male this can increase to 1200-1400 progeny [57]. Males have different anatomy and can be identified by their slimmer body and blunt tail with a fan for mating [58]. *C. elegans* contain an intestine which spans from the mouth to the anus and have muscles, glands, and a nervous system like humans [55].

After a *C. elegans* egg is laid, four larval stages follow known as L1, L2, L3 and L4 after which the nematode develops into an adult as seen in Figure 3 [57]. At 20°C the larval stages take around three days if there is an abundance of food and will survive as adults for approximately three weeks [57]. L1 worms will develop into Dauer

worms if there is a harsh environment as these are more adapted for survival and will develop into L4 worms once favourable conditions has been located [57].

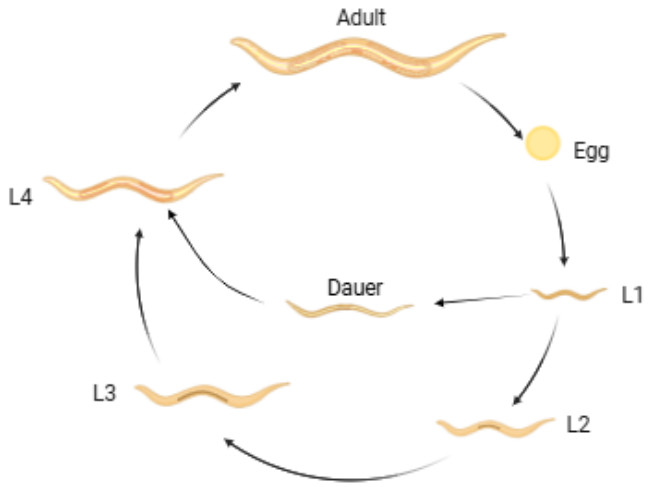

Figure 3: C. elegans Life Cycle. Schematic of the C. elegans life cycle from embryo to the four developmental stages - L1, L2, L3 and L4 - to adulthood including the alternate Dauer pathway. Created using BioRender [14].

1.4.2 *C. elegans* amyloid beta strains:

C. elegans have been a useful model for the investigation of Alzheimer's since transgenic lines were created that were able to produce amyloid beta in the cell walls [59]. Amyloid beta generation can be driven by different conditions in *C. elegans* such as temperature. As the worms are exposed to a certain temperature the amyloid beta accumulation increases, and the rate of paralysis increases. Therefore, *C. elegans* can be given different food sources, compounds, and conditions to see if they influence the paralysis rate caused by the amyloid beta in the cell walls. This can then be extrapolated into potential therapeutic techniques for humans.

For example, a study investigated the potential of silymarin, which is an antioxidant compound, to slow down neurodegenerative disorder progress in age-related diseases such as Alzheimer's disease [60]. This involved using the *C. elegans* CL4176 transgenic model containing the induced expression of amyloid beta protein in muscle tissues [60]. These were expose to temperatures higher than 23°C resulting in the paralysis of the worms [60]. Once treated with silymarin there was observed to be delayed paralysis due to the enhance resistance to oxidative stress, suggesting the potential that this compound has as a preventative measure for Alzheimer's disease [60]. This highlights how these worms can be used in the identification of compounds to aid in the prevention of aging and age-related disease and the benefits they can show.

1.5 Aims and Objectives

The purpose of this research was to explore the relationship between microbiota and amyloid beta toxicity using *C. elegans* as a model organism. Neurological diseases such as Alzheimer's disease are often associated with the accumulation of amyloid beta plaques in the brain, resulting in cognitive decline and other neurological symptoms [61]. There has been emerging evidence that the gut microbiota can influence these processes.

This study examined interactions between microbiota and amyloid beta toxicity in *C. elegans* with the aim to identify protective mechanisms for Alzheimer's disease therapies. Previous unpublished work by the Ezcurra laboratory showed *Stenotrophomonas terrae* (St1) and MYb57 protects against amyloid beta toxicity in GMC101 *C. elegans.* The *C. elegans* strains CL2006 and CL4276 were evaluated

using different microbiota as food to see if protection against amyloid beta by microbiota is broad and generalizable.

To ensure there are no detrimental effects on *C. elegans* health, development assays were conducted on worm length and L4 development speed when fed on different microbiota.

St1 transposon insertion mutants were generated to identify the genes associated with the protection against amyloid beta toxicity by St1.

Supplementation assays using vitamin B12 and NAC were performed due to previous studies suggesting they protect against amyloid beta toxicity [1].

To see whether mitochondrial fission (through *drp-1*) is involved in the protection given by St1 and MYb57 against amyloid beta, a *C. elegans* strain (unc-54p::Aβ-1-42::unc-54 3'-UTR + mtl-2p::GFP; drp-1(tm1108) IV) was created.

This study aims to identify mechanisms by which microbes influence amyloid beta toxicity and reveal any key metabolic or signalling pathways involved. The study conducted aims to contribute to the field of microbiota research and its implications for human health, specifically in developing interventions, such as probiotics or gene therapy, which could modulate the gut microbiota to prevent or treat neurodegenerative diseases like Alzheimer's disease.

Chapter 2 Method:

2.1 *C. elegans* strains genotypes

Five different strains of *C. elegans* were used, N2, GMC101, CL2006, CL4176, and CU6372. N2 is the wild type of *C. elegans* [62]. GMC101 genotype is dvIs100 [unc-54p::Aβ1-42::unc-54 3'UTR] [63]. This means the unc-54 promotor drives Aβ1-42 expression in the body wall muscle cells. The genotype of CL2006 is dvIs2 [pCL12(unc-54::Aβ1-42::unc-54 3'UTR) + pRF4(rol-6(su1006))] [64]. This strain also uses the unc-54 promotor to drive Aβ1-42 expression in the body wall of muscle cells but also contains a rol-6(su1006) marker for selection. CL4176 genotype is dvIs27 [myo-3p::A-Beta (1-42)::let-851 3'UTR) + rol-6(su1006)] [65]. This has the same rol-6(su1006) marker for selection as CL2006, but the myo-3 promotor drives Aβ1-42 expression in the muscle cells. The genotype for CU6372 is drp-1(tm1108) IV, meaning the drp-1 gene has been deleted from chromosome IV [66]. CU6372 *C. elegans* has a deletion mutation causing *dynamin-related protein 1* (*drp-1*) to lose its function. The *drp-1* gene is involved in mediating mitochondrial fission and amyloid beta has been shown to disrupt this mitochondrial function and accelerate neuronal death [67]. These strains were chosen to assess the generalizability of microbial protection across different *C. elegans* Alzheimer's disease models and to explore potential mechanistic pathways such as mitochondrial involvement through *drp-1* deletion.

2.2 Worm maintenance

C. elegans were maintained on Nematode Growth Medium (NGM) plates that were seeded with the standard bacterial food source *E.coli* OP50, as per the standard protocol [68]. The worms were transferred using a platinum wire pick and maintained in incubators. The GMC101, N2 and CU6372 strains were kept in the 20°C incubator to maintain stable growth and gene expression levels. The CL4176 and CL2006 strains were kept in the 15°C incubator to prevent premature amyloid beta expression, which is temperature sensitive. The NGM plates were prepared using the standard protocol [68].

For 1L of NGM 2.5 Bacto TM Peptone, 17g of Agar and 3g of NaCl were topped up with 1L of dH20. This was autoclaved and cooled to 60°C. Once cooled, 25ml of KH_2PO_4 (1 M) and 1ml of cholesterol (12.93mM in ethanol), 1ml of MgSO4 (1 M) and 1 ml of $CaCl \cdot 2H_2O$ (1 M) was added. 10ml of NGM was poured into 6cm plates and dried overnight. These was seeded with the appropriate bacteria as required.

2.3 Synchronisation

The solutions prepared for synchronisation can be found in Table 2. A bleaching technique was used to synchronise and sterilise *C. elegans* for the assays to create uniform age populations. Between six and eight L4 worms were transferred from a maintenance plate to an empty seeded plate (four-five plates prepared). These plates were populated in three/four days and contain a multitude of adults with embryos in their uterus. These plates were washed with 1ml M9 to collect the worms and were transferred into Eppendorf tubes. The Eppendorf tubes were centrifuged

for approximately seven seconds to collect the worms into a pellet. The M9 supernatant was gently removed to avoid disturbing the pellet. 1ml bleaching solution was added to the tubes for two/three minutes, until the bodies started to visibly breakdown under the microscope. These were centrifuged again, and the supernatant removed. The worms were washed with M9, centrifuged and the supernatant discarded. This was repeated twice. The remaining eggs/embryos were resuspended in 1ml M9 and left in the appropriate incubator (15°C or 20°C) to hatch overnight. The L1 worms were centrifuged with approximately 700ul of the supernatant removed to increase the concentration of worms found within a smaller volume of M9. Approximately 200 L1 were placed onto a seeded plate with the food conditions as per the assay requirements.

Table 2: Synchronisation solutions protocols

Solution	Protocol
M9	Dissolve 3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl and 1ml (1M) $MgSO_4$ in H_2O to 1L and autoclave to sterilise
Bleaching solution	3ml bleach (10-15% sodium hypochlorite), 2ml NaOH (5M) and 7ml dH_2O

2.4 Bacterial culturing

Using a sterile loop, a small amount of bacteria (from streaked lysogeny broth agar plate) was transferred into a 15ml falcon tube containing approximately 8ml of lysogeny broth (LB). The bacterial strain details can be found in Table 3. The *Stenotrophomonas* strains including MYb 57 are incubated in a 30°C shaking incubator overnight. The *E. Coli* strains are incubated in a 37°C shaking incubator

overnight. The remaining MYb strains were cultured overnight at 25°C in a static incubator.

Research into wild type *C. elegans* gut microbiota has been completed with bacterial strains being isolated and strains being linked to health and longevity [69]. Using this research, the Ezcurra lab selected 11 species found in the natural microbiota of *C. elegans* and mixed them together to create an Experimental Microbiome. The selected 11 MYb species strain details can be found in Table 3. This Experimental Microbiome enables the effect of a more complex microbiota on health in comparison to the standard OP50 food source to be assessed. The Experimental Microbiome was created by adding 1ml of each LB culture of MYb 9, MYb 10, MYb 11, MYb 27, MYb 45, MYb 56, MYb 57, MYb 71, MYb 83, MYb 120 and MYb 218 to a falcon tube.

NGM plates were seeded with 250µl of the bacterial solution and left to grow over two/three days.

Table 3: Bacterial Strains used abbreviations and species details.

Bacterial ID	Species
OP50	*Escherichia coli*
St1	*Stenotrophomonas terrae*
St2	*Stenotrophomonas ginsengisoli*
St3	*Stenotrophomonas pavanii*
St4	*Stenotrophomonas indicatrix*
St5	*Stenotrophomonas sp. Leaf70*
St6	*Stenotrophomonas bentonitica*
St7	*Stenotrophomonas lactitubi*
MYb 9	*Achromobacter sp. MYb9*
MYb 10	*Acinetobacter sp. MYb10*
MYb 11	*Pseudomonas lurida*
MYb 27	*Brevundimonas sp. MYb27*
MYb 45	*Microbacterium sp. MYb45*
MYb 56	*Bacillus sp. MYb56*
MYb 57	*Stenotrophomonas sp. MYb57*
MYb 71	*Ochrobactrum sp. MYb71*
MYb 83	*Leuconostoc sp. MYb83*
MYb 120	*Chryseobacterium sp. MYb120*
MYb 218	*Pseudomonas sp. MYb218*

2.5 Paralysis

After synchronisation approximately 200 L1's are placed from the M9 solution onto the appropriate food conditions. CL2006 and CL4176 were incubated at 15°C. GMC101, CU6372 and the unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV strain were incubated at 20°C.When these are L4, approximately 110 worms are transferred onto fresh food of the same condition. For CL2006 and CL4276, six plates were set up with two of each food condition (OP50, St1, and MYb57). For each food condition of CL2006 and CL4176 one plate was upshifted to 20°C and one plate was upshifted to 25°C. For the *drp-1* paralysis assay one plate of OP50, St1, MYb57 and The Experimental Microbiome, was for the GMC101 worms and another set was made for the unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV strain. This made eight plates in total for this assay.

These eight plates were upshifted to 25°C. For the full paralysis screen of mutant St1, one plate per mutant St1, one OP50 and one St1 was used and were upshifted to 25°C.

For the full paralysis screening, the number of healthy, paralysed, and dead worms were counted and recorded at each day of the experiment, at the same time. Paralysed animals were identified by the inability to move away when lightly tapped. Paralysed and dead worms were removed from the assay plate to prevent them being counted again on day two. On day two, the original still healthy worms were transferred onto fresh food. This was completed to prevent starving and prevent younger worms that had been hatched from being mistaken as the original L4's and then counted on further days. These worms were counted until day three of the experiment.

2.6 Supplementation

Supplementation assays using Vitamin B12 and NAC were performed due to previous studies suggesting they protect against amyloid beta toxicity [1]. These eight conditions were set up to see if by supplementing OP50 with N-Acetylcysteine (NAC) or Vitamin B12 the protection against amyloid beta paralysis would increase to level provided by St1 or increase St1 protection.

Eight conditions were set up for this assay as seen in Table 4. Vitamin B12 and NAC were diluted in sterile dH_20 for the concentrations as seen in Table 4. The dilutants were then poured and spread evenly onto the seeded NGM food plates as

appropriate and left to dry. These plates then underwent the paralysis assay protocol.

These eight conditions were selected to evaluate the individual and combined effects of Vitamin B12 and NAC on amyloid beta paralysis. OP50 and St1 were used as bacterial food to compare their baseline protective effects, while supplementation with B12 and NAC allowed for assessing whether these compounds could enhance protection against paralysis. Two concentrations of Vitamin B12 were evaluated to determine any dose dependent effects. These supplements were used due their antioxidant properties, which may counteract amyloid beta toxicity. This test ensures a comprehensive analysis of potential protective interactions against amyloid beta.

Table 4: Food types and supplementation concentrations used for plates in assay.

Food	Supplement	Supplement concentration
OP50	N/A	N/A
OP50	B12	1.488nm
OP50	B12	7.44nm
OP50	N-Acetylcysteine	5mM
St1	N/A	N/A
St1	Vitamin B12	1.488nm
St1	Vitamin B12	7.44nm
St1	N-Acetylcysteine	5mM

2.7 Transposon insertion protocol

Reagent protocols can be found in Table 5. First, LB agar plates were prepared. 16g of Agar and 25g of LB were topped up to 1L and autoclaved. Once this cooled to 60°C in a water bath, it was poured into 10cm plates. LB agar with kanamycin plates were prepared by adding 1ml of 50mg/ul kanamycin to the cooled autoclaved LB agar and then poured into 10cm plates. These plates were labelled to differentiate

those containing kanamycin. LB agar + Diaminopimelic acid (DAP) solution plates were prepared by adding 500ul of LB+DAP to an LB agar plate and tilting the plate to spread evenly. These were left to dry.

Table 5: Transposon insertion protocol reagents used and preparation details.

Reagents	Preparation
DAP (plate) 60mM stock Diaminopimelic Acid 11.4mg/ml:	Added 200ul to 20ml LB agar
LB+DAP (liquid) 5mM stock Diaminopimelic Acid 11.4mg/ml:	Added 10mg DAP in 10ml dH_2O (for use 1ml DAP stock solution + 9ml LB and filter sterilise) (final 500uM final concentration)
Isopropyl β-D-1-thiogalactopyranoside (IPTG) Mr= 238.31g/mol	100mM stock e.g. 23.83mg/ml
Blotting paper	Cut Western blot paper into squares of approximately 2cm x approximately 2cm and autoclave

2.7.1 Preparing Strains:

The donor strain pJA1 was streaked from frozen stock onto dried LB agar with dried LB + Diaminopimelic acid (DAP) solution added to the surface of the plate. pJA1 is a DAP-auxotrophic strain of bacteria meaning it requires DAP to grow. This was incubated overnight at 37°C. The recipient strain St1 was streaked from frozen stock onto LB agar and incubated overnight at 30°C. Once these grew, 8ml LB+DAP solution was inoculated with a colony from the donor plate and incubated overnight at 37°C. 8ml LB was inoculated with a colony from the recipient plate and incubated overnight at 30°C.

2.7.2 Conjugation:

8 squares of blotting paper per plate were transferred onto dry LB agar and LB agar + DAP plates. Donor and recipient cultures were centrifuged at 4500rpm for five minutes. These were resuspended at 1/10th volume (800ul). Concentrated donor and recipient cultures were transferred to respective labelled 50ml tubes (reduced chance of contamination pipetting from 50ml tube than from 15ml falcon tube). 50ul-100ul was added as a drop on to the middle of each square of blotting paper for the conditions of DONOR + DAP, RECIPIENT, and DONOR + RECIPIENT + DAP. The plates were incubated at 37°C for one day and then incubated at 30°C for one day.

2.7.3 IPTG Induction:

Using sterile forceps, the squares of blotting paper (with bacteria) from the LB agar plates were transferred into sterile 50ml tubes for donor, receiver, donor + receiver. LB+IPTG was prepared. 0.5ml-1ml LB+IPTG per square of blotting paper was added, e.g. add 3ml to 3-6 squares of blotting paper with conjugated bacteria. This was vortexed for one minute. It was not vortexed for too long as blotting paper would disintegrate. 200ul-1000ul of the culture was plated on to LB agar + Kanamycin (Kan) plates and spread evenly by tilting the plate. These were incubated for two days at 30°C. The plates were removed from the incubator and the control plates were assessed to ensure there was no contamination.

2.7.4 Manual Colony Picking:

LB agar +Kan plates were prepared and divided into approximately 20 squares. The colonies from the mutagenesis plate were restreaked as individual colonies into the

new LB agar + Kan plates (for secondary selection with Kan). These were incubated at 30°C for two days.

2.7.5 Preliminary Paralysis Screen:

Each St1 mutant was inoculated into individual tubes containing 4ml LB + Kan. St1 and OP50 were individually inoculated into tubes with 4ml LB (controls). These were incubated in a shaking incubator for one day at 30°C. Per mutant and control, Two NGM plates were seeded with 250ul of the culture and left to grow for 2-3 days. Approximately 50 L1 GMC101 worms were placed onto each plate and incubated at 20°C. Once the worms reached L4, these plates were shifted to a 25°C incubator. Two days later, the paralysis of the plates was assessed. The worms on control plates were counted and categorised as healthy, paralysed, or dead. The OP50 worms typically paralysed significantly more than the St1. The mutant plates would be tapped lightly against a surface to encourage the worms to move. These would be assessed visually to determine if the mutant plates were OP-like (lots of paralysed worms) or St1- like (small number of paralysed worms). Those that had similar paralysis rates to OP50 were restreaked onto a new LB + Kan plate and incubated at 30°C. These would then undergo a full paralysis screen using GMC101's to check if the protective effect of St1 against paralysis had been lost. If it had these mutants would then be stored and sent off for genome sequencing to see how this mutant is genetically different from the unmutated St1.

2.7.6 Storing Mutants:

Glycerol stocks were set up from St1 mutants. 50ul of the mutant bacteria and 50ul 50% glycerol was added in a 96 well format. This was covered with a foil sheet.

2.8 *drp-1* transgenic strain cross

The lysis solution was created by adding 10µl of proteinase K to 1ml of worm lysis buffer.

2.8.1 Single worm lysis:

25µl of the lysis solution was pipetted into the PCR tube lid. One worm was picked and placed into the solution in the lid. The PCR tube lid was secured onto the tube and centrifuged for a second to bring the solution and the worm to bottom of tube. A microscope was used to check that the worm was in the solution at the bottom of the PCR tube.

2.8.2 Multiple worms' lysis:

The plate with worm was washed with lysis solution with 50µl. The lysis solution that has been used to wash the plate was pipetted back up and 25µl placed into a PCR tube. The PCR lid was secured, and the microscope used to check the worms were in the lysis solution.

2.8.3 Lysis conditions:

The PCR tubes with the lysis solution and worms were placed at -80°C for 30 minutes. After this, the PCR tubes were placed into the PCR machine at 60°C for one hour, then 95°C for 15 minutes then a 12°C hold.

2.8.4 Amyloid beta PCR:

For PCR amplification 1ul of DNA produced from each of the crossed worm lysis's was added to create respective 25 µl reaction mixtures containing OneTaq 2X Master Mix (defrosted on ice), forward primer (CCGACATGACTCAGGATATGAAGT)

[66], reverse primer (CACCATGAGTCCAATGATTGCA) [66], and nuclease-free H_2O. The primers had a concentration of 10 µM of which 0.5 µl was used of each per OneTaq reaction PCR reaction. The positive control used 1µl of GMC101 worm lysis DNA in replace the crossed worm lysis DNA. The negative control used 1ul of N2 worm lysis DNA in replace the crossed worm lysis DNA. After initial 30s denaturation at 94°C, 30 amplification cycles were performed as followed: 30s at 94°C, 20s at 52°C. 20s at 68°C. This was followed with a final five minutes at 68°C and a 12°C hold.

2.8.5 CU6372 PCR:

For PCR amplification 1ul of DNA produced from each of the crossed worm lysis's was added to create respective 25 µl reaction mixtures containing OneTaq 2X Master Mix (defrosted on ice), forward primer (CGAAGGCCAAGTAGACTATC) [67], reverse primer (GAGGTTAAGCCCATGCAATA) [67], and nuclease-free H2O. The primers had a concentration of 10 µM of which 0.5 µl was used of each per OneTaq reaction PCR reaction. The positive control used 1µl of CU6372 worm lysis DNA in replace the crossed worm lysis DNA. The negative control used 1ul of N2 worm lysis DNA in replace the crossed worm lysis DNA. After initial 30s denaturation at 94°C, 30 amplification cycles were performed as followed: 30s at 94°C, 20s at 48°C. Two minutes at 68°C. This was followed with a final five minutes at 68°C and a 12°C hold.

2.8.6 Agarose Gel Electrophoresis:

The 1% agarose gel was created by adding 1g of agarose to 100ml of 1xTAE buffer. This was microwaved with the occasional swirl till there were no crystals left in solution. The solution was cooled using water on the outside of the flask. 10µl of

SYBR Safe (at 10000x) was added to the solution and mixed. This solution was added to the gel tray and clamped at the top and bottom with plastic combs placed inside to create the wells. It was then checked to ensure there were no bubbles. This was left to cool for 20-30 minutes. The plastic combs and clamps were then removed, and the gel was placed into the electrophoresis tank/gel tank. TAE buffer was added to fill above the level of the gel.

2.8.7 Preparing solutions and running Gel Electrophoresis:

5µl of the 1kb DNA ladder was added to 5µl of 6x blue loading dye and transferred into the first and last wells in the gel. 10µl of the PCR solution was added to 5µl of blue loading dye and transferred into the other wells. The gel was run at 110V for 20 minutes (Amyloid beta PCR) or 30 minutes (CU6372 PCR).

2.8.8 Male Worm Generation:

Six L4 GMC101 worms were picked and placed onto a new OP50 NGM plate. 2-3 plates were prepared. These were placed in the 30°C incubator for approximately five hours. These were then move to the 20°C incubator for normal growth. Three to four days afterwards the progeny was assessed for males as they started reaching the L4 stage.

2.8.9 Cross generation:

One CU6372 hermaphrodite was placed with seven/eight GMC101 males on a fresh OP50 NGM plate. Repeat for 5-10 plates. These were assessed every day and once progeny of these was seen, multiple worm lysis was performed using a positive control of GMC101 and a negative control of N2. These underwent amyloid beta

PCR, and the products ran on a gel electrophoresis. Those plates that displayed the 100bp band had four worms selected and placed on individual fresh OP50 plate and incubated again. These suggest these worms are heterozygous or homozygous for amyloid beta. Once these produce progeny, single worm lysis is performed using the same controls as before. Four worms from each plate are selected. These will undergo amyloid beta PCR and be run on the gel electrophoresis. The plates that produced eight 100bp bands were selected and four/five worms picked and placed onto individual fresh OP50. These would be homozygous for amyloid beta. Four worms from the progeny from each plate were selected for single worm lysis with positive controls of CU6372 and negative controls of N2. These then underwent CU6372 PCR and a gel electrophoresis. Those that produced four smaller bands per plate of approximately 1614bp instead of approximately 2020bp were identified to be homozygous of CU6372 and four worms from each of these successful crosses were placed on individual new OP50 plates and maintained.

A paralysis assay was then completed using the new strain with the genotype unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV, GMC101 and CU6372 on the food condition of OP50, St1, M57, and the Experimental Microbiome.

2.9 Sizing & Development

After synchronisation of N2 worms, approximately 200 L1's were placed from the M9 solution onto the appropriate food conditions. The food conditions used for this assay were OP50, M57, St1, St2, St3, St4, St5, St6 and St7. These were then incubated at 20°C. When these were L4, approximately 110 worms were transferred onto fresh

food of the same condition. These worms and food conditions were used for the sizing assay and development assay.

2.9.1 Sizing assay:

The food plate of L4 worms was placed under the WormLab Imaging System with the camera set to the height of seven. The image was focused and 5-10 photos of different sections of the plate were taken to ensure a variety of different worms to be available to measure. ImageJ was used with the segmented line tool to draw along the length of the worm from head to tail. The measure tool was used to size approximately 50 individual worms. This was repeated for all food conditions. This process was repeated for day one adults (D1ad).

2.9.2 Development assay:

2.5% agarose gel was heated in the microwave for 10s. A small dot was added to slide, and a slide was placed on top to flatten agarose gel and make it even. The top slide was removed once the gel had cooled. 4ul of 25mM tetramisole was added to the gel in centre. Approximately 50 L4 N2 worms from one food condition were placed into the tetramisole. A coverslip was added, and the slide labelled with the food condition. This was repeated for the other food conditions. Under a dissecting microscope the worms were recorded as either early l4, mid l4 and late l4 of each worm from one food condition. This was repeated for each food condition. These were determined using vulval staging as seen in Figure 4 [72].

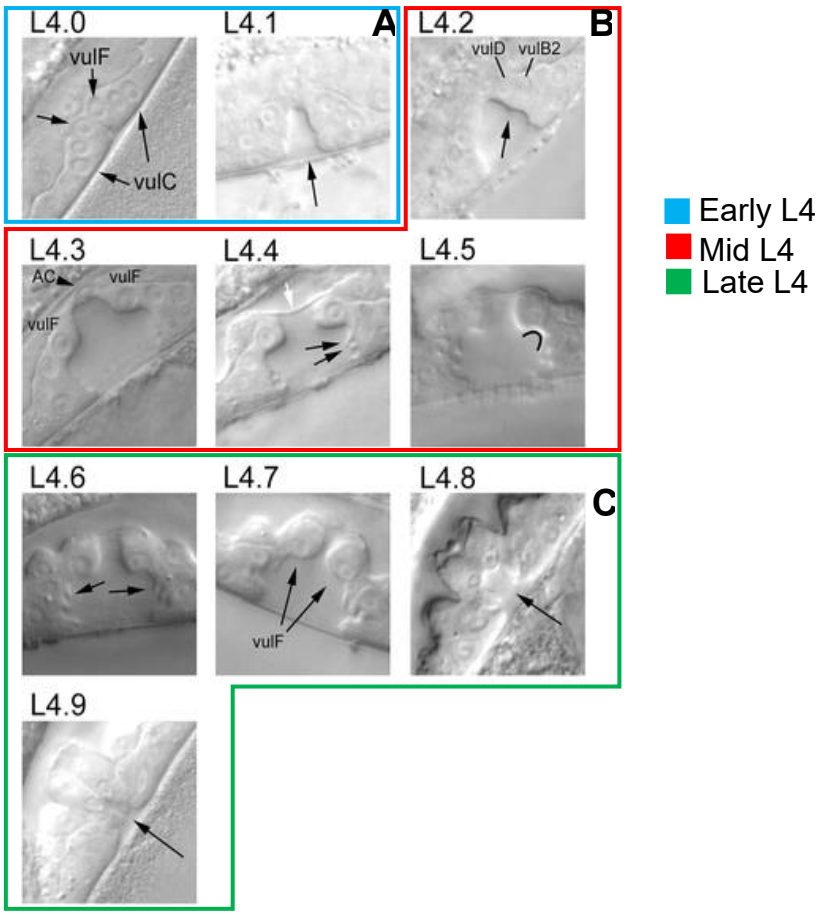

Figure 4: Normaski images of distinct stages of *C. elegans* vulval stages with annotations displaying how these stages were grouped to create the classification used in this study of *C. elegans* development staging [68]. (A) illustrates early L4 vulval development. (B) illustrates mid L4 vulval development. (C) illustrates late L4 vulval development.

2.10 Statistical analysis

The programs used for statistical analysis were Excel and GraphPad Prism.

Statistical analysis was conducted to assess the significance of differences between experimental conditions. A two-way ANOVA was performed to evaluate the effects bacterial diet on paralysis rate, as well as potential interaction effects.

Multiple comparisons tests were performed with either Tukey's multiple comparisons or Dunnett's multiple comparisons. Dunnett's multiple comparisons test was applied when comparing experimental bacterial diets to the OP50 control, whereas Tukey's

multiple comparisons test was used to compare all bacterial food groups to each other. Statistical significance is indicated in figure legends.

All experiments included three biological replicates. The number of worms used per condition per replicate depended on the assay being assessed. Statistical significance was set at $p < 0.05$ unless otherwise specified. Statistical significance is indicated in figure legends. A two-way ANOVA was performed to identify main effects and interactions between bacterial diets and *Aβ* toxicity. Multiple comparison tests ensured statistical robustness.

Chapter 3 Results:

3.1 Different C. elegans paralysis models and protective effects

Previous unpublished research in the Ezcurra laboratory using GMC101 has shown that St1 and MYb57 protect against proteotoxicity, supporting that the microbiota can have a protective effect. To see whether this protective effect is specific to the GMC101 model, other *C. elegans* amyloid beta models were assessed. GMC101 expresses amyloid beta in body-wall muscle using the *unc-54* promotor [63]. CL2006 also expresses amyloid beta using *unc-54,* while CL4176 uses the *myo-3* promotor, which also drives expression in body-wall muscle [64] [65]. These strains of worms were incubated at 15°C and not the typical 20°C as they are known to be more temperature sensitive and will start amyloid beta expression at temperatures above 20°C [73]. Due to this they were upshifted at L4 and to either 20°C or 25°C. Both temperatures were assessed to determine if the

different bacteria have protective effects across *C. elegans* models and temperatures.

For CL2006, worms incubated at 25°C on St1 and MYb57 paralysis were statistically less paralysed ($p < 0.05$, Two-way ANOVA, Tukey's test) than those grown on the OP50 control on D1ad as seen in Figure 5.A. This suggests that the protective effect of St1 and MYb57 transfers across strains and is not strain specific. This is not seen on the remaining days since the worms paralysed faster and were mostly all paralysed by day two adults (D2ad). The CL2006 strain produced high variability in paralysis rates and sometimes paralysed slower, although the trend of St1 and MYb57 worms paralysing slower than OP50 worms still was present.

For CL4176, Three biological replicates were completed. No statistical difference was observed at 25°C between St1 and MYb57 compared to control. However, at 20°C, there was a statistical reduction in paralysis rate of St1 and MYb57 ($p < 0.05$) when compared to OP50 over the three days. As shown in Figure 5.B, St1 and MYb57 produced protection against amyloid beta paralysis, further suggesting that the protective effects of the microbiota against amyloid-beta are not specific to the amyloid-beta model.

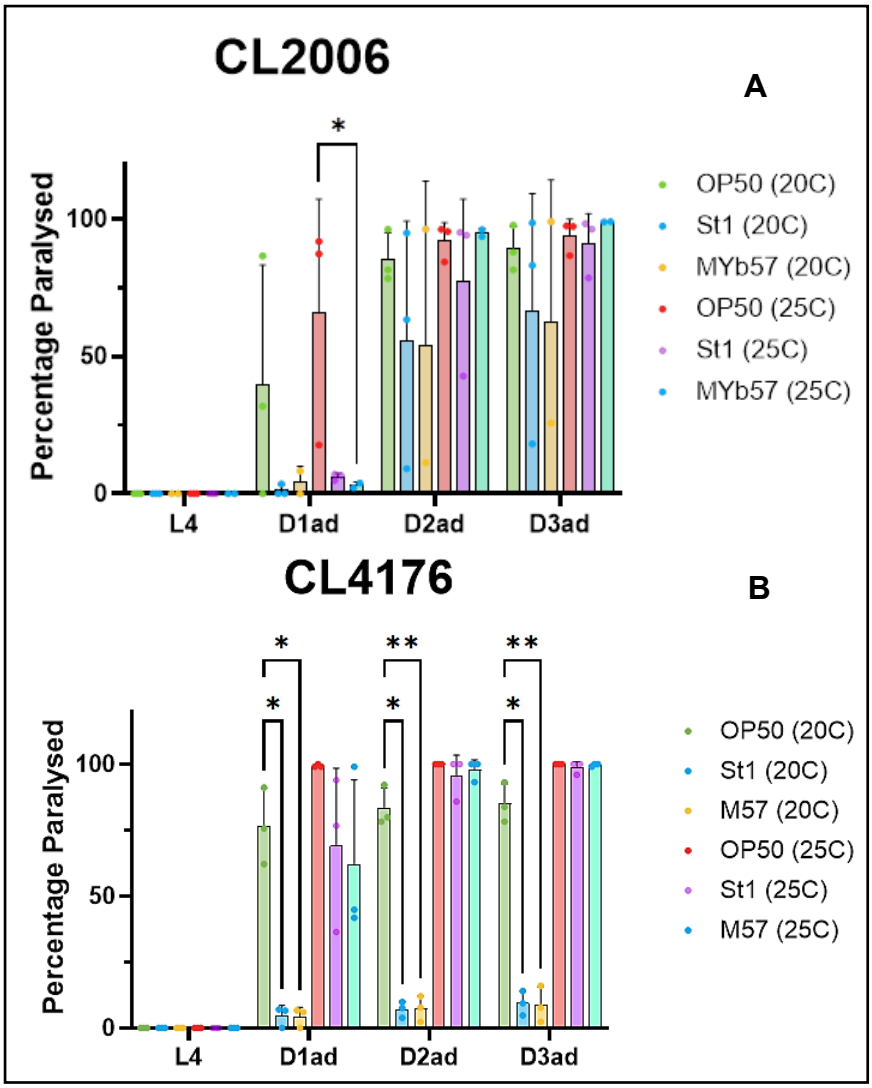

Figure 5: Percentage of paralysed *C. elegans* each day. ~110 worms placed on each food condition (OP50, St1, MYb57) and incubated at either 20°C or 25°C from L4. (A) CL2006 data includes three biological replicates. (B) CL4176 data includes three biological replicates. Asterisk (*) represent statistical significance using two-way ANOVA with Tukey's multiple comparisons test – P-value *. All comparisons were made at corresponding time points relative to the control OP50 *E. coli* diet. P values: *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001. CL2006 Two-way ANOVA: F(6, 8) = 1261, p = <0.0001, n = ~ 110 per replicate per condition (~300 per condition total). CL4176: Two-way ANOVA: F(1.58, 3.16) = 21.42, p = 0.0152, n = ~ 110 per replicate per condition (~300 per condition total). No asterisk indicates no statistical significance.

3.2 Effects of different microbiota diets on C. elegans development

Whilst *C. elegans* are typically a robust organism that can survive of most bacteria, there are some bacteria that will have a detrimental effect on *C. elegans* healthy development [74]. Previously in the Ezcurra lab, research was conducted that showed St1, St2, St3, St4, St5, St6, and St7 produced a protective effect against amyloid beta paralysis in GMC101 *C. elegans*. To test whether these strains had any detrimental effects on N2 *C. elegans* health, a sizing and a developmental assay were completed as per Chapter 2.9.

As seen in Figure 6, some bacterium did not influence the rate of development in N2 *C. elegans* from early L4 to late L4. There was no statistical difference seen between the number of worms that reached each size in comparison with the control (OP50) for those grown on St1, St4, St5 and St7 ($p > 0.1$, Two-way ANOVA). MYb57, St2, St3 and St6 had more early L4 worms than the control ($p < 0.1$, Two-way ANOVA). Additionally, St2 had less worms that were mid L4's ($p > 0.01$, Two-way ANOVA). and late L4 ($p > 0.1$, Two-way ANOVA). Due to the increase in early L4's ($p > 0.001$, Two-way ANOVA). MYb57 also had less late L4's ($p < 0.1$, Two-way ANOVA) than the control but no statistical difference in the number of mid L4 worms ($p > 0.1$, Two-way ANOVA). This suggests that MYb57, St2, St3, and St6 all could lack either nutrients and metabolites needed for healthy growth or produce inhibitor metabolites that have delayed this development.

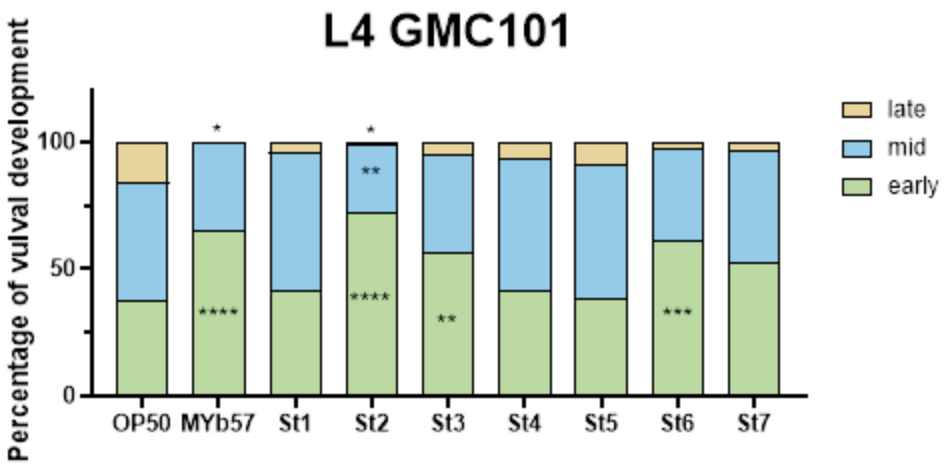

Figure 6: Percentage of GMC101 worms in each L4 development stage using the classification as seen in Figure 2. Each food condition had ~40 worms. Data uses three biological replicates. All worms were incubated in 20°C. Asterisk (*) represent statistical significance using two-way ANOVA with Dunnett's multiple comparisons test comparing to the OP50 control. All comparisons made were based on corresponding vulval stages to the control, OP50 E. coli diet. P values: *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001. Development stage: Two-way ANOVA: F(8, 54) = 6.214e-008, p = >0.9999, n = ~40 worms per replicate per condition. The F-value reported reflects the main effect of food. Interaction effects (*food* × *stage*) were not provided under the selected analysis with Dunnett's multiple comparisons test. No asterisk indicates no statistical significance.

3.3 The effect of different microbiome diets on C. elegans length

The different bacterial food for the *C. elegans* was seen to affect the size of the worms as seen in Figure 7. The worms grown on different food to the control (OP50) worms were seen to be statistical smaller. The effect can be seen from the L4 stage (Figure 7.A) and in Figure 7.B with the worms staying statistically smaller. Whilst some St2 L4 larger worms were the size of the smaller L4 OP50 worms, by D1ad all St2 worms were smaller than OP50. This suggests all these bacteria lack the requirements for normal *C. elegans* growth.

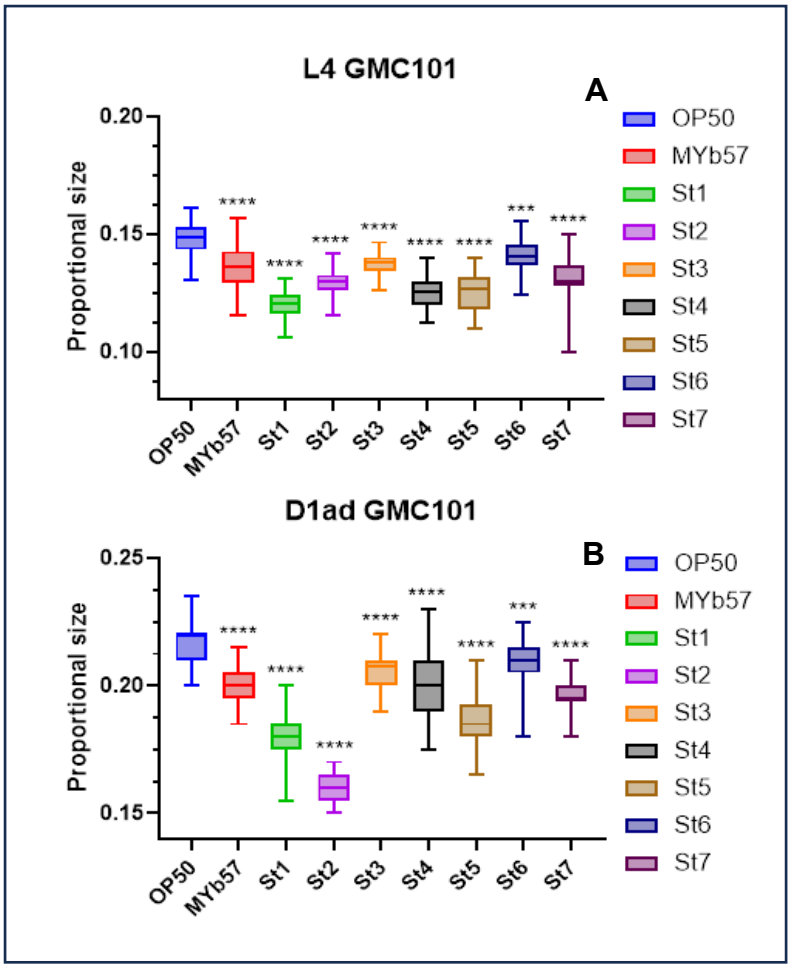

Figure 7: Proportional lengths of GMC101 *C. elegans* that were incubated on different microbiomes using measuring on ImageJ. (A) Worms measured at L4 from each condition. (B) Worms measured at D1ad from each condition. All worms were incubated at 20°C. All images used for ImageJ measuring were taken using WormLab with camera set to height seven. ~50 worms were measured for each food condition. Two biological replicates completed and used. Worms used for L4 sizing were the same worms used for the D1ad sizing. Asterisk (*) represent statistical significance using Mixed-effect analysis test – P-value *. All comparisons made were based on corresponding vulval stages to the control, OP50 E. coli diet. P values: *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001. No asterisk indicates no statistical significance. The mixed-effects analysis followed by Dunnett's multiple comparisons test focuses on pairwise comparisons to the control. Therefore, an overall F-value for fixed or interaction effects is not reported under this analysis.

3.4 St1 mutants generated did not remove protective Stenotrophomonas effect against paralysis in *C. elegans* paralysis model.

To understand how *Stenotrophomonas* protects against amyloid beta toxicity, mutants were generated to knock out gene function in the bacteria. These mutants were generated using transposon insertion protocol and assessed for the desired phenotype as seen in Figure 8. The St1 mutant desired would result in *C. elegans* having reduced protection to amyloid beta toxicity than worms grown on unmutated St1. Approximately 400 mutants St1 underwent preliminary paralysis screening. This involved visually assessing each plate of food condition to determine if the mutant plates were OP-like (high percentage of paralysed worms) or St1- like (low percentage of paralysed worms). However, most of these showed no difference in paralysis protection to the unmutated St1 fed worms as seen in Table 6.

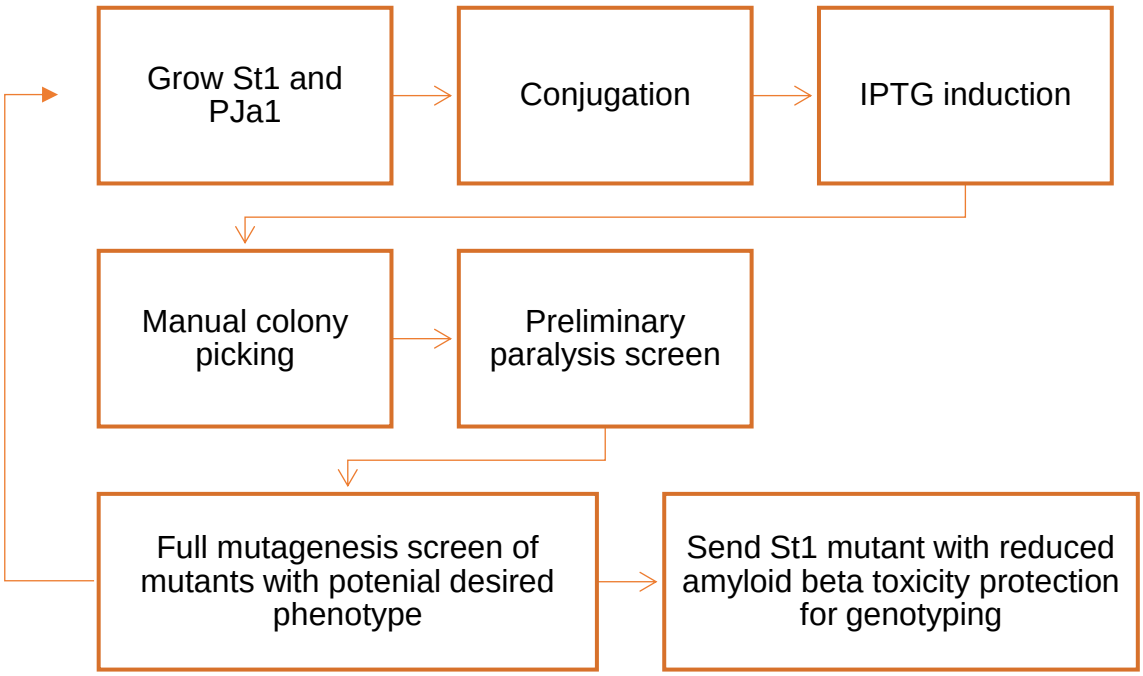

Figure 8: Brief overview of generation of *Stenotrophomonas* mutants with knocked out gene functions and protocol following to assess for desired phenotype.

Table 6: List of mutagenesis batches with how many mutants were isolated. The St1 mutants that displayed reduced amyloid beta toxicity protection in *C. elegans* in comparison to those grown on unmutated St1 can be seen.

Mutagenesis batch no.	Total number of mutants in batch	Mutants that showed reduced amyloid beta paralysis protection
1	40	-
2	30	-
3	21	15,17
4	200	1, 18, 43, 104, 192
5	13	-
6	80	-
7	80	48, 51, 52, 53, 57, 58, 60, 65

The few that initially showed some reduced protection underwent full paralysis screening with three replicates. As seen in Figure 9, the two potential candidates given the number 104 and 192 had no protective significant reduction in protection against amyloid beta toxicity to the unmutated St1 fed worms.

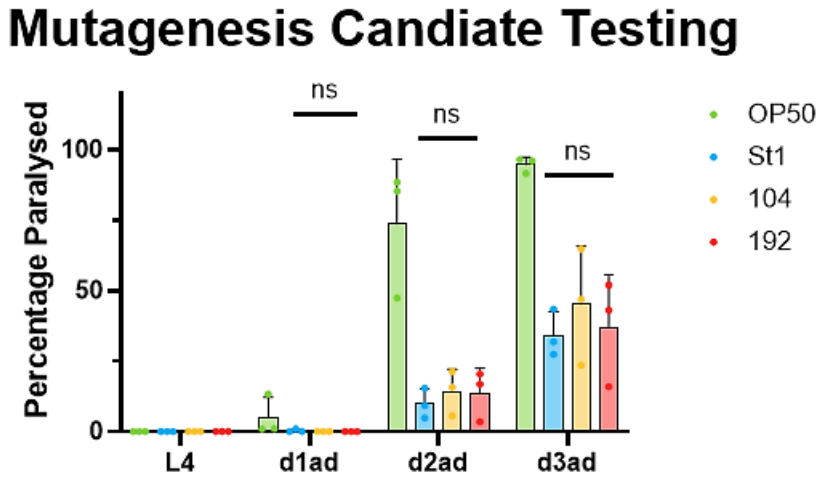

Figure 9: Percentage paralysed GMC101 *C. elegans* populations under different food conditions across days. ~110 L4 worms used per condition. Data uses three biological replicates. OP50 and St1 work as the controls and reference points of

normal protective effects against amyloid beta. 104 and 192 are St1 mutants. NS = non-significant. Mutagenesis candidate testing: Two-way ANOVA: $F(9, 24) = 8.74$, $p = <0.0001$, n = ~110 per replicate per condition.

Chapter 3 Discussion:

This chapter focuses on establishing mechanisms by which the microbiome might affect Alzheimer's disease by using a *C. elegans* model. As well as understanding whether these microbiota effects are specific or whether they have potential to be applied to other scenarios.

3.5 Amyloid beta as a Therapeutic Target for Alzheimer's Disease

To understand how different microbiomes that *C. elegans* are exposed effect proteotoxicity, three different amyloid beta *C. elegans* models were examined. The GMC101, CL2006 and CL4176 strains all produce amyloid beta which is a peptide linked to Alzheimer's disease. By using these models, the influence on amyloid beta toxicity and aggregation can be examined. GMC101 and CL2006 produces amyloid beta using the *unc-54* promotor whereas CL4176 uses the *myo-3* promotor [64] [65]. By testing different strains that use different promoters and contain different mutations the protective effects of microbiomes can be observed, and it can be determined if they are strain specific or generalisable. Furthermore, by testing temperature sensitive strains with the microbiomes we can evaluate the robustness of the protective effect under different physiological conditions.

As seen in Figure 5 St1 and MYb57 showed protective effects against amyloid beta for both the CL2006 and CL4176 strains. Since this protective effect by St1 and MYb57 is seen in different strains including GMC101 despite different promotors for

amyloid beta, it suggests these effects are not strain specific. Furthermore, significant effects were seen at 20°C for CL4176 but not 25°C highlighting the importance of testing at multiple temperatures. Therefore, St1 and MYb57 may produce this protection by either through provision of essential nutrients or reduction of inhibitory compounds that encourage amyloid beta toxicity. Understanding how these microbiotas offer this protection can lead to therapeutics for neurodegenerative disease. Insights are gained on potential microbiota-based treatments for neurodegenerative diseases by understanding the microbiome influence on amyloid beta toxicity. By investigating gut bacterial impact on amyloid beta expression this can be eluded into the gut-brain axis role in Alzheimer's disease. The potential for translation research into human neurodegenerative diseases is increased from this study by comprehensively assessing interactions between *C. elegans,* microbiota and amyloid beta.

3.6 Developmental delays consequences for research

Since bacteria were seen to affect the phenotype that was being investigated, further tests were conducted to assess overall growth and development affects. Normal development is crucial when using *C.* elegans as a biological model as variations in size and development could lead to confounding variables effecting result interpretation. Especially, as there are many assays that are conducted from when *C. elegans* should be L4 as based on OP50 growth. Furthermore, delayed L4 development may influence the reproductive timeline of *C. elegans* and could result in reduced output or lower growth rates [75] This can affect assays using genetics, ageing or reproductivity. A study showed a strain of *C. elegans* that produced smaller

worms also had a reduced lifespan [76]. The reduction in size may be related to poor nutrition which can affect the longevity and influence aging studies. Figure 4 shows worms grown on MYb57, St2, St3, St6 had delayed L4 development. Figure 5 showed that the average worms fed on MYb57, St1, St2, St3, St4, St5, St6, and St7 were smaller. Therefore, these reductions in size and delayed development may highlight further issues in the worm's health. These developmental delays could mean that timings for assays are not technically correct as the timing used are based on worms grown on the standard OP50. However, the effects seen for most microbiota was not dramatic.

3.7 Generation of mutants for targeting genes in microbiota for amyloid beta protection

After deciding that the microbiomes did have some effects on growth and bacterial genes that might affect the phenotype. This was done using transposon insertion of PJa1 into St1. Mutants are created by introducing genome-wide random disruptions of loci into the bacterium [77]. The aim was to find a St1 mutant that loses its protective effect against amyloid beta. This mutant would then be sent off for sequencing. The results of this we would compare to the normal St1 sequence and identify the genetic differences. This would identify the genes/pathway linked to amyloid beta toxicity protection. Despite generating approximately 400 mutants, only a few initially showed promise in the preliminary paralysis screen. The ones that did show promise had no significant difference to the normal St1 protective effect against amyloid beta when three full paralysis screens were completed. Therefore, none of the mutants generated met the criteria for genomic sequencing as the phenotype

desired was not found. This gene that confers this protection may be an essential gene which is why it has not been located. It may be that by knocking out the protective gene, that the mutated St1 is unable to grow. Due to this, a more targeted approach was taken during the rest of the study through supplementation and *C. elegans* strain crosses.

3.8 Pathways and metabolite effects on development

There are critical indicators for *C. elegans* health and physiological fitness. These were examined in Chapter 3.1 and 3.2. These indicators were assessed to see if feeding the worms different bacterial food would negatively or positively impact their health. Reduced size of the worms or delayed development would indicate that necessary nutrition or environmental stresses are now present. For example, it was found that enriching the *C. elegans* diet with glucose shortened their lifespan due to changing the glycerol metabolism [78]. Changing the bacterial food source from the standard OP50 to a different type has also been linked to reduced size of the worms. This suggests that this bacterium may be lacking nutrients or contain inhibitory compounds for the *C. elegans* to grow [75].

One study explains how due to environmental stressors such as lack of nutrients, *C.* elegans can enter three diapause stages [74]. These are L1 arrest, Dauer diapause and adult reproductive diapause. They explained how *C.* elegans will alter their developmental rate to enhance fitness in environmental stress and ensure survival. *C. elegans* require a wide range of nutrients to develop normally including lipids, carbohydrates, and proteins [79]. This might explain the reduction in size and delayed development seen in the worms in Chapter 3.1 and 3.2.

3.9 Implications of neurodegenerative disease research

Understanding how different metabolites affect amyloid beta can lead to potential treatment or understanding of Alzheimer's. Using *C. elegans* these properties can be examined. One study found the metabolite resveratrol reduces amyloid beta induced toxicity in a *C. elegans* model [80]. It was determined that it reduces aggregated amyloid beta by targeting specific proteins in proteostasis [80]. It is important to assess these metabolites and bacterium on a biological model as whilst they may have beneficial effects against amyloid beta, they could have detrimental effects on other health aspects. For example, smaller or slowed development worms may indicate that the metabolites or nutrients being evaluated could cause stress or toxicity. Furthermore, the interactions between *C. elegans* and microbiomes may give greater understanding if gut health and its influences on neurodegenerative disease.

3.10 Strengths and weaknesses

Overall, this study showed the potential development variations that different microbiota can have on *C. elegans*. By assessing multiple aspect, including L4 development and length, a holistic and comprehensive view of different microbiome effects is created. This is an essential component to understand as it can affect future assays and ignoring it could offer confounding variables. By using different strains such as CL2006 and CL4176, it enables the generalisability of the results to be increased and allows a broader perspective of impacts of microbiomes. The use WormLab allows reproducible data since the method was standardise and allowed for accurate measurements of worms since they could be measured from images.

Furthermore, the use of ImageJ ensured accurate measurements as segmented lines could be drawn along the body wall and measured using their tool.

However, this study only has two replicates of the CL2006 assay which affects the statistical value. Due to this strain producing such variability more replicates are necessary to check whether these results generate are accurate and reproducible. A further weakness is that whilst categories were created for vulval development using the stages identified, categorising the worms is subjective [72]. Determining what categories, the worms belong in can be difficult when they are about to close to reaching the next stage.

Finally, the use of transposon insertion has the potential to find the gene/pathway associated with St1 quickly since it has the potential to probe the entire genome of the microbiome [81]. However, this can result in the phenotype you desire not appearing as the insertion may not occur in the genomic region that causes this protection.

3.11 Future Research

In the future, more assays could be tested using the nine food conditions seen in Chapter 3.1. These assays could be lifespan or a brood size assay. A lifespan assay could inform whether these microbiotas have any toxic effects in worm health and a brood size assay would determine if reproduction is increased or decreased. These would give more information about how these microbiotas affect the health and development of the worms grown on these conditions. Further experiments could be done evaluating the protective effects of St1 and MYb57 on more *C. elegans* strains.

For example, LSD1091 strain could be used [82]. In this strain the amyloid beta is secreted into the extracellular space unlike the strains in this study where it was in the body wall [82]. This would determine if these microbiota effects were able to influence amyloid beta paralysis if it is in a different region. Generation of mutants through transposon insertion could be continued to find the gene/pathway responsible for the protective phenotype.

3.8 Summary

This chapter focused on understanding how different microbiomes that *C. elegans* are exposed to effect proteotoxicity. St1 and MYb57 induced protective effects against amyloid beta paralysis across different *C. elegans* strains, suggesting these effects are not strain specific or promotor specific. Testing at different temperatures for the CL2006 and CL4176 strains highlighted the importance of temperatures conditions as the results significantly differed in temperature conditions. Different microbiotas were found to reduce size and delay L4 development in N2 *C. elegans.* If this effect had been more dramatic it could have introduced confounding variables if not considered in assays such as aging and reproduction. These delays may be due to nutritional deficiencies or environmental stressors. This highlights the importance of specific nutrients and how inhibitory compounds in bacterium can impact development in worms. Understanding how these metabolites influence amyloid beta is crucial for therapeutics for Alzheimer's. The pathways and genes involved in the amyloid beta protection were attempted to be identified through transposon insertion of PJa1 into St1. However, despite approximately 400 mutants, none lost their protective effect meaning the genes/pathways responsible for the

protection did not get disrupted. This study had a comprehensive view by examining various aspects that define *C. elegans* health such as vulval development and length. However, some of the assays has limited replicates reducing accuracy. Further research into lifespan and brood size would enhance understanding on microbiota effects on *C. elegans* health. Finally, assessing more strains of *C.* elegans with amyloid beta being produced in different regions would increase the applicability of preserved protective effects seen to be produced by St1 and MYb57 against amyloid beta paralysis. To target the genes and pathways responsible for the protective effects seen by different microbiota in *C. elegans* beta amyloid models, a more direct approach was taken during the rest of the study using supplementation and *C. elegans* strain crosses.

Chapter 4 Results:

4.1 Vitamin B12 and NAC did not produce a protective paralysis effect in C. elegans on an OP50 diet.

Assays with supplementation of vitamin B12 and NAC were performed as previous literature has shown it to be protective against amyloid beta toxicity [1]. The hypothesis was that St1 may protect by producing these molecules. One study showed that low levels of vitamin B12 can result in progressive cognitive impairment [83]. Vitamin B12 is needed for synthesis of DNA, red blood cell formation, maintaining nervous system health, homocysteine metabolism and energy production [84]. N-acetylcysteine (NAC) has been used in treatments in mice with intracerebroventricular applications of Aβ and improved memory and learning [85]. NAC is an antioxidant and is anti-inflammatory [86].

These were selected as potential metabolites of interest that St1 may produce causing the protective effect against amyloid beta and were supplemented onto GMC101 worms' food. As shown in Figure 10 the supplementation of these did not have any significant effects on paralysis rates for those grown on the St1 food throughout the duration of the assay ($p > 0.05$, Two-way ANOVA, Dunnett's test). However, on day three we observed a non-significant reduction in paralysis in the worms on the St1 supplemented food in comparison to those on the non-supplemented St1 food. Therefore, the supplemented B12 and NAC may have reduced paralysis rates past d3ad and may be worth investigating. Whilst OP50 + 7.44nM B12 significantly reduced paralysis on d1ad worms in comparison to supplemented OP50, this effect was lost in the days after.

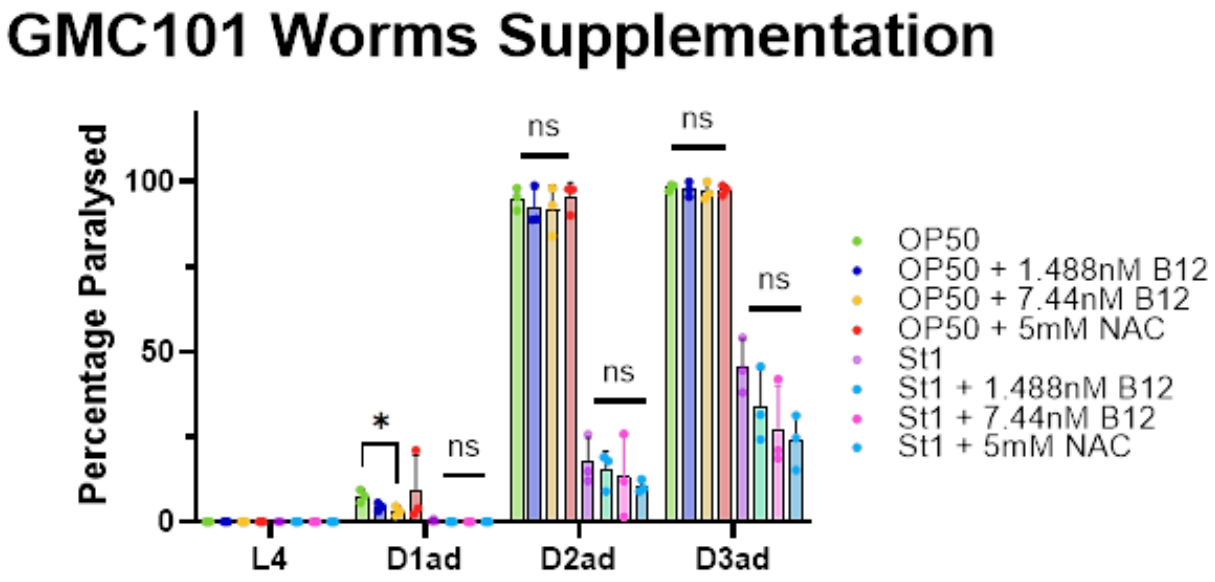

Figure 10: Percentage of paralysed GMC101 *C. elegans* each day. ~110 worms placed on each food condition (OP50, OP50 + 1.488nM B12, OP50 + 7.44nM B12, OP50 + 5mM N-acetylcysteine (NAC), St1, St1 + 1.488nM B12, St1 + 7.44nM B12, and St1 + 5mM NAC) and incubated at 25°C from L4. Data uses three biological replicates. (B) CL4176 data uses three biological replicates. Asterisk (*) represent statistical significance using two-way ANOVA with Tukey's multiple comparisons test – P-value *. All OP50 conditions were compared to the OP50 with no supplementation. All St1 conditions were compared to the St1 with no supplementation. P values: *p<0.1. Ns = no statistical significance. GMC101 worm supplementation: Two-way ANOVA: F(21, 56) = 75.28, p = <0.0001, n = ~110 per replicate per condition.

4.2 Knocking out *drp-1* gene function does not remove *Stenotrophomonas terrae*, MYb57 and Experimental Microbiome protection against amyloid beta toxicity.

A targeted approach to knock out a gene function was used to understand how microbiota protects against amyloid beta toxicity. CU6372 *C. elegans* has a deletion mutation causing *dynamin-related protein 1* (*drp-1*) to lose its function. The *drp-1* gene participates in mediating mitochondrial fission and amyloid beta has been shown to disrupt this mitochondrial function and accelerate neuronal death [71].

Mitochondria are dynamic organelles that undergo fission and fusion which are essential for maintaining mitochondrial function, distribution, and quality control in cells. During cell division *drp-1* ensures that the mitochondria are correctly distributed to daughter cells [88]. Furthermore, cellular stress can lead to *drp-1* activity causing mitochondrial fragmentation [88]. This can maintain cellular health as it can help to isolate damage mitochondrial components for degradation [88]. The *drp-1* gene creates this mitochondrial fragmentation by promoting mitochondrial fission by wrapping around and constricting the mitochondrion leading to the organelle being divided into two smaller mitochondria [85].

St1, MYb57 and the Experimental Microbiome may protect against proteotoxicity by altering mitochondrial dynamics. By crossing the *drp-1* mutant with GMC101 the effect of this genes function in relation to amyloid beta toxicity could be established. The *drp-1* gene was selected due to previous unpublished work in the Ezcurra laboratory showing that the Experimental Microbiome induced changes in the mitochondrial dynamics in the body-wall muscle. If this gene is responsible for the protective effect by St1, MYb57 and the Experimental Microbiome against amyloid beta toxicity in *C. elegans*, the unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV strain would be expected to lose protection as the gene does not function. As shown in Figure 11 the lack of *drp-1* function in the unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV strain does not appear to have any difference on St1 protection in comparison to GMC101 St1 worms' protection ($p < 0.05$, Two-way ANOVA). Additionally, it does not seem to affect the paralysis rate in MYb57 or Experimental Microbiome worms ($p < 0.05$, Two-way ANOVA). More

assays need to be completed to verify the accuracy and reproducibility of these results as this data is only from one biological replicate. Since there was only one biological replicate, statistical analysis was not performed.

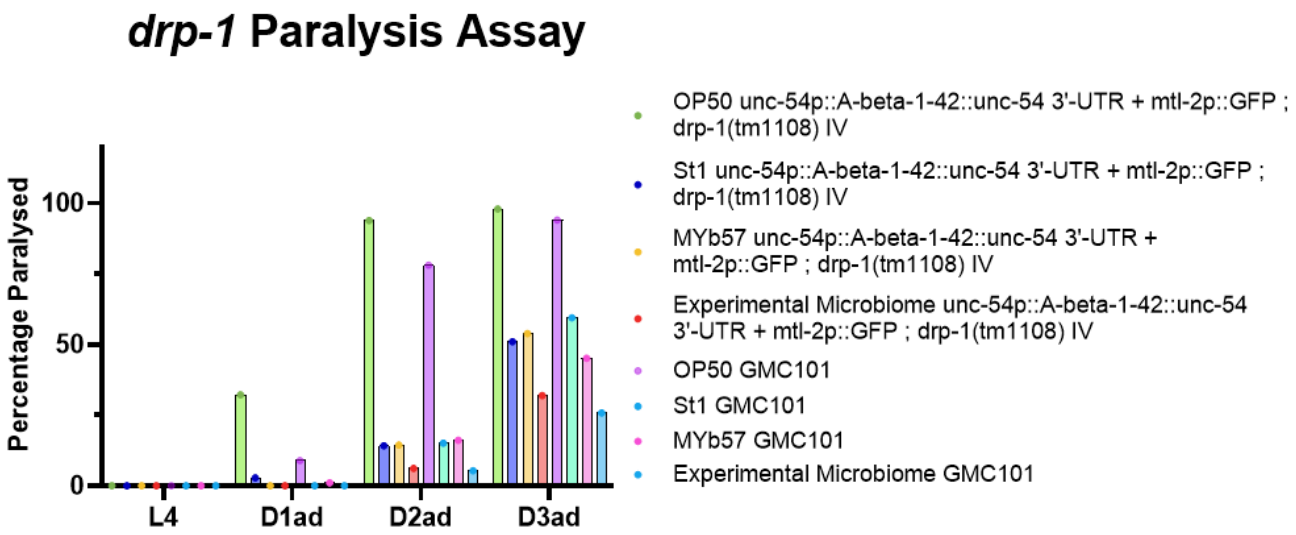

Figure 11: Percentage of paralysed *C. elegans* each day. unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV *C. elegans* were assessed as well as normal GMC101 *C. elegans*. ~110 worms placed on each food condition (OP50, St1, MYb57, and the Experimental Microbiome). Worms incubated at 25°C from L4. One replicate. No statistical analysis due to only one replicate.

Chapter 4 Discussion

Chapter 4 focuses on a hypothesis-driven approach to understand how microbiota protects against amyloid beta toxicity. This is accomplished using a strain of *C. elegans* with mutations that knock out gene function and by supplementing different metabolic pathways.

4.3 Microbiomics pathways effect on Alzheimer's disease

By using supplementations of metabolites, it can be assessed whether St1, MYb57 and the Experimental Microbiome produce these inducing the protection against amyloid beta toxicity. In Chapter 4.2, the protective effect seen in the reference study

was unable to be replicated and both vitamin B12 and NAC were not seen to have significant on paralysis rates due to amyloid beta toxicity in this assay [1]. This could be due to different laboratory conditions and protocol variations. Whilst these did not reduce paralysis rate in this assay, St1 d3ad that were supplemented did seem to start to differentiate more from the supplemented St1 paralysis rates. Increasing the duration of this assay may show vitamin B12 and NAC to slow down amyloid beta toxicity longer term. In a longitudinal study looking at Vitamin B12, Folate, and Sulphur Amino Acids levels and Brain Magnetic Resonance Imaging Measures in older adults, lower Vitamin B12 was associated with reduced brain volume and increased white matter lesions [90]. Furthermore, higher Homocysteine levels, often due to lower B12 and folate, were linked to greater brain atrophy and more white matter damage [90]. The study suggests that maintaining adequate vitamin B12 and folate levels is crucial for brain health in older adults, potentially reducing the risk of Alzheimer's disease by preventing brain atrophy and white matter damage [90].

There are many different metabolites that can be supplemented. It was found that by supplementing betaine, homocysteine levels were reduced in *C. elegans* as well as amyloid beta toxicity, highlighting the potential of supplementation for reducing amyloid beta toxicity [91]. Supplementing metabolites could identify any metabolites produced by St1 inducing protection against amyloid beta and could lead to therapeutic treatments of Alzheimer’s disease.

4.4 Exploring gene functions influencing Alzheimer's disease.

St1 has been shown to have a protective effect against amyloid beta in *C. elegans*. By identifying the genes in St1 which produce this effect, biological insights into

Alzheimer's development can be gained. As seen in Chapter 4.1, *C. elegans* that lost *drp-1* function did not alter the rate of paralysis of *C. elegans* grown on St1, MYb57 and the Experimental Microbiome. This suggests that mitochondrial fission may not the pathway responsible for the protective effect against amyloid beta toxicity given by these bacteria. Research into these pathways can provide insight and understanding on preventions and therapeutics for Alzheimer disease. Despite this, it was found that in Alzheimer's disease, one major mitochondrial abnormality is a reduction in mitochondrial energy production [92]. This leads to decreases ATP levels in neurons which is critical for synaptic function and plasticity maintenance [92]. Therefore, the energy reduction can accelerate neuronal death and impair cognitive functions.

Additionally, studies have highlighted that the balance between the mitochondrial fission and fusion process is crucial for maintaining mitochondrial function and integrity and Alzheimer's has been seen to disrupt this [93]. The dysregulation of *drp-1* can lead to excessive mitochondrial fragmentations which has been linked to synaptic loss and neuronal death [94].

The mitochondrial fission pathway did not appear to be linked in the protective effect given by St1 to *C. elegans* against amyloid beta toxicity within this assay. Identifying the genetic pathway responsible for this protective effect induced by St1 could improve diagnosis and predictability of Alzheimer's through biomarkers. It could also lead to therapeutic treatments of Alzheimer's disease through drug treatments or gene therapy.

4.5 Microbiota effects and therapies for Alzheimer's disease

There are many different microbiome targeted therapies for Alzheimer's disease [95]. Some of these include the use of probiotics, prebiotics, symbiotic, postbiotics and faecal microbiota transplantation [95]. A study transplanting faecal microbiota from Alzheimer's patients into microbiota-depleted rats found that the gut microbiota does influence Alzheimer's generation [96]. The rats developed impairments in behaviours related to the hippocampal neurogenesis and these impairments reflected the cognitive scores of Alzheimer's patients [96]. This confirms that alterations to the gut microbiota can contribute to Alzheimer's symptoms [96].

Faecal microbiota transplantation has been seen to improve symptoms of Alzheimer's disease [97]. This was seen in an 82-year-old man with Alzheimer's disease and recurrent *Clostridioides difficile* infection [97]. Once the patient underwent this transplantation using stool from his healthy wife, within two months his cognitive function improved, and his Mini-Mental State Examination score rose [97]. This highlights how the gut microbiota can influence Alzheimer's disease.

A meta-analysis assessing animal studies and the role of probiotics and prebiotics in reducing neurodegenerative disease found that those that had dietary supplementation of probiotics had improved cognitive function [98].

4.6 Strengths and weaknesses

The vitamin B12 and NAC supplementation paralysis assay used three biological replicates increasing the reproducibility of this study. Any effects that vitamin B12 could have produced was comprehensively analysed as two different concentrations were assessed. However, even the higher concentration of vitamin B12 was unable

to induce the protective amyloid beta toxicity effect in *C. elegans* grown on OP50. This assay duration could have been extended further to get a better understanding on its effects of the supplementation onto St1 grown *C. elegans* since most were still unparalysed by D3ad.

More biological replicates need to be completed for the *drp-1* cross paralysis assay. Whilst the initial results do not indicate that the *drp-1* pathway is activated in St1 amyloid beta protection, more replicates are required to confirm. More replicates would allow for statistical analysis to compare those with a functioning *drp-1* gene and those without.

4.7 Future Research

Completing more amyloid beta paralysis assays with different crossed *C. elegans* strains with mutations could lead to identifying the gene responsible for protection by St1, MYb57 and the Experimental Microbiome. This could identify new drug treatments for Alzheimer's disease. More supplementations of different metabolites could be continued to identify this protective pathway triggered by St1. Understanding how St1 induces protection from Alzheimer's is crucial for developing better treatments and therapeutics.

4.8 Summary

This study investigated whether the *drp-1* gene, which participates in mitochondrial fission, is responsible for the protective effect of *Stenotrophomonas* against amyloid beta toxicity in *C. elegans*. CU6372 *C. elegans* with a nonfunctional *drp-1* gene was crossed with GMC101's which expressed amyloid beta. The paralysis rates were

assessed, and the loss of *drp-1* function did not affect the protective effects provided by *Stenotrophomonas* suggesting this gene is not responsible for the protection. The research aimed to identify the genes involved in protection against amyloid beta toxicity by microbiota which would provide insights into Alzheimer's disease mechanisms. Assessing other genes may identify these pathways since mitochondrial fission did not seem to be involved.

Vitamin B12 and NAC supplementation was explored to see if they would replicate the protective effects given by St1 against amyloid beta toxicity in OP50. However, these did not reduce the rate of paralysis suggesting these are not the pathways St1 exerts its protective effect. However, an increased duration of this assay could be done to see if paralysis rates are affected longer term by these supplements onto St1 as a minor difference was seen on day three in comparison to the control with no supplementation. This metabolite supplementation was used to identify the St1 derived compound that protects against amyloid beta toxicity. Despite vitamin B12 and NAC being ineffective in reducing paralysis, there are more supplementations that can be performed to find any potential metabolites produced by St1 for amyloid beta toxicity protection.

Further research could identify how St1 triggers these protective effects against amyloid beta toxicity. More *C. elegans* gene knock out strains identify the genes associated with the protective effect. Further metabolic supplementation could help determine the metabolites produced for protection. These could potentially lead to a better understanding of Alzheimer's and lead to more diagnostic and therapeutic treatments.

Chapter 5 Conclusion:

This study explored interactions of bacterial species and amyloid beta toxicity in *C. elegans* with a focus of identifying protective mechanisms that could be used in developing therapeutic strategies for Alzheimer's disease. First, due to previous unpublished work in the Ezcurra laboratory showing St1 and MYb57 protects against amyloid beta toxicity in GMC101 *C. elegans*, assays were run to see if these microbiotas protect in other models. CL2006 and CL4176 worms were fed OP50, St1 or MYb57 and the paralysis rates from amyloid beta expression were assessed. St1 and MYb57 were shown to offer protection against amyloid beta toxicity across different *C. elegans* strains increasing the generalisability of these findings. To ensure that these microbiotas and similar microbiotas did not have any detrimental effects on *C. elegans,* development assays were performed comparing length and L4 development speed was assessed when fed on different microbial species. The effect of different microbiota on *C. elegans* size and L4 development was assessed. This study found that different microbiomes reduced the size and delayed the L4 development of *C. elegans* Whilst the effect was not dramatic it could introduce confounding variables in assays focused on aging and reproduction if not properly accounted for. Furthermore, it could highlight that these bacteria produce metabolites or nutrients that could cause stress or toxicity within the host.

Through transposon insertion of pJA1 into St1 400 mutants were generated to try to identify the genes and pathways responsible for the protective effects against amyloid beta toxicity. The mutant of interest would lose its amyloid beta protective effect against amyloid beta and would be sent off for genotyping to see what genes

had been disrupted. However, none of the St1 mutants lost their protective effect, so the gene potentially responsible this protective effect was not isolated and identified. This gene that confers this protection may be an essential gene which is why it has not been located. It may be that by knocking out the protective gene, that the mutated St1 is unable to grow.

A more targeted approach was then implemented supplementation assay were performed with vitamin B12 and NAC as previous literature has shown it to be protective against amyloid beta toxicity [75]. However, the results were unable to be replicated in this study.

Finally, a *C. elegans* strain was created with the genotype *unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP ; drp-1(tm1108) IV.* The creating of this allowed for the involvement of mitochondrial fission to be assessed to understand whether it induces the protective effect provided by St1, MYb57 and the Experimental Microbiome against amyloid beta toxicity. The *drp-1* gene was selected due to previous unpublished work in the Ezcurra laboratory showing that the Experimental Microbiome induced changes in the mitochondrial dynamics in the body-wall muscle.

The *drp-1* gene engages in mediating mitochondrial fission and amyloid beta has been shown to disrupt this mitochondrial function and accelerate neuronal death [67]. Initial results suggested that *drp-1* is not involved in this protection, however more biological replicates are required to confirm this.

These findings highlight the potential of microbiome-based interventions preventing amyloid-beta toxicity and offer new insights into the complex interactions between

microbiomes and neurodegenerative processes. However, further research is needed to identify the specific genetic and metabolic pathways involved, for innovative therapeutic strategies such as probiotics, prebiotics, or gene therapy, to be developed against Alzheimer’s disease.

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