A model system to investigate microbiome effects on age-related health in *C. elegans*

Brigita Kudzminskaite

Master of Research: Microbiology

2021-2022

University of Kent

Abstract

With the advancement of modern medicine, our aging population is rising sharply. In recent years there has been more focus in increasing healthspan, as well as lifespan, to improve the quality of life of older adults. The microbiome has been shown to play a huge role in human biological functions in both health and disease. This study focuses on two major healthcare concerns (a) Alzheimer's disease and (b) Sarcopenia, using the model organism Caenorhabditis elegans. The research aims to investigate a microbiome approach towards treatment or prevention of these diseases.

When using a *C. elegans* AD model, an experimental microbiome (EM) diet was shown to be protective against Aβ toxicity. From this EM, one bacterial species – *Stenotrophomonas sp.Myb57* – became a focus due to its significant protective effects. It was found that this protection was genus-specific, with some species releasing a compound in its cell-free media which elicits this effect. Further study will hopefully allow us to isolate the gene or compound that is causing the protection, as a potential for an Alzheimer's disease treatment.

C. elegans was additionally used as a model for aging muscles. When testing the effects of an experimental microbiome on muscles, results showed that it made no difference to the body wall muscle cell morphology in early adulthood or the muscle contractability throughout aging. When muscle function was assessed, however, it seems that worms fed the EM had lower muscle strength in early adulthood, but were much more able to maintain this strength as they aged compared to the control.

Overall, there is very promising evidence that the microbiome may be great source of research for therapeutic techniques against Alzheimer's disease, Sarcopenia and many more.

Contents Page

Abbreviations

Abbreviation	Meaning
AD	Alzheimer's disease
APP	Amyloid Precursor Protein
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
C. elegans	Caenorhabditis elegans
CFM	Cell free media
CFU	Colony-forming unit
CNS	Central nervous system
CTFα	C-terminal fragment alpha
CTFβ	C-terminal fragment beta
D1ad	Day 1 adult
EM	Experimental microbiome
GABA	γ-Aminobutyric acid
GF	Germ-free
GFP	Green fluorescent protein
GI	Gastrointestinal
H	Healthy individuals
L1, L2, L3, L4	Larval stage 1,2,3,4
LB	Luria broth
LPS	Lipopolysaccharides
MybMix	Mix of the 11 Myb/M strains
n	number of sample
OP	OP50/E.coli OP50
PF	Pathogen-free
sAPPα	Soluble Amyloid Precursor Protein alpha
sAPPβ	Soluble Amyloid Precursor Protein alpha
SCFAs	Short-chain Fatty acids
SD	Standard Deviation
Aβ	Amyloid beta

Chapter 1: Introduction

The human microbiota

Communities of microbes that harbour all multicellular organisms make up the microbiota. The human microbiota is comprised predominantly of bacteria, but also include archaea, protozoa, viruses and microscopic eukaryotes that live symbiotically on and within various locations of the human body, including the oral cavity, respiratory tract, genital organs and the gut [1, 2]. There is huge interest in the human microbiota as it has been suggested that the microbiome is a 'massive untapped source of drug targets' due to its ever-growing influence on health [3]. For the last 14 years, since the Human Microbiome Project began, microbiologists have been fascinated by the interactions between the microbiota and host, and how the bacteria living within us can impact our health [4]. The Microbiome Project wanted to determine whether 'healthy' microbiomes had any common elements and how these differed in people with specific disease states [4].

The human gastrointestinal (GI) tract is harboured by nearly 10^{14} microorganisms which make up complex and dynamic population of the gut microbiota [5]. PCR-amplified 16S rRNA gene sequencing is a form of bacterial analysis that targets a 16S short variable regions that allow taxonomic resolution at species and strain level [6]. This technique along with more recent metagenomic analysis has allowed scientists to identify the composition of the human microbiota to good detail, but there is a lot more research to be done to gain an even better understanding. The collective genomes of these microbial species make up the microbiome, which is estimated to contain 22 million genes in the gut microbiome alone [7]. This is compared to only around 22,000 protein-coding genes in the human genome [6]. These

microbial genes provide traits that humans did not need to evolve themselves, helping to produce important metabolites and biological compounding within the human body [8].

A healthy microbiota

It is extremely challenging to define what a 'healthy' microbiota constitutes [9]. As studies are using progressively larger numbers of human participants, there seems to be great variation in the microbial composition of supposedly healthy individuals [10]. Using genetic sequencing techniques, scientists have shown that the majority of healthy microbiomes are dominated by two main phyla, *Bacteroides* and *Firmicutes* [2]. More specifically, individuals vary in their *Firmicutes/Bacteroides* ratio. When this ratio was investigated in the context of obesity, both human and mouse studies revealed that obese participants have a higher *Firmicutes/Bacteroides* ratio, showing an interesting correlation between bacterial composition and obesity [11, 12]. Other examples of taxa that have been associated with human health include *Bifidobacterium, Lactobacillus* and *Eubacterium* [13]. Species such as *Butyrovibrio crossotus* and *Anaerotruncus colihominis* have been shown to be possibly protective against weight gain, through pathways involved in the biosynthesis of vitamins and carbohydrate metabolism [13, 14]. Another important consideration in a healthy microbiota is microbial diversity. High bacterial diversity is usually an indicator of good gut health [13, 2]. Dysbiosis and low microbial richness of the gut microbiota have been associated with a range of metabolic diseases including inflammatory bowel disease and diabetes [15, 16].

The gut microbiota have many biological functions

Study of the microbiota has revealed that gut microbes have great potential to impact all human physiology, in both health and disease [17]. In fact, recent studies have shown that hardly any part of human physiology is untouched by commensal bacteria [5]. The gut microbiota plays a crucial role in forming barriers against colonisation of pathogenic bacteria, as well as the maturation and education of the host immune response [5, 18]. It has an influence on metabolic function and the biosynthesis of vitamins, neurotransmitters and Short-chain Fatty Acids (SCFA's) [17, 19] (Figure 1.1).

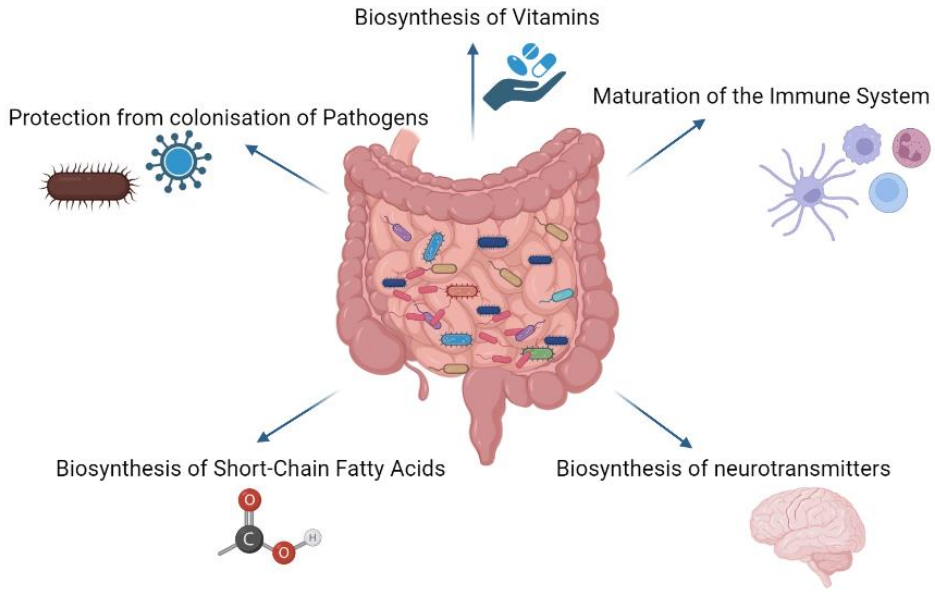

***Figure 1.1 - Microbiome Functions**. Illustration annotating some of the biological functions that the microbiome has. Created using BioRender.com [52].*

SCFA's are an essential energy source for the intestinal mucosa and are synthesised by gut microbes using indigestible dietary fibres [17]. Some SCFA's have the potential to have beneficial health effects whereas an excess of others have been associated with disease. Diversity in the gut microbiome is important for health due to the different functions that individual bacterial genera and species hold. As an example, enterotype 1 – which contains a high proportion of Bacteroides – has been shown to contain many genes involved in the biotin

and riboflavin biosynthesis pathways, while enterotype 3 - with a high level of Prevotella – has a higher frequency of genes involved in the synthesis pathways of thiamine and folate [13].

The aging microbiota

Throughout our lifetimes, the composition of our microbiota changes from birth as we develop and age, with an overall reduction in microbial diversity [20]. Aging is associated with an increase in pathogenic and opportunistic bacteria within the gut. Age-related changes in the microbiota includes an increase in the number of facultative anaerobes and changes in the dominance of species [21]. A noticeable difference in one study showed a decrease in the levels of *Bifidobacteria* and *Lactobacillus* compared to younger individuals [22]. They also showed that *Bacteroides* species diversity had increased in healthy elderly people, while *bifidobacterial* species diversity decreased – with *B. adolescentis* and *B. angulatum* being the most common [22]. Furthermore, *Faecalibacterium prausnitzii* and multiple member of the *Firmicutes* have been shown to be lower in older adults, while *Staphylococcus* and *E. coli* often increase [13, 23]. This data reveals that there is a clear shift in the composition of the gut bacteria as individuals age, which could be linked to the increased risk of age-related diseases.

The microbiome approach to treatments

Knowledge of individual bacterial effects within the microbiome could lead to innovative and effective therapies. The knowledge gained from the management of Helicobacter pylori induced peptic ulcers revealed that certain chronic disease can be caused by growth of particular bacteria within the gut [24]. Initial considerations for therapeutic targets were bacterial species with known genome sequences and biochemical functions [25]. *Bacteroides thetaiotaomicron*, for example, obtains a variety of genes that have beneficial effects for the

host and other commensal bacteria, through elaborate apparatus dedicated to the hydrolysis of indigestible dietary polysaccharides [26]. Several species of *Lactobacillus* increase mucin glycoprotein production which are important macromolecular constitutes of epithelial mucus that have a major role in modulating the immune system and protection against pathogens [27].

Faecal transplantation

One suggested strategy for microbiota-targeted therapies is the way in which *H. pylori* peptic ulcers are often treated. This involves the use of antibiotics and faecal transplantation to re-introduce healthier bacterial strains into the patients gut [25]. Another common use is for the treatment of *C. difficile* infections, for which it has been an inexpensive and effective method, with a success rate of around 90% [28]. The faecal material used is highly screened and from a healthy donor to ensure that it leads to a diverse gut ecosystem [29]. However, this method is unpredictable and could lead to long-term disruption of gut ecology and the destruction of beneficial microbes. It is also not a comfortable procedure for the patients, with common side effects including abdominal cramps, transient diarrhoea and constipation [28]. There is also the risk of pathogenic strains being transferred as well, leading to other future problems that may not be recognised [30]. Some long-term adverse effects that have been reported by patients include obesity, rheumatoid arthritis and irritable bowel syndrome [28]. This shows the need for therapeutic interventions that use the microbiome approach in a more specified and accurate way.

Probiotics and Prebiotics

Another strategy is the use of probiotics and prebiotics to manipulate the gut microbiota and create a beneficial bacterial community within the host [25]. Probiotics are live microorganisms that beneficially affect the host by promoting gut microbial balance and homeostasis [31]. These can be in tablet form, like vitamins, or in food products such as natural yogurt and fermented vegetables. Some probiotic bacterial genera include Lactobacilli, Bifidobacterium and Streptococcus [25, 31]. Probiotic products often claim to strengthen the immune system, reduce inflammation and help improve digestion. On the other hand, prebiotics are a group of nutrients that feed the microbiota, promoting the growth of healthier bacterial species and the production of beneficial short-chain fatty acids [32]. Oligosaccharide carbohydrates are a type of prebiotic that help stimulate *Bifidobacterium* and to a lesser extent *Enterobacteria* and *Firmicutes* [33]. It is also interesting to note that intestinal microbes have also been shown to affect the efficacy of some drugs [34]. For example, gut bacteria has the ability to affect individuals response to cancer immunotherapies, so microbiome-nutrition based interventions alongside treatment have been suggested for more effective treatment results [34]. Although this is an interesting and effective approach, there is concern around the use of pro- and prebiotics as their effects in vivo could potentially be unpredictable [3]. Also, there is a lack of understanding behind the mechanisms that act during probiotic and prebiotic use, which becomes a slight grey area for scientists, and can lead to very differing effects in different individuals [35].

The microbiome as a drug target

Within the pharmaceutical industry, it has been suggested that the human microbiome gives rise to potentially millions of microbial drug targets, due to the extensive number of genes

[3]. This could lead to a better and more specific way of using a gut microbiome approach to treatment. This brings its own challenges, with there being such a wide number of different bacterial strains, it is difficult to find specific targets that have a specific mode of action [35]. Creating microbiome-based therapies requires microbiologists to determine a causal mechanism between microbe and host [24]. Although there is now a lot of data about the microbiota, there is still a lack of understanding behind the genes, pathways or compounds responsible for the effects that specific bacteria may have. The benefit, however, is that development of drugs could be a more specified and traceable approach. Researchers are beginning to focus on small molecules that gut microbes produce that have a beneficial effect in health [3, 36]. For example short-chain fatty acids such as butyrate, acetate and propionate, all of which have important roles within the body, with butyrate thought to be the most important for human health [36].

As of yet, this research is still in early stages as drug developers are still confronted by the complexity of the microbiome [35].

C. elegans as a model organism

In microbiology, model organisms provide powerful experimental opportunity to investigate the biological effects of bacterial species in vivo [37]. In terms of the complexity of the microbiome, traditional mammalian models can be expensive, time consuming and inherit challenges of biological complexity, especially for early research [35, 38].

The nematode *Caenorhabditis elegans* is a central model organism used across various biological disciplines [39]. *C. elegans* was discovered as a model system by Sydney Brenner in 1965 in order to study animal development and behaviours [40]. They can be isolated from soils and rotten organic materials rich in diverse bacterial communities [41]. Although they

are deceptively simple, their cellular complexity and conservation of disease pathways with higher organisms makes *C. elegans* an effective model for biological research [42]. Further advantages include their short life-span, ease of laboratory cultivation and small size. *C. elegans* are self-fertilising and monoclonal, allowing a single worm to populate a whole plate with genetically identical progeny [43].

The genetic code of the nematode is well known, with homologs found for around 60-80% of human protein-coding genes [51]. Moreover, they are a whole organism with multiple organs and biochemical pathways without the ethical complications that are involved with mammalian models [35]. This allows scientists to harbour informed theories of how drugs and compounds may affect humans as well. The nematode is highly amendable to genetic manipulation and being transparent, it allows microbe colonisation and behavioural changes to be monitored easily using microscopy [39]. Researchers are also able to insert fluorescent tags to specific areas of their body to visually identify targeted biological processes [42]. This gives rise to genetic analysis and genetically modified strains of the worm. There are a range of diseases that have been researched using *C. elegans*, including aging, muscular dystrophy and Parkinson's disease [42]. There are various ways in which *C. elegans* disease models are generated, some of the most common being (1) mutant knock out or RNAi knock down of human disease gene homologues to investigate phenotypes, and (2) expressing human genes to induce phenotypes that are disease-related in worms [42].

Its benefits, alongside the physiological understanding of *C. elegans*, has increased their use in drug discovery, making them a potentially huge part of future drug studies that have the potential to change patients' lives.

C. elegans anatomy

The anatomy of *C. elegans* has been well studied and is understood in nice detail. Once a *C. elegans* embryo is laid, its development proceeds through 4 larval stages – L1, L2, L3 and L4 – after which it molts into an adult worm (Figure 1.2) [44]. At 20°C, the development stages take around 3 days as long as there is abundant food, and the adults survive for around 3 weeks [44]. Under a harsh environment, L1 stage worms are able to proceed via an alternative dauer pathway. Dauer are adapted for long-term survival, and are able to molt into L4 stage when a more favourable environment has been found, in order to proceed with the rest of its life cycle [44].

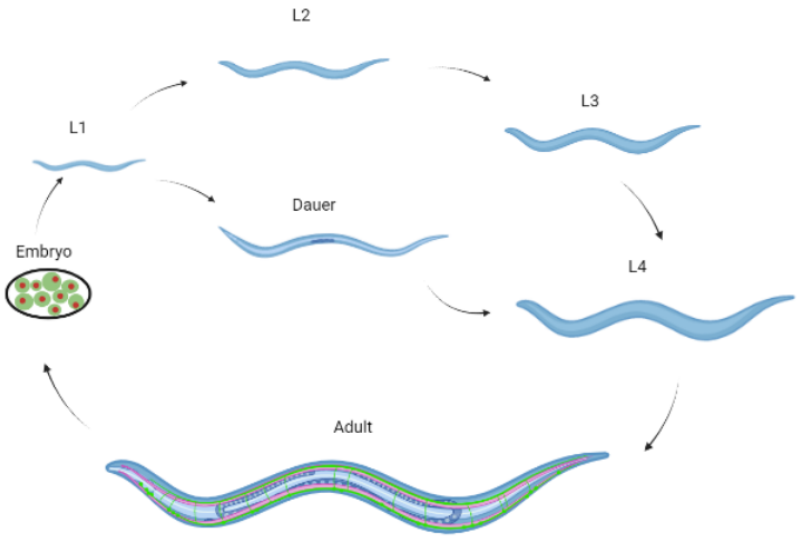

Figure 1.2 - C. elegans Life Cycle. *Schematic of the C. elegans life cycle from embryo to the 4 developmental stages - L1, L2, L3 and L4 - to adulthood. Includes the alternate Dauer pathway. Created using BioRender.com [52].*

C. elegans feed through a two-lobed pharynx [44]. Like humans, they have an intestine which spans from the mouth through to the anus, as well as muscles, glands and a nervous system [42]. There are 2 *C. elegans* sexes – hermaphrodites (XX) and males (XO). Hermaphrodites are

self-fertilising and produce homozygous genetically identical progeny [44]. Their reproductive system consists of somatic gonad, their germline and the vulva through which they lay eggs (Figure 1.3). Germ cells from the distal cells pass sequentially through the mitotic, meiotic prophase and diakinesis stages [44]. The oocytes get fertilised by sperm in the spermatheca to form diploid zygotes which are stored in the uterus and laid through the vulva [44]. A hermaphrodite is able to produce around 300 progeny in the first 3-4 days of adulthood, however, if they mate with a male this number can increase to 1200-1400 progeny [44]. Males are mostly important to provide better survival in harsh conditions through increased progeny and increased genetic variation for enhanced survival rates. They are anatomically different to hermaphrodites, with a thinner body and a blunt tail with a fan and rays to allow for mating [45].

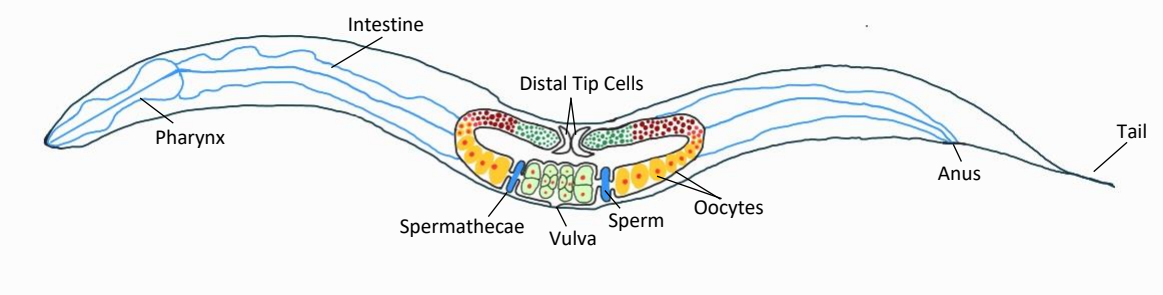

Figure 1.3 - C. elegans Hermaphrodite Anatomy. *Simple schematic representing the pharynx and intestinal system in blue and the reproductive system of the hermaphrodite in the centre. Created using Samsung Notes.*

<u>*C. elegans* as a model for microbiome studies</u>

Traditionally, *E. coli* has been used as the standard food source in *C. elegans* cultivation. However, more recently, scientists have found that in the wild, *C. elegans* eat a variety of bacteria and thus, have a diverse microbiome [41]. Therefore, *C. elegans* are especially ideal for studies into host-microbe interactions. Evidence has shown that bacterial signals affect the development and aging of *C. elegans* [38]. For example, *Comamonas aquatica* is

important in the production of B12 in the nematode, which affect their development and fertility [46]. Furthermore, *Bacillus subtilis* biofilm has been shown to increase *C. elegans* longevity through the downregulation of the insulin-like signalling pathway [47]. In contrast, folate synthesis by *E. coli* decreases worm lifespan by accelerating animal aging [47]. These and many more important host-microbe interactions present *C. elegans* as a model organism with various pathways and mechanisms that are conserved with higher organisms.

A range of *C. elegans* assays can be used to observe the effects of microbes on their health and behaviour, making them a powerful biosensor for the study of microbe-host interactions [49]. They allow phenotypes to be captured at great quality, and the effects of bacterial strains can be measured both quickly and easily [35]. Since the physiological make-up of *C. elegans* is well understood, these assays can be well targeted to certain tissues such as the digestive system, muscles, and nervous system [43]. Originally, in the use of *C. elegans* as a model organism, the worms were fed *E. coli* as their laboratory food. Since their use in microbiome studies, their natural microbiome was examined using 16S sequencing and found they have a very diverse microbiome dominated by *Bacteroidetes, Proteobacteria, Firmicutes* and *Actinobacteria* [39]. Studies have revealed that there is strong individual variation of bacterial communities within the *C. elegans* gut, with only a small core microbiome being observed [41].

Research has revealed the composition of the *C. elegans* microbiota can have impact on health and longevity [39]. Through extensive research into the *C. elegans* wild microbiome, many bacterial strains isolated from their microbiota have been genotyped [50]. Of these strains, 11 species were chosen (See table 1.1) to be mixed and create an experimental microbiome. This experimental microbiome is fed to the worms and compared to their

laboratory food source, *E. coli*, allowing us to observe the effect of the microbiome on a range of behaviours and health indicators.

Table 1.1 - Experimental microbiome - *The 11 Myb strains that were used to create the experimental microbiota (MybMix) in the Ezcurra lab.*

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*

The microbiota and gut-brain axis

In recent studies, there has been a major focus on the relationship between the gut microbiota and the function of the central nervous system, commonly referred to as the gut-brain axis [20, 53]. There have been links made between peripheral intestinal functions and the emotional and cognitive centres of the brain [54]. In particular, the influence of the gut microbiota through signalling involving neuro-immuno-endocrine mediators has been shown to have a central biological function [54]. There are many pathways of communication between the gut and the brain, including neurological pathways, endocrine pathways and immune pathwayss [55]. The gut microbiota provides bidirectional communication through cytokines, hormonal, and neuronal signals [20, 55].

For example, the microbiota has an important role in the metabolism of Tryptophan [55]. Tryptophan is an important amino acid that is a precursor to metabolites such as melatonin, niacin and serotonin [56]. In fact, approximately 90% of the serotonin in our bodies is produced in the gut by the enterochromaffin cells [55]. Serotonin is a monoamine neurotransmitter that modulates the majority of human behaviour, affecting mood, memory and motor control [57]. These are all areas that Alzheimer's disease disturbs, showing a clear link between this and balanced serotonin levels that are influenced by the gut microflora. Furthermore, gut bacteria – such as Lactobacillus and Bifidobacterium – are actively involved in γ-Aminobutyric acid (GABA) production [21, 58]. GABA is also an essential inhibitory neurotransmitter in the CNS that has a direct effect on human personality and stress management [58]. It has been shown that the levels of aminobutyric acid in the intestine correlates with levels in the central nervous system, thus, decreased numbers of the bacteria that produce it have been associated to brain dysfunction [21].

Overall, the bacteria within our guts have an important role in producing metabolites that are used within the brain. In turn, the central nervous system also has a major role in regulation of the permeability, immunity and motility of the gastrointestinal tract via the enteric nervous system [21].

Alterations in the microbiota have been associated with neurological diseases such as multiple sclerosis, autism and Alzheimer's disease [59]. Due to the communication between the gut and CNS, there has been growing evidence that modulation of the microbiota through diet and probiotic intervention, could become a new treatment for managing such diseases.

Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia, affecting nearly a million people in Europe [60]. It is a neurodegenerative disease in which the neuronal cells within the brain are slowly destroyed impairing patients' memory, language and behaviour [61]. This leads to the loss of ability to perform daily tasks and a substantially decreased quality of life. The main risk factor of Alzheimer's disease dementia is age, with 1 in 14 people over the age of 65 suffering from the disease [62]. Incidence increases with age, affecting 32% of people aged 80 and above [21]. With an aging world population, this prevalence is continuing to grow, increasing the need for effective treatment options and interventions. One example of an Alzheimer's treatment is cholinesterase inhibitors, which have been shown to enhance the quality of life when prescribed at the appropriate time, however, they do not improve the rate of decline [63]. As of yet, there are no curable treatments and due to its degenerative nature Alzheimer's causes rapid decline in patients [5]. Research into different approaches to treat or prevent such debilitating symptoms is essential to help our aging population lead healthier and happier lives.

Alzheimer's Disease Pathophysiology

The pathophysiology of Alzheimer's Disease is still not fully understood, however, today it is believed that amyloid beta (Aβ) (Figure 1.4) and tau (Figure 1.5) are the two main drivers in its pathology, making them focal targets for research models and therapeutic development [21, 64].

Amyloid Precursor Protein (APP) is a transmembrane protein that functions to regulate cell growth, neurite outgrowth and motility [65]. Nonamyloidogenic processing of APP includes α-secretase cleaving the protein into a C-terminal fragment, CTFα, and the sAPPα fragment

that is soluble and diffuses away [64, 65]. Then, λ-secretase cleaves CTF and releases the Aβ intracellular domain and extracellular p3 fragment [64]. In the amyloidogenic pathway, however, beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is involved. BACE1 has been confirmed as the neuronal β-secretase, cleaving APP to form CTFβ and sAPPβ [64, 66]. Following this, CTFβ is cleaved by λ-secretase at one of several sites to generate Aβ peptides - with the most common forms being 1-40 and 1-42 – and also form Aβ intracellular domain [64, 65].

Aβ monomers are flexible, soluble and have high aggregation propensity [67]. In the brain, they begin to stick together and oligomerise causing Aβ deposits to build-up as plaques and intracellular neurofibrillary tangles accumulate in and around the nerve cells within the brain, disrupting neuron to neuron signalling [59, 67, 68]. It is believed that the Aβ42 isomer is more neurotoxic and hydrophobic, forming the core of the senile plaque, which are highly insoluble pro-inflammatory lesions [67]. The accumulation of Aβ further triggers the inflammatory cascade leading to damage to neuronal axons and synaptic loss, and thus, cognitive impairment [69].

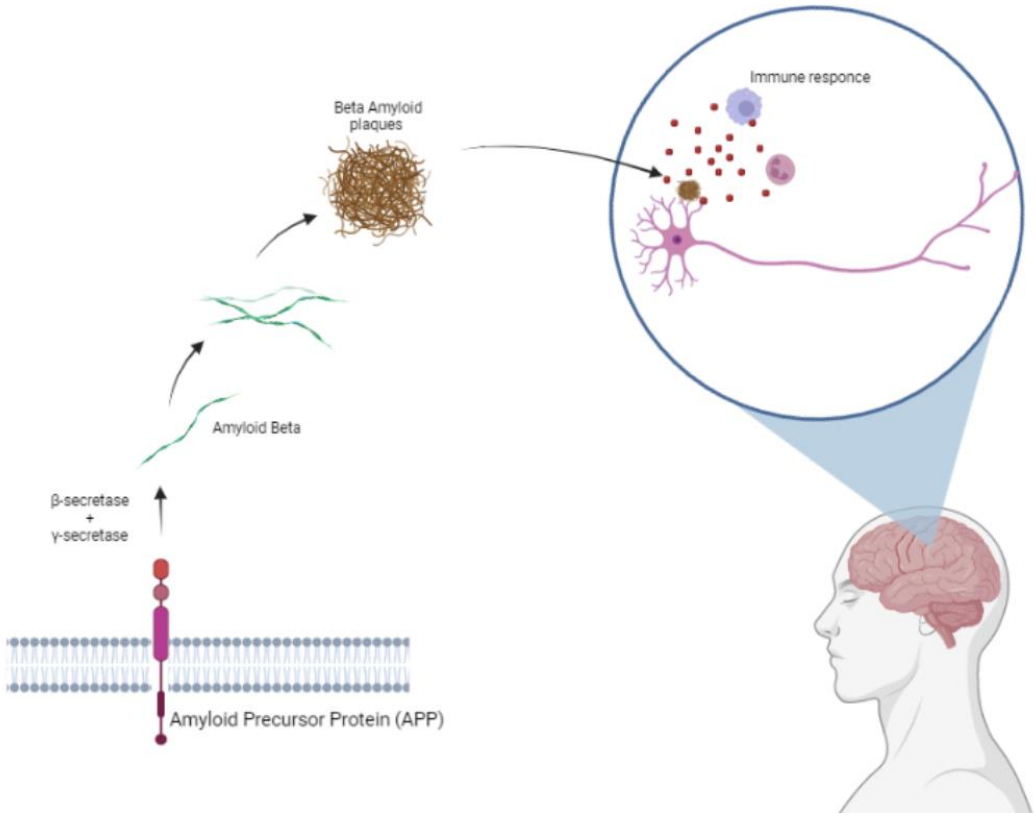

Figure 1.4 - Alzheimer's Disease Amyloid Beta Pathology *- Showing the Amyloid Precursor Protein (APP) being cleaved by beta-secretase and gamma-secretase, leading to insoluble amyloid beta monomers forming. These monomers oligomerise to for beta amyloid plaques. These plaques get in between the neuronal cells within the brain causing interruption of cell to cell signalling and causing and immune response. Created using Biorender.com [52].*

Tau is a protein associated with neuronal microtubules involved in the promotion of microtubule self-assembly by tubulin and stability [70]. Just like all cells within the body, the cytoskeletal microtubules are essential for cell signalling, chemical transportation and regulation of cell growth. It is believed that the build-up of these β-amyloid plaques may trigger the activation of kinase within the neuronal cells [71]. This leads to phosphorylation of tau, causing them to aggregate to form oligomers and higher order fibrils, creating the distinct tangles found in AD patients [72]. Alzheimer's patients are often reported to have axonal transport deficits and defective microtubule assembly [64].

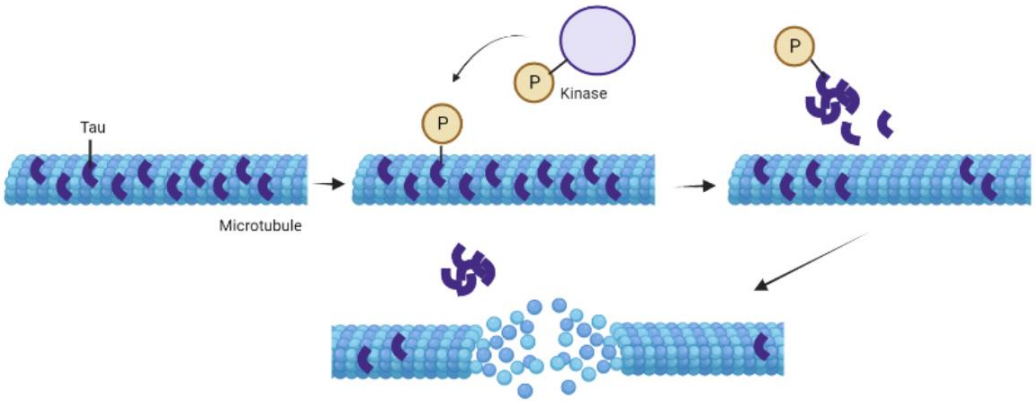

***Figure 1.5 - Alzheimer's Disease Tau Pathology**. Within the cytoskeleton of neuronal cells contain the protein tau essential for their strength and stability. Kinase can be activated within the cells which transfers a phosphate group onto the tau. the tau protein then changes shape, stops supporting the microtubules and gets clumped up with other tau to produce neurofibrillary tangles. This leads to a lack of support of the microtubule structure meaning they can't function well. Created using Biorender.com [52].*

The Microbiota and Alzheimer's disease

Many studies have shown the links between the human microbiota and Alzheimer's disease. it has been suggested that the gut microbiota may be involved in the pathogenesis of Alzheimer's disease [73].

Analysis has shown that Alzheimer's patients have a less diverse and compositionally differing gut microbiota compared to control age- and sex-matched individuals [59]. *Vogt et al.'s* study showed the bacterial distinctions between Alzheimer's patients (AD) and healthy individuals (H). Their data has shown that Alzheimer's patients have a significantly increased relative abundance of *Bacteroides* (median, AD:~12% vs. H: ~7%), as well as *Phascolarctobacterium* (median, AD: ~1.2% vs. H: ~0.2%) and *Blautia* (median, AD: ~13% vs. ~8%) within the *Firmicutes* [59]. In contrast, there is a significant decrease in the abundance of *Bifidobacterium* (median, AD: ~0.1% vs. ~1.25%) [59].

This contradicts results from Zhuang *et al.'s* study which found AD patients to have a lower abundance of Bacteroides and showed almost no difference in the abundance of Firmicutes

[73]. Despite this, they still showed a difference in the Bacteroidetes/Firmicutes ratio [73]. They further showed an increased relative abundance of Actinobacteria and lactobacillus compared to controls [73].

Although the figures aren't all consistent, they do all confirm that Alzheimer's disease is associated with dysbiosis of the gut microbiota. These results confirm that there is disease associated alterations of the microbiota making it a possible target for therapeutic interventions.

Further research shows that certain gut bacteria may produce proteins that could modify the interaction between the immune and nervous systems triggering the disease [60]. For example, high levels of short-chain fatty acids such as acetate and valerate have been associated with more amyloid deposits within the brain, whilst butyrate has been associated with lower levels of amyloid plaques [60]. It has also been suggested that lipopolysaccharides (LPS), which make up the membrane of gram-negative bacteria (such as *Bacteroides*), are able to trigger systemic inflammation and produce pro-inflammatory cytokines after translocation from the gut to circulation [74]. This has also been associated with the link between the gut microbiota and metabolic diseases including obesity and diabetes [74]. Furthermore, a mouse study showed that injections of LPS induced an increase in Aβ1-42 in the hippocampus and led to cognitive impairment [75]. This is associated with Alzheimer's disease in human studies, which have shown AD patients to have a 3-fold higher level of LPS levels in their plasma (H: 21 ± 6 pg/ml vs. AD: 61 ± 42 pg/ml) [76].

As well as LPS, various bacterial strains have been associated with the accumulation of β-Amyloid [77]. Species of gram-negative bacteria, such as Streptomyces, Pseudomonas and Bacillus, have been found to have functional amyloid systems [78]. Due to this, a large number

of microbiome bacteria have the capability to produce high quantities of amyloids, potentially exposing our bodies to a systemic amyloid burden, particularly during aging due to increased permeability of the gut [78]. However, on the opposite side, a mouse study showed that feeding wild-type mice with Aβ1-42 triggered changes in their gut microbiome composition [79].

Further research into this ever increasing link between Alzheimer's disease and gut health may pave the way for unique protective strategies, including bacterial cocktails, informed dietary intervention and even target-specific drugs.

A *C.elegans* amyloid β toxicity model for Alzheimer's Disease

The transgenic *C. elegans* model, GMC101, contains a temperature sensitive mutation which causes full length Aβ_{1-42} transgene expression within the body wall muscle cells of the nematode [80, 81]. These beta-amyloid oligomerise and aggregate in the muscles of the worm leading to an age progressive paralysis phenotype that is induced by a temperature upshift to 25℃ [81]. A great advantage of using the GMC101 Alzheimer's model is that it has a 4 day turnover, allowing many repeats of assays to be done in a short time leading to extensive data and effective troubleshooting. Overall, this model provides a valuable tool for investigating the mechanisms of Aβ_{1-42} toxicity and testing innovative therapeutics against Alzheimer's Disease.

Sarcopenia

The human skeletal muscle system is essential for movement, balance and posture. Skeletal muscle is a type of straited muscle, and is composed of myofibrils which are made up of

sarcomeres. Within the sarcomere, two main fibres – actin and myosin – work together in order to perform voluntary contraction and relaxation of the muscles [82].

As we age, skeletal muscle mass is lost, which leads to a loss of strength and function [83]. In many older adults, this has adverse effects such as impaired mobility and increased risk of falls and hip fractures [83]. Sarcopenia is a skeletal muscle disorder in which there is a progressive accelerated loss of muscle mass and function [84]. It is a common age-related process that occurs in older adults [84]. With increasing age, there is a reduction in the number motor units and muscle fibres and a reduced size of type 2 fibres, leading to a gradual decrease in muscle volume [83]. This is a result of aging disturbing the homeostasis of the skeletal muscles and an imbalance between the anabolic and catabolic pathways of muscle proteins [84]. Sarcopenia also affects the integrity of mitochondria in myocytes, and these molecular changes in the muscles alter the complex signalling pathway that includes insulin-like growth factor and mTOR transcription factor [84].

Since aging has been associated with a reduction in the microbiota diversity, studies have suggested that the presence of a gut-muscle axis that may regulate the onset and progression of sarcopenia and related physical frailty [85, 86]. There is evidence that alterations in the gut bacterial composition could promote inflammation and anabolic resistance, which leads to reduced muscle mass and impaired function [86]. Furthermore, a reduced production of SCFA – associated with an aging microbiota – could reduce mitochondrial fatty acid oxidation and promote insulin resistance [85]. This can lead to increased deposition of intramuscular fatty acids, resulting in lower muscle strength [87].

C. elegans as a model for muscle studies

Various vertebrates and invertebrates have been used as whole model organisms to investigate the structure and function of muscles and neuromuscular diseases. Using whole-organisms to assess muscle function is important because it allows the investigation of overall muscle function in animals while they are moving as well as any impairments in muscle structure. The overall structure and function of the sarcomere – the basic functional unit of the muscle – is highly conserved between nematodes and vertebrates [88].

C. elegans have both striated and non-striated muscles. Striated are similar to human skeletal muscle while non-striated in hermaphrodites resemble the smooth muscle in vertebrates [89]. An adult worm contains 95 diamond-shaped body wall muscle cells which are arranged in four longitudinal bands forming a single layer of cells, running from head to tail [88] (Figure 1.6). The majority of studies are performed on these striated body wall muscle cells [88]. Structurally, the *C. elegans* body wall muscles are made up of myosin containing a thick filament associated with M-lines and actin containing thin filaments known as dense bodies [88]. These are functionally analogous to Z-band and M-lines in vertebrate muscles [90]. The majority of their muscle force is transmitted laterally through M-lines and dense bodies, which are directly anchored to the membrane making the nematode an ideal model for studying muscle function and its age-related decline [90].

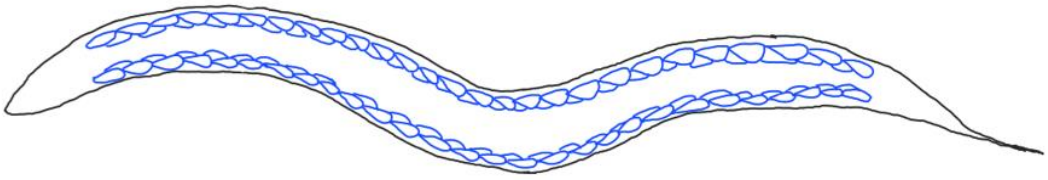

Figure 1.6 - C. elegans Body Wall Muscle *- Simple schematic illustrating the positioning of the body wall muscle cells (in blue) within C. elegans (outline in black). Created using Samsung Notes.*

A *C. elegans* strain for visualisation of the body-wall muscle

RW1596 (myo-3p::GFP) is a transgenic *C. elegans* strain in which green fluorescent protein GFP) is expressed in body-wall muscle using the myosin promoter myo-3 (strain number RW1596) was used to visualise the muscle sarcomeres [91, 92]. Due to myosin being one of the main muscle fibres, under green fluorescent microscopy, the induvial sarcomeres and entire body wall muscle cells could be clearly identified, showing the shape and size of each muscle cell. This strain allowed for observation of muscle cell structure and allowed for comparisons between dietary effects.

Aims and objectives

In regards to the microbiome, paralysis assays in the Ezcurra lab have revealed that GMC101 worms being fed the experimental microbiome show reduced onset of paralysis when compared to those that are fed *E. coli* OP50. Further, when each of the eleven strains was tested individually, it was clear that *Stenotrophomonas MYb57* was one of the strains which significantly reduced paralysis over a 4 day period. Furthermore, when the respective cell-free media of each of the strains was tested, *Myb57* was the only bacteria to show protection. This suggests that *MYb57* – and possibly compounds that it releases - are having a protective effect on GMC101 *C. elegans* and is inhibiting or reducing beta-amyloid plaque formation. *Stenotrophomonas* is a gram-negative bacteria, of which species are commonly found in soils and plants [93]. *MYb57* contains 4164 protein-coding genes within it genome, a small number of which are having this protective effect seen in *C. elegans*.

To research further into *Stenotrophomonas MYb57*, this project aims to narrow down or possibly discover which gene(s) may be responsible for the protective effect that this bacteria has, in order to possibly reveal the mechanism behind it. This could be used to create a

therapeutic method against Alzheimer's disease. In order to achieve this, seven strains of *Stenotrophomonas* that are genetically similar to *MYb 57* will be tested using the GMC101 paralysis assay, as well as using the cell free media of each bacteria. This will reveal whether any of these strains lack the protective gene against amyloid-beta aggregation. If so, these lacking *Steno* strains will be genetically compared to *MYb57* using bioinformatic tools. The cell free media of all the *Stenotrophomonas* strains will further reveal whether there is a compound produced by the bacteria that may be having this protective effect. This research could bring us one step closer to figuring out a therapeutic approach for humans that may slow down onset of Alzheimer's and impact those suffering from the disease in a positive and possibly life changing way. It could also lead to a preventative approach by manipulating the gut ecology of at risk individuals.

In regards to aging muscle health and sarcopenia, this project also investigates the effects that a microbiome diet may have on *C. elegans* muscle cells. Previous work in the lab has shown that the thrashing rate of C. elegans fed on an OP50 diet has a rapid decline as they age. However, when fed the experimental microbiome, they had a lower thrashing rate on day one of adulthood, but showed very little decline with age. Our aims were to explore body wall muscle cells through fluorescent microscopy, to identify any differences in the morphology of the cells when comparing the microbiome diet to the control. We further plan to test the effects of this microbiome diet on the muscle function in *C. elegans* by studying the rate of burrowing through a layer of gel, while induced by a chemoattractant. This has the potential to give us a better understanding of how the microbiome may affect muscle health throughout aging, leading to possible intervention in older adults.

Chapter 2: Materials and Methods

Worm husbandry

The *C. elegans* were maintained on agar plates seeded with a bacterial food source, standard laboratory food being OP50 *E. coli*, as per the standard protocol [94]. The worms were transferred using a platinum wire pick or eyelash pick, and maintained in 20°C incubators.

E. coli was cultured in Luria broth (LB) (Table 2.1) overnight in a 37°C shaking incubator (180rpm).

Nematode Growth Medium (NMG) is prepared using standard protocol [94]. For 1.6L of NMG 27g of Agar, 4g of $\mathrm{Bacto}^{\mathrm{TM}}$ Peptone, and 4.8g of NaCl are topped up with 1.6L of $\mathrm{dH_2O}$. This is autoclaved and cooled to 55°C. Then 40mL of $\mathrm{KH_2PO_4}$ (1 M) and 1.6ml each of cholesterol (12.93mM in ethanol), $MgSO_4$ (1 M) and $\mathrm{CaCl \cdot 2H_2O}$ (1 M). This was pouring in 6cm plates with 10mL of NGM in each and dried overnight. These plates could then be seeded with bacteria as necessary.

Synchronisation

To synchronise the *C. elegans* for the assays, a bleaching technique was used. 5-10 adult (usually day 1) worms were transferred from an maintenance plate to an empty seeded plate (prepared 3-4 plates). This will populate the plates in 4 days. There will be many adult that embryos in the uterus. These worms are washed with M9 (Table 2.1) which gets transferred into Eppendorf tubes. The Eppendorf tubes are centrifuges to collect all worms into the pellet. The M9 supernatant is then removed gently, avoiding the pellet. The bleaching solution (Table 2.1) is then added onto the worms for 3-5 minutes, until the bodies are visibly breaking under a dissecting microscope. This is centrifuged again and supernatant removed. Then the worms are washed with M9 3 times to remove any remaining bleach. After the washes, the remaining

eggs/embryos are resuspended in M9 and left on a rocker overnight, this allows the eggs to hatch and synchronise to L1 stage in the M9. Finally, the L1's are seeded onto seeded plates, as per the conditions of the assay.

***Table 2.1 - Protocol for general solutions**. Includes protocol for making LB Broth, M9 and bleaching solution.*

Solution	Protocol
LB	(1L) Dissolve 25g of LB granules in 1L of dH_2O and autoclave.
M9	(1L) 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 [94].
Bleaching solution	(12ml) Mix 3ml bleach (10-15% sodium hypochlorite), 2ml NaOH (5M) and 7ml dH_2O.

Table 2.2 - List of Reagents.

Name	Supplier	Serial number
Agar	Melford	9002-18-0
Agarose	Melford	MB1200
Bacto™ Peptone	Becton, Dickinson and Company	211677
Sodium Chloride (NaCl)	Fisher Scientific	S/3160/60
Miller LB Broth	Fisher Scientific	BP9723/500
Potassium Phosphate (KH_2PO_4)	Melford	P41200-1000.0
Cholesterol	Sigma-Aldrich	A6848-50MG
Magnesium sulphate ($MgSO_4$)	Melford	M24300-1000.0
Calcium Chloride (CaCl)	Fisher Scientific	S/3160/60
Pluronic F-127	Sigma-Aldrich	P2443-250G

<u>*C. elegans* strains</u>

N2 - C. elegans wild type isolate

GMC101 - dvIs100 [unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP]

RW1596 - myo-3(st386) V; stEx30. stEx30 [myo-3p::GFP::myo-3 + rol-6(su1006)]

Bacterial strains

Table 2.3 – The bacterial species

Abbreviation	Species
OP50	*Escherichia coli*
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*
St 1	*Stenotrophomonas terrae*
St 2	*Stenotrophomonas ginsengisoli*
St 3	*Stenotrophomonas pavanii*
St 4	*Stenotrophomonas indicatrix*
St 5	*Stenotrophomonas sp. Leaf70*
St 6	*Stenotrophomonas bentonitica*
St 7	*Stenotrophomonas lactitubi*

Culturing the bacterial strains

The *Stenotrophomonas* strains (including Myb57 as control) were cultured overnight ~18h in a 30°C shaking incubator. The Myb strains were cultured overnight at 25°C in a static incubator.

A small amount of bacteria (streaked on LB agar plate) is transferred, using a sterile pick, into 15ml falcon tubes containing ~8ml of LB.

NGM plates were seeded with 250µl of the bacterial solution and left on a bench to grow over 2-3 days.

Making the cell free media

To make the sterile cell free media (CFM) of the bacterial strains, the grown culture in LB is cetrifuged to contain all of the cells in the pellet. The supernatant of the solution was then filtered through a syringe into a fresh falcon tube to remove any debris or cells left over (Fig. 2.1). 250µl of the CFM was added on top of OP50 seeded plates and left to dry before use.

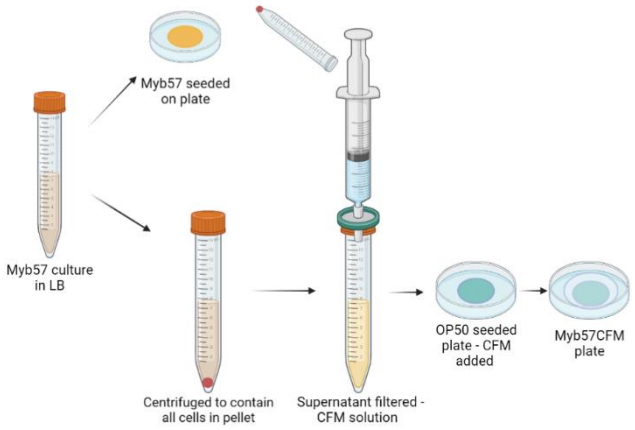

Figure 2.1 - Schematic of the protocol to seed plates and make cell free media. *Shows how the CFM and seeded plates were made, using Myb57 as an example, for use in assays. Created with Biorender.com [52].*

Paralysis Assay

The paralysis assay was done using the GMC101 *C. elegans* strain. Figure 2.2 shows a simple outline of how the paralysis assay was done. After synchronisation, the L1's (~200) were transferred from the M9 solution to plates, with each of the food conditions. They were incubated at 20°C until they reached L4 stage. Then 100 L4's were transferred to a plate with fresh food of the same condition. This plate is then upshifted to a 25°C incubator (or any controls may stay at 20°C). Each day the number of healthy adults and paralysed worms was counted and recorded (Figure 3.1 in the results section shows what paralysed worms looked like and how they were distinguished from healthy worms). Every other day, the healthy adults were transferred onto fresh food to prevent starving and so young worms that had

hatched would not grow to adults and be counted on further days. The counting was done up until day 3 adults.

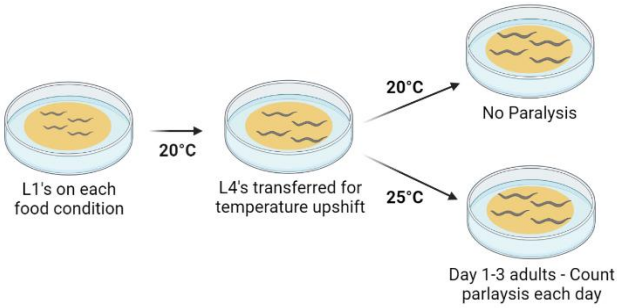

***Figure 2.2 - Schematic presenting the burrowing assay protocol**. A simple step by step of how the paralysis assay was done using GMC101's. Created using Biorender.com [52].*

Microscopy of RW1596 (myo-3p::GFP)

Differential interference contrast microscopy under fluorescence was used to image the RW1596 strain worms. To prepare the slide, day 1 adults on the plate were checked and only roller worms were transferred as these were the worms tagged with green fluorescence expressed by myosin.

An even thin layer of 2.5% agarose was cushioned between 2 clean slides. One of the slides is removed and a 5µl drop of Tetramisole (diluted 1:4 Tetramisole:M9) is added in roughly the centre of the agarose layer. ~30 worms are then transferred from the plate into tetramisole drop which will immobilise the worms. Then use a pick to gently move the worms close together and gently add a cover slip. once the slide is labelled it can be taken for imaging. Imaging was done at 40x oil immersion and photos taken were under fluorescence with a green filter (examples of images can be seen in figure 4.2).

Burrowing Assay

The burrowing assay used N2 wild-type strain worms and was designed using Lesanpezeshki *et. al.'s* study for guidance [95]. Figure 2.3 presents a schematic summarising the protocol.

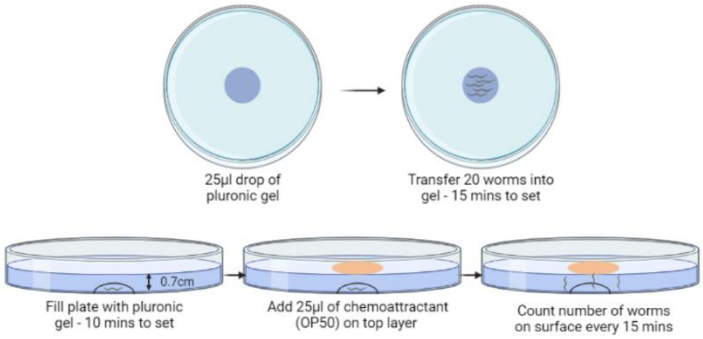

Figure 2.3 - Schematic of the burrowing assay protocol.
Created with BioRender.com [52].

To prepare, a 25µl drop of 26% w/w Pluronic gel F-127 is added in the centre of a 3.5mm plate. To prevent too much food transfer, worms on each of the food conditions (OP50 control and MybMix) were transferred to an intermediate NGM plate containing no food. The plate was sometimes tapped lightly on the surface to encourage the worms to move away from residual food. Then, an eyelash pick is used to transfer 20 worms (at different days of adulthood) from the intermediate plate into the drop of gel (Fig 2.4). Once the drop of gel is set (~15 mins), 6ml of Pluronic gel is poured on top of the drop, forming a gel layer that is 0.7mm thick, this is also left to set (~10 mins). 25µl drop of a concentrated solution of OP50 (cultured in 8ml of LB and concentrated 10x fold) is added on top of the gel layer above the gel drop containing the worms. The OP50 acts as a chemoattractant, encouraging the *C. elegans* to burrow towards it.

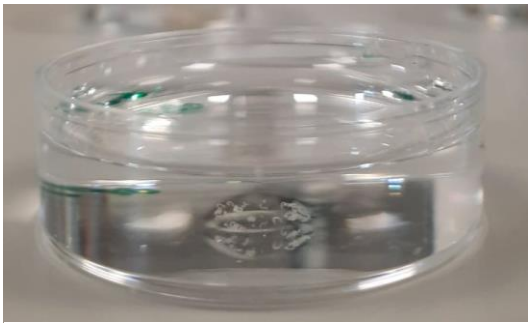

Figure 2.4 - Photo of C. elegans in a drop of Pluronic gel with 0.7cm layer of gel on top.

A timer starts once the chemoattractant is added. From here, the number of worms that have burrowed up to the OP50 drop are counted and removed from the plate using an eyelash pick. This is done for 4h 15m.

Pluronic gel

26g of Pluronic gel (solid) is added into a 250ml glass bottle and dH_2O is added to 100ml. This is incubated at 4°C to dissolve, while intermittently mixing with a magnetic stirrer for around 10 minutes to help to dissolving process. This is left overnight at 4°C to fully dissolve, producing a clear viscous gel. At room temperature (~20°C) the gel begins to solidify. During preparation, the Pluronic gel is kept in an ice bucket to maintain viscosity and ensure easy pouring of the liquid.

Contractability assay

The contractability assay was done alongside the burrowing assay, using N2 worms at the same stages of life (day 1, 4 and 7 of adulthood). For this 2 plates were set up with a copper ring melted into each of them. The copper ring was heated under the Bunsen burner and placed and help on the NGM plates (with no food). The copper rings were used as they contain the worms in a small area, making them easier to track and record. One of the plates was

clean with just NGM and nothing added. The other plate had a 100µl of 75µM levamisole added on top and spread with a glass spreader. Once soaked into the plate, the copper ring was added.

To begin, 10 N2 worms were picked from the original plate of worms and transferred into the copper ring on the plate with no levamisole. The worms were then recorded using worm tracker for around 30s as they crawl around the area within the copper ring. Next, those same 10 worms were transferred into the copper ring on the plate that has been soaked in levamisole and quickly recorded using worm tracker for around 4-5 mins or until all the worms had paralysed.

For the analysis, the videos were screenshotted at the point where the worms were seen the most clearly (and separated) on the pre-levamisole plate and when they were all paralysed on the post-levamisole plate. The lengths of the worms where measured using image J (example of an image can be seen in figure 4.7)

Statistical analysis

All analysis done is outlined in more detail in the results section. Programs used for analysis included excel, ImageJ and GraphPad Prism. The statistical tests for each of the results are specified in the figure legends of any graphs and statistical figures.

Chapter 3: Investigating the effects of the microbiome on a *C. elegans* Alzheimer's Model

Introduction

The paralysis phenotype

The GMC101 strain used for the paralysis assays produces $A\beta_{1-42}$ strands within the *C. elegans* muscles, which elicits a progressive paralysis phenotype that could be monitored. Paralysed worms were identified when the worm was immobile, or unable to form the classic S-shape with their bodies as the crawl on the plate (Fig. 3.1a). To confirm that the worms were not in a quiescent state or dead, the plate was firstly lightly tapped on a flat surface to cause vibration and encourage any healthy worms to crawl. Then when identified, the potentially paralysed worms would be prodded lightly on the head with the platinum pick. If the worms were paralysed they would usually wiggle their heads but be unable to move their bodies - any healthy worms would usually crawl away. Finally, as can be seen in figures 3.1b and 3.1c paralysed worms would often have eggs surrounding them because they were able to lay eggs through the vulva but unable to crawl away due to the paralysis.

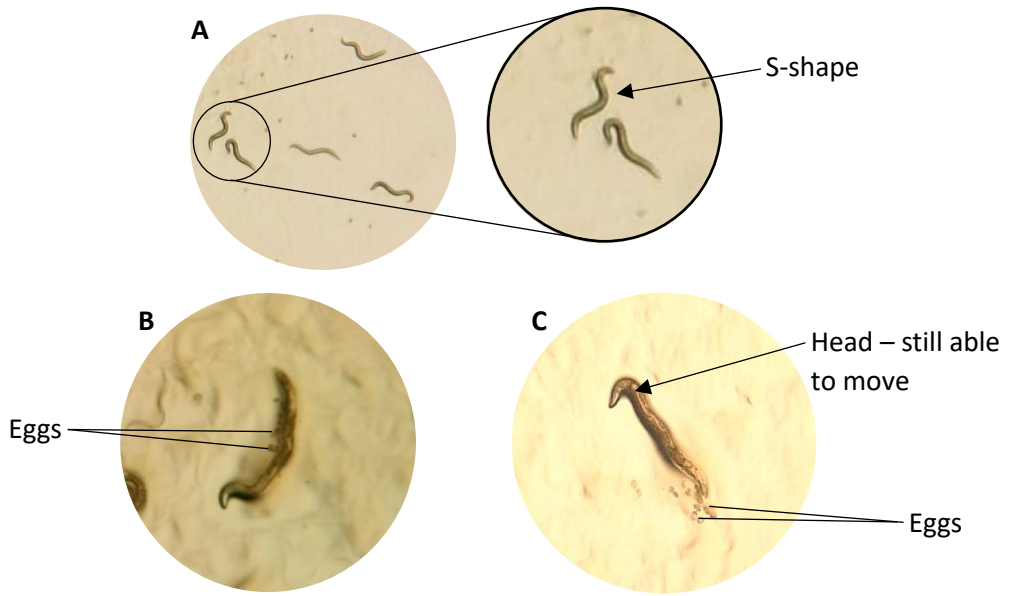

Figure 3.1 – Healthy and paralysed C. elegans*. (a) N2 day 2 adults representing healthy C. elegans forming the S-shape with their bodies as they crawling on the plate. (b) and (c) GMC101 day 3 adults that have paralysed, images taken from two different plates. All Images were taken through a dissecting microscope.*

The effects of an experimental microbiome diet on an Alzheimer's model (GMC101)

There have been many investigations to explore the effects of an experimental microbiome diet on various health and disease parameters in the Ezcurra lab. Previously, the GMC101 Alzheimer's disease model was used to study how a microbiome diet may influence onset of the progressive paralysis phenotype. Data revealed that *C. elegans* grown on the experimental microbiome showed a reduced rate of paralysis when compared to the control (OP50 *E. coli*) (Fig. 3.2a). In particular, there were much lower levels of paralysed worms on day 2 and 3 of adulthood. The sterile cell free media of the experimental microbiome, added on top of OP50, also showed a reduction in paralysis rate, although not as strong as the bacterial strains themselves (Fig. 3.2a).

To test these results in further detail, the same paralysis assay was done using each of the individual strains from the experimental microbiome. This gave some varying results, with a

four of the strains showing a reduction in paralysis rate including, MYb27, MYb56, MYb45 and MYb57.

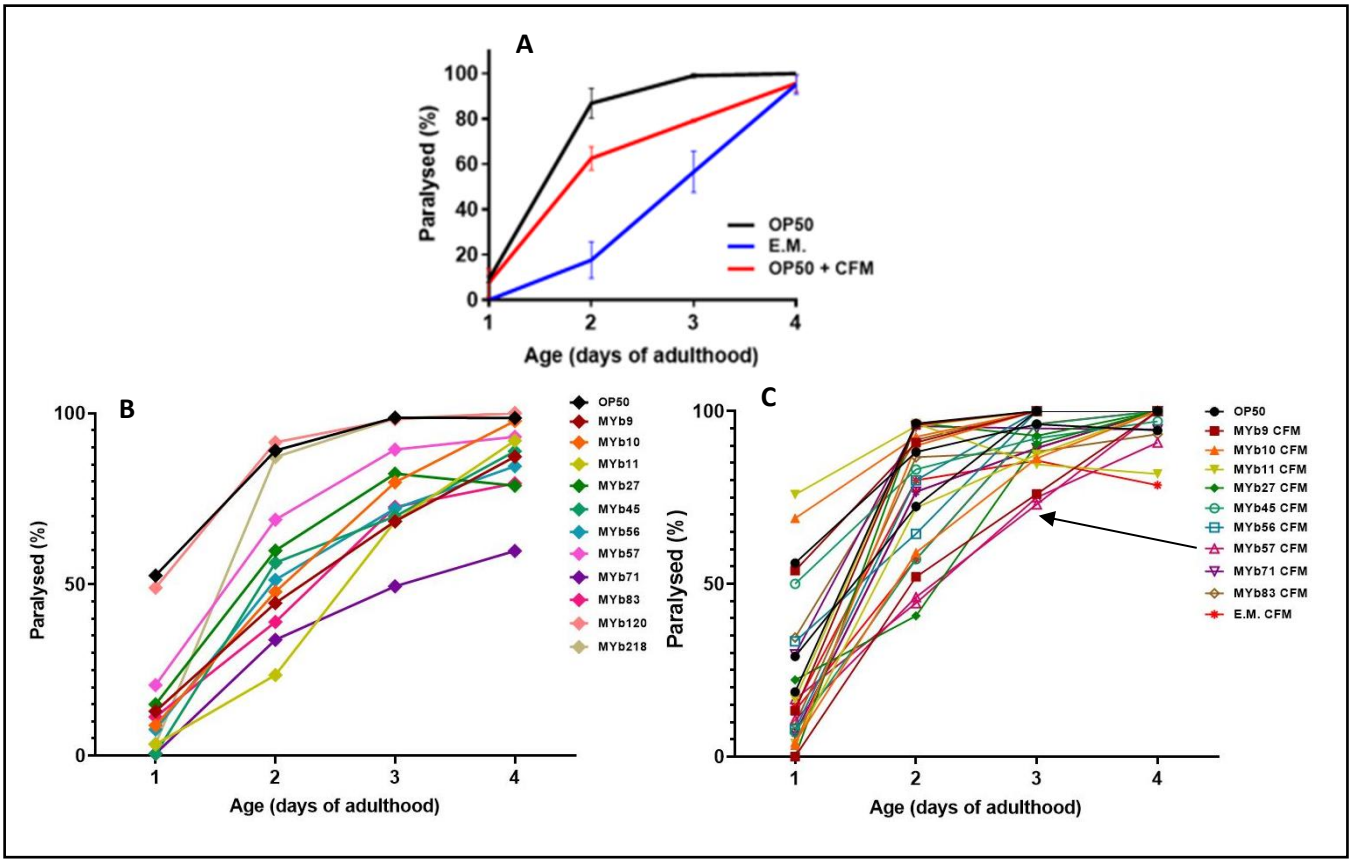

Figure 3.2 - Paralysis rate of GMC101's on different food conditions. *(a) Showing the rate of paralysis of worms fed OP50 as the control compared to the experimental microbiome and its cell free media added on top of an OP50 seeded plate. (b) The paralysis rate of worms fed each of the individual 11 strains within the experimental microbiome and (c) their respective cell free media. All these results and graphs were made by Mireya Vazquez-Prada Ansaldo, a member of the Ezcurra lab.*

Due to speculation that *C. elegans* are unable to grow on certain individual bacteria, three of the strains were tested again, observing their development. It was found that the worms fed on MYb27, MYb56 and MYb45 were all still considerably under developed when the OP50 and MYb57 controls had grown to L4 stage. Continuing to monitor this over a 4 day period, there was no observable development in the *C. elegans* on those 3 conditions. Figure 3.3 shows images of worms that should have been day 2 adults, as seen in figure 3.3d which shows the control worms fed on MYb57. The size of the worms does not clearly show that they are under developed especially since the images were taken at different heights on the

dissecting microscope and so are at different magnification, however, the most obvious difference between the 3 strains and the control is that there are no eggs laid on the plates. As seen in the control, on a plate with day 2 adult worms, you would expect many eggs to be laid, suggesting that MYb27, MYb45 and MYb56 fed worms at this point had not yet even developed to day 1 adults. This would suggest that those bacteria were not a viable option as a protective strain even though previous results may have shown otherwise.

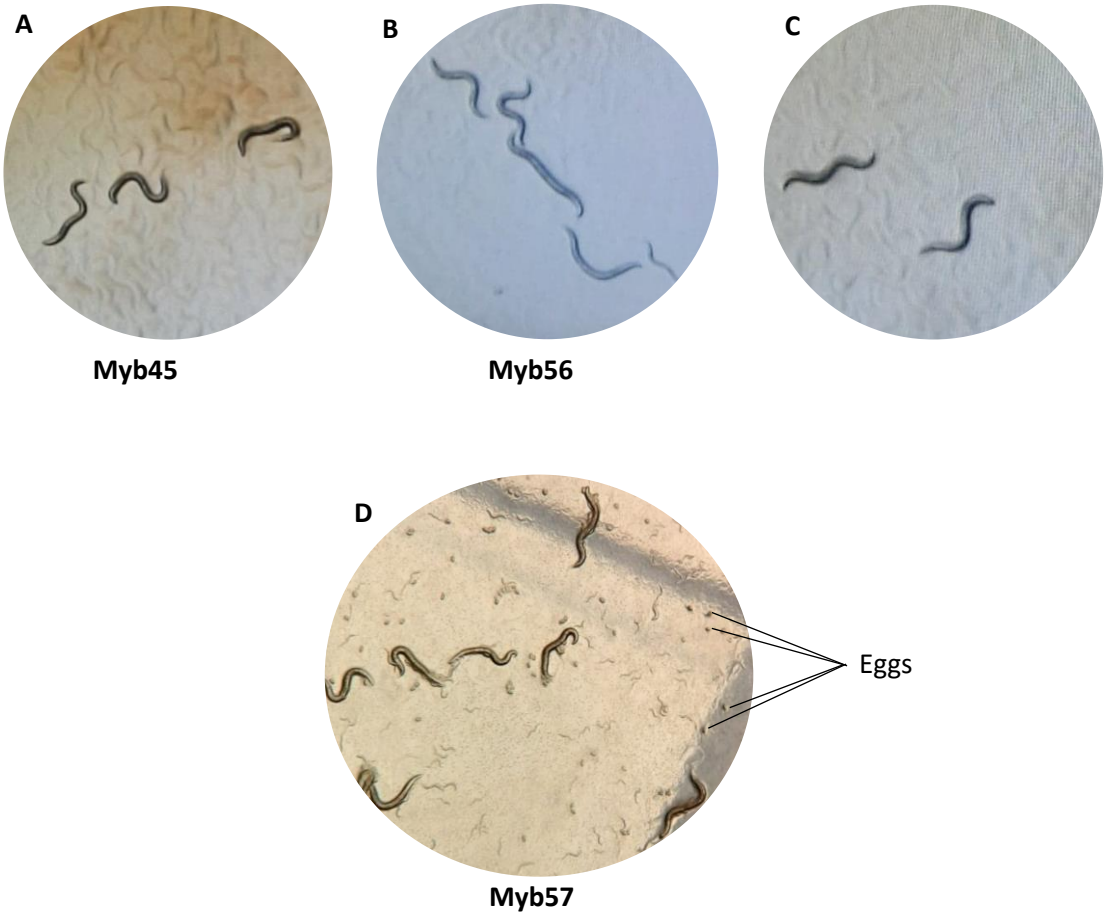

Figure 3.3 - Conditions that C. elegans did not develop on. *Worms on the fourth day after seeding L1's onto plates fed (a) Myb27, (b) Myb45 and (c) Myb56 on which no eggs can be seen as the worms have not developed to adulthood. The control (d) are worms fed on Myb57 which are Day 2 adults with many eggs laid on the plate.*

Figure 3.2c further presents the effects of the individual cell free media of each of the respective bacterial strains that were tested for paralysis. MYb57 CFM added on OP50 was

one of the conditions to show a reduction in rate of paralysis of the GMC101 worms. This would suggest that this bacteria is producing and releasing a compound that is having a beneficial biological effect on paralysis. This made *Stenotrophomonas MYb57* a very interesting strain in regards to a possible approach to treatment of Alzheimer's Disease.

Results

The experimental microbiome and its CFM is protective against paralysis

Confirming those results, my data showed that compared to the standard laboratory food OP50, an experimental microbiome diet showed significantly reduced paralysis on day 2 (EM:∼5% vs. OP: ∼40%, mean percentage of paralysed worms, standard deviation (SD) presented in fig. 3.4a), day 3 (EM: ∼20% vs. ∼75%) and day 4 (EM: ∼40% vs. OP:∼90%) adults (Fig. 3.4a). When testing Myb57 individually as the food source, it also showed significantly reduced paralysis on day 2 (M57: ∼10% vs. OP: ∼34%, mean percentage of paralysed worms, SD presented in fig. 3.4b) and day 3 (M57: ∼35% vs. OP: ∼65%) adults when compared to OP50 (Fig 3.4b.). Although not as strong, Myb57 CFM also showed a significant reduction in paralysis on day 2 (M57CFM: ∼15% vs. OP: ∼34%) and 3 (M57CFM: ∼15% vs. OP: ∼65%) adults (Fig. 3.4). This data would suggest that *Stenotrophomonas Myb57* protects GMC101 *C. elegans* against paralysis, containing a gene that leads to this protection. It further indicates that a protective compound is being produced by Myb57 which it releases into its Cell Free Media. When consuming this compound(s), *C. elegans* are somewhat protected against the paralysis caused by the aggregation of Amyloid-beta in their muscles.

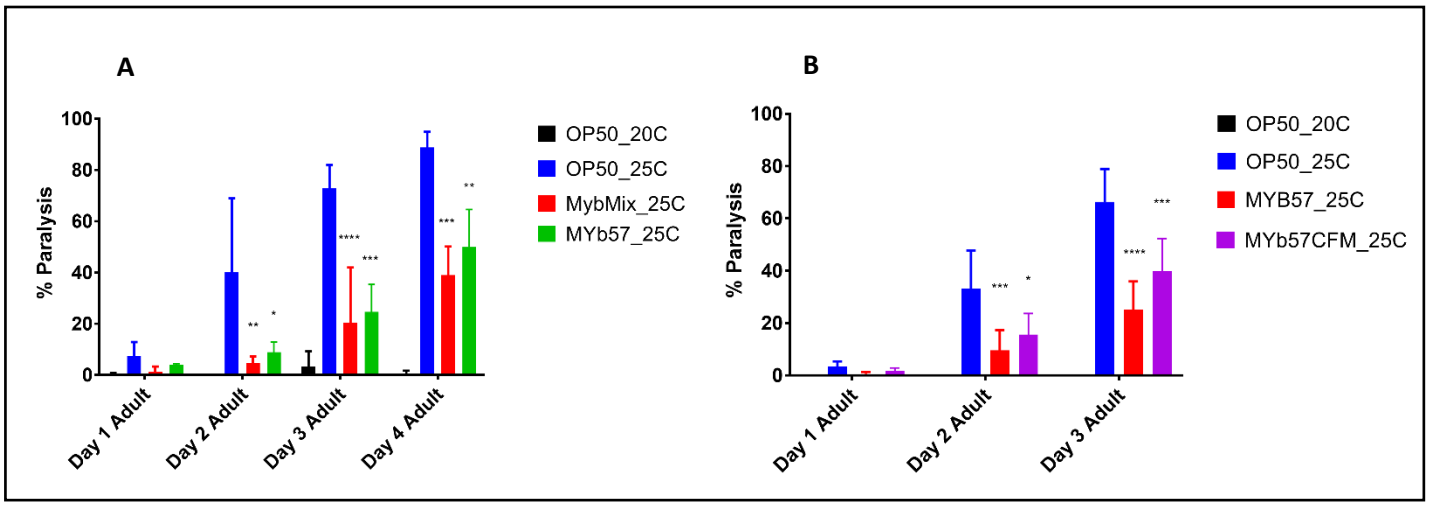

Figure 3.4 - Paralysis Rate of GMC101 C. elegans on differing conditions. (a) *Graph showing the percentage of paralysed worms (n~100 per condition) on day 1 to 4 of adulthood on 4 conditions (1) Fed OP50 E. coli, incubated at 20°C at L4 stage (2) Fed OP50 E. coli, incubated at 25°C at L4 stage (3) Fed MybMix – experimental microbiome – incubated at 25°C at L4 stage (4) Fed Myb57 Stenotrophomonas strain, incubated at 25°C at L4 stage.* ***(b)*** *Graph showing the percentage of paralysed worms on day 1 to 3 of adulthood on 4 conditions (1) Fed OP50 E. coli, incubated at 20°C at L4 stage (2) Fed OP50 E. coli, incubated at 25°C at L4 stage (3) Fed Myb57, incubated at 25°C at L4 stage (4) Fed OP50 E. coli with Myb57 cell free media added on top. Both graphs include data from 3 biological repeats. Asterisk (*) represent statistical significance using Turkey's multiple comparisons test – P-value *<0.0332, **<0.0021, ***<0.0002, ****<0.0001, all comparisons made were to the control, OP50 E. coli diet.*

The *Stenotrophomonas* strains

In order to narrowing down which gene(s) are having this protective effect on the *C. elegans* GMC101 Alzheimer's model, we wanted to use comparative genomics. For this approach, the aim was to investigate whether this gene is species or genus specific. In the case of being species specific, other strains within the genus – that do not possess this protection – could be compared using bioinformatic tools. Since the genomes are highly conserved between species of the same genus, it may allow for large chunks of the genome to be cancelled out, thus, narrowing down which possible gene or group of genes are causing this protection.

Table 3.1 shows the 7 *Stenotrophomonas* strains we decided to compare to MYb57. These strains were all available to order from The Leibniz Institute DSMZ [96]. They were chosen because they all had their genomes sequenced, allowing for comparisons. They were also all sourced from places that *C. elegans* may also be found in the wild, such as soils and plants, increasing the chances of the bacteria being found in the natural *C. elegans* microbiome. The

strains are non-pathogenic to humans, making them safe to use within the laboratory, without putting any extra precautions in place.

***Table 3.1 - The Stenotrophomonas strains**. A summary of the 7 strains of Stenotrophomonas that were chosen to investigate, including strain name, the source from which they were extracted and the medium within which they grow ideally in. All strains and details were found on DSMZ from which they were ordered [96].*

	Strain	**Source**	**Medium**
St 1	*Stenotrophomonas terrae R-32768* [97]	soil	Trypticase Soy Broth Agar
St 2	*Stenotrophomonas ginsengisoli DCY-01* [98]	soil of ginseng field	Nutrient Agar
St 3	*Stenotrophomonas pavanii* [99]	stems of sugar cane (Brazilian variety widely used in organic farming)	Nutrient Agar
St 4	*Stenotrophomonas indicatrix WS40, LMG 29942,* [100]	TSA-contact plate sample of dirty dishes	Nutrient Agar
St 5	*Stenotrophomonas sp. Leaf70*	leaf of Arabidopsis thaliana, wild-type genotype	Trypticase Soy Broth Agar
St 6	*Stenotrophomonas bentonitica BII-R7* [101]	soil, bentonitic formation	Nutrient Agar
St 7	*Stenotrophomonas lactitubi M15* [100]	Milking machine biofilm	Trypticase Soy Broth Agar

The protection of *Stenotrophomonas* is genus specific

To test whether the protection was genus or species specific, the paralysis assay was done using these 7 *Stenotrophomonas* strains to investigate how they affect the paralysis rate of the GMC101's.

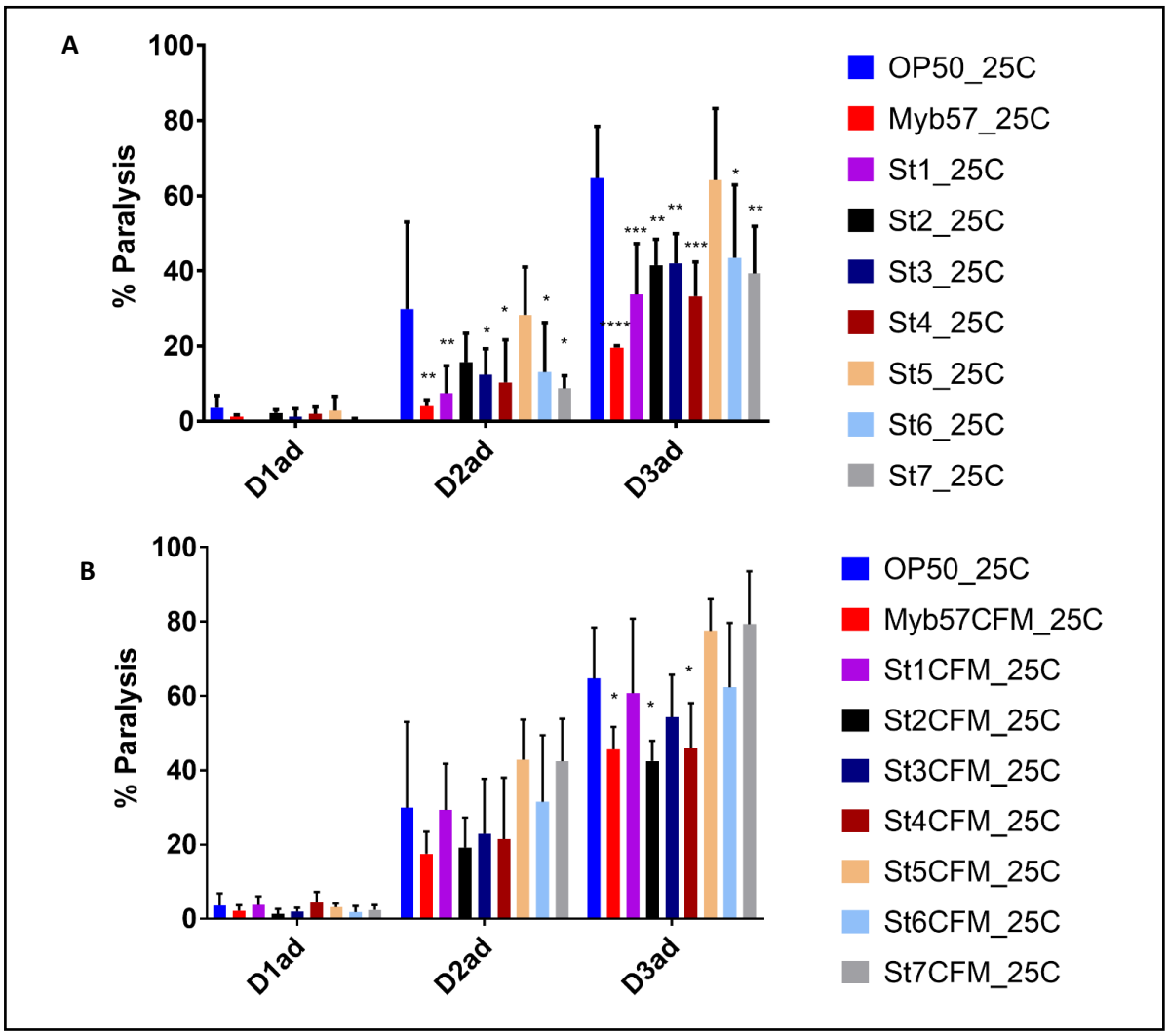

Figure 3.5 - Paralysis Rate of GMC101 C. elegans with the 7 Stenotrophomonas Strains (a) and their Respective Cell Free Media on OP50 (b) as the Food Source.. *Graph showing the percentage of paralysed worms from day 1 to 3 of adulthood when incubated at 25°C at L4 stage.* ***(a)*** *The worms were fed each of the Stenotrophomonas strains with OP50 and Myb57 as the controls. This includes data from 3 biological repeats.* ***(b)*** *The worms were fed on OP50 seeded with the cell free media of each of the respective Stenotrophomonas strains. The two controls were OP50 and Myb57 cell free media. This includes data from 3 biological repeats. The Asterisk (*) represent statistical analysis using Fisher's LSD test – P-value *<0.0332, **<0.0021, ***<0.0002, ****<0.0001. No asterisk indicates no statistical significance. All comparisons were made to the OP50 diet.*

The results showed that all of the *Steno*- strains are protective against paralysis to some extent (Figure 3.5a). MYb57 still has the strongest protection on day 2 (M57:~5% vs. OP:~30%) and 3 (M57: ~20% vs. OP: ~65%) adults. On day 2 of adulthood, St1 (~8%), St3 (~12%), St4 (~10%), St6 (~14%) and St7 (~10%) fed worms all show significantly lower levels of paralysis compared to OP50 (~30%) fed worms. On day 3, the differences are even more substantial, with St1 (~30%), St2 (~40%), St3 (~42%), St4 (~32%), St6 (~44%) and St7 (~39%) showing significant protection compared to OP50 (~65%). The *Steno* 5 strain shows slight protection but not enough for statistical significance. This may suggest that this strain may be lacking in the protective gene that the other strains have. However, considering the rest of the results, and the fact that only 3 repeats have been pooled, it is more likely that the assays did not show St5 as protective by chance. The results overall would suggest that this protection against paralysis is genus specific. To confirm this further, other colleagues within the Ezcurra lab that have repeated this assay using the St5 strain have observed it to be protective.

The cell free media of each of the *Stenotrophomonas* strains showed some more varied results . The *Stenotrophomonas* strains, including MYb57, showed no significant reduction in the percentage paralysis of the GMC101's in day 2 adults. Looking at figure 3.5b, there does seem to be some general reduction in percentage paralysis of worms fed OP50 with added MYb57 CFM (~18% vs. ~30%, mean percentage of paralysis vs. the control OP50, SD can be seen in fig. 3.5b), St2 CFM (~19% vs. ~30%), St3 CFM (~22% vs. ~30%) and St4 CFM (~20% vs. ~30%).

On day 3 of adulthood, as expected OP50 with MYb57 CFM added did show significance in the reduction of paralysis (~44% vs. ~65%). In addition, St2 CFM (~40% vs. ~65%) and St4

CFM ($\sim 44\%$ vs. $\sim 65\%$) were the two *Stenotrophomas* strains whose cell free media showed a significant protection against paralysis. Although not significant, St5 CFM (~54% vs. ~65%) also showed a good reduction in percentage paralysis when compared to OP50. This would suggest that adding the cell free media of some (St2 and St4) of the *Stenotrophomas* strains, including MYb57, on OP50 has a protective effect against paralysis. However, the protection from cell free media is not as strong as from the bacterial strains themselves.

From these results, it could be hypothesised that *Stenotrophomonas MYb57* contains a gene(s) that have protection against the formation of Beta-Amyloid in the *C. elegans* GMC101 strain, or protect against the paralysis phenotype that the Aβ results in. These result would suggest that the protective gene is conserved within the *Stenotrophomonas* genus. Since the cell free media also protects to some extent, there must be a compound that is released by MYb57, that when consumed by the *C. elegans*, provides this protection.

Discussion

Chapter 1 focused on researching how an experimental microbiome effects a *C. elegans* Alzheimer's disease model, with a particular interest in the *Stenotrophomonas* bacterial genus. The aim was to investigate whether there was any potential in using a microbiome approach towards a therapeutic intervention against Alzheimer's disease.

Targeting β-Amyloid for treatment against Alzheimer's disease

Induced expression of β-Amyloid in the muscles of the GMC101 *C. elegans* strain allows for a rapid and reproducible paralysis phenotype that can be used to monitor treatments that modulate Aβ [80]. The paralysis assay was used to quantify the effects of multiple bacterial strains (or mix of bacteria) as a dietary source on Aβ toxicity. Since Aβ toxicity is believed to be a major cause of Alzheimer's disease in humans, it makes it a key therapeutic target [102]. However, as of yet, targeting amyloid-beta towards a therapeutic intervention against Alzheimer's disease have been unsuccessful [103]. Evidence has shown that amyloid is often deposited in the early stages of Alzheimer's disease, even before symptoms are noticed [104]. Therefore, it has been suggested that these trials have failed as they have been insufficient to prevent further progression of the disease, even in mild-to-moderate patients [104]. The tau protein is another target towards a therapeutic approach. As part of the AD pathology, phosphorylation of tau leads to them dissociating from the microtubules and forming aggregates into neurofibrillary tangles [105]. This has led to many therapeutic approaches towards AD to focus on kinases and phosphatases that cause these abnormal tau phosphorylation [106]. More modern approaches have been towards immunotherapies that target Aβ inflammation [107]. Solenezumab is an example of an anti-Aβ monoclonal antibody which targets the $A\beta_{13-28}$ region, and has been shown to improve cognitive defects in some

mouse models of AD [107, 108]. Overall, although there is strong evidence that Aβ has an important role in AD pathology, there is a strong debate on whether Aβ is an appropriate therapeutic target. It has been suggested that more inclusive perspective are needed for an effective Alzheimer's disease treatment, due to it being a multi-factorial and complex disease [104].

A microbiome approach towards therapeutic interventions against AD

A microbiome approach was taken because there have been multiple studies that have shown the important role that the microbiota has on the majority of human biological pathways [109]. There has been convincing evidence that there is a link between Alzheimer's disease and the diversity and composition of the gut microbiota [110]. This led to the hypothesis that there may be specific bacteria within the microbiome that have a beneficial effect in preventing or slowing down onset of AD symptoms. Recent research has been focusing on the manipulation of the gut microbiota as a way of treating Alzheimer's disease, mainly through faecal transplants and probiotic/symbiotic use [111,112]. One mouse study investigated how faecal transplants affected Alzheimer's symptoms and pathologies, using an AD-like pathology with amyloid and neurofibrillary tangles ($\mathrm{ADLP}^{\mathrm{APT}}$) transgenic mouse model [113]. Their results showed that AD-mice that were exposed to a healthy microbiota over a 4 month period showed improved cognitive function, Aβ burden and tau pathology [113]. Another investigation tested a symbiotic as a potential treatment using a *Drosophila melanogaster* AD model. Three probiotic strains - *Lactobacillus plantarum, Lactobacillus fermentum* and *Bifidobacteria longum spp. Infantis* (1.0 x 10^9 CFU (colony forming unit)/ml each) – combined with 0.5% of TFLA powder increased the motility and survivability of the flies and also rescued Aβ deposition [114]. Their study suggested that optimised symbiotic

formulations could be an effective preventative therapy for Alzheimer's disease without harmful side effects [114]. Probiotics have also been investigated on human AD patients in a randomised, double-blind, controlled clinical trial [115]. This study showed that probiotic milk contain *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum* and *Lactobacillus fermentum* (2.0×10^9 CFU/g each) over 12 weeks showed a improvement in a mini-mental state examination score compared to controls [115]. All this research shows very promising approaches in AD treatment. *C. elegans* has proved to be a powerful model organism towards this goal. They have complex biochemical pathways, many of which are conserved through their mammalian counterparts [116]. They have genes that are correlated with AD including an APP-related gene, apl-1, and a tau homolog, ptl-1, which open the door for more specified models of AD [116]. *C. elegans* could be used to test more of the bacteria found in probiotics - such as those mentioned in the studies discussed – that could be more closely related to humans and show which strains could have the strongest effects in preventing pathologies of Alzheimer's disease. However, finding a potential probiotic bacterial species may not be most effective potential intervention, mainly because a whole bacterial species has a complex and varied biology, with multiple functions and pathways. We wanted to possibly find and isolate a compound, which was shown to be produced and released into the *Stenotrophomonas* Myb57 cell free media. This could lead to a more specific drug that targets only Aβ leading to more efficient and consistent beneficial results.

Results from the paralysis assay showed that the experimental microbiome (MybMix) had a significant protective effect against the paralysis phenotype compared to the control, *E. coli* OP50 (Fig. 3.4). The sterile cell free media of the experimental microbiome, added on top of OP50, showed a less potent, yet still significant protection. From previous results, it was decided that one of the 11 bacterial species, *Stenotrophomonas sp. Myb57*, had the most

interesting effect against paralysis and therefore became the focal bacteria in this project. Myb57 alone as a food source showed a significant reduction in the paralysis of GMC101 worms, and similar to the MybMix, its cell free media had a significant – but less potent – protective effect on paralysis (Fig. 3.4b).

These results would imply that this species contains a gene and also releases a compound (as would be found in the cell free media) that causes protection against Aβ toxicity when ingested. Students in the Ezcurra lab attempted to isolate the biological compound within the CFM that was having this effect, however, this proved challenging due to the loss of bioactivity of the bioactive compound during biochemical extraction. Therefore, on joining the lab, the approach was to narrow down the possible functioning gene.

Seven other *Stenotrophomonas* strains were tested against the paralysis phenotype, with Myb57 and OP50 as the controls. All the strains showed protection against paralysis – although not as strong as Myb57 (Fig. 3.5a). This would suggest that the protective gene is conserved throughout the *Stenotrophomonas* species. Their respective cell free media, however, did not show as strong a protection. Only 2 strains showed significant reduction in paralysis, St2 (*Stenotrophomonas ginsengisoli*) and St4 (*Stenotrophomonas indicatrix*). The rest of the *Stenotrophomonas* cell free media's showed varying protection, with some showing no protection at all (Fig. 3.5b). A possible reason for it being a less strong effect may be because only a limited amount of the compound is released into the CFM. When the *C. elegans* eat the bacteria itself, they grind it releasing the maximum amount of the compound, which in turn provides the maximum effect. Since the cell free media of some of the strains, such as St5 and St7, showed no protection at all, it could be suggested that there is a non-conserved transporter that allows for the compound of interest to be released.

Other studies have shown interesting results in a *Drosophila melanogaster* Alzheimer's disease model. This transgenic AD model causes expression of the human $A\beta_{1-42}$ in the eyes of the *Drosophila*, leading to eye degeneration characterised by the rough eye phenotype [117]. This is used as a way of showing varying degrees of the neurotoxic effects of degeneration [117]. Tan *et. al's* study showed *Stenotrophomonas* to have a beneficial role in preventing neurodegeneration [118]. They also showed that administering *Lactobacillus plantarum* DR7 as a probiotic restored the microbiota diversity, which lead to an increase in *Stenotrophomonas* and *Acetobacter* [118]. Other Lactobacillus species were also shown to reverse the effects of Aβ toxicity in this model, showing another potential bacterial focus that may be isolated from the microbiome [117]. These results would suggest further that *Stenotrophomonas* is an interesting genus to investigate in regards to its beneficial effects towards AD. Much of the literature testing the microbiota as a therapeutic source against Alzheimer's and other degenerative diseases focus on different bacterial strains, however, *Stenotrophomonas* has shown to be a potential for novel therapeutic techniques [118]. Looking at our experimental microbiome, it still had the most powerful protective affect against paralysis. Showing the potential for other bacterial strains that may also have interesting effects against AD.

Summary

To summarise, these results would suggest *Stenotrophomonas* as a bacterial genus of interest when searching for innovative therapeutic interventions towards Alzheimer's disease. In a *C. elegans* AD model, it has shown significant protection against the progressive paralysis phenotype observed. Some species within the genus also release a compound that shows protection when added on top of their usual laboratory food, *E. coli*. This compound could

possibly be produced by *Stenotrophomonas* as a defence mechanism. Many bacterial species produce amyloid in high quantities [67]. These amyloids form aggregates that have a functional role in the bacteria [119]. For example, Curli are amyloids that are components of the extra-cellular matrix which is required for formation of biofilms [119, 120] Furthermore, in some pathogenic species – such as *E. coli* and *Salmonella spp.* – generate amyloid which gives them an advantage in tissue invasion and colonisation [119]. However, when unchecked, these aggregates can become toxic and lead to rapid cell death due to a loss of function of essential proteins [119]. Therefore, bacteria have developed different strategies for detoxifying these aggregates and ensuring sophisticated protein quality control [119]. This could therefore be a reason for bacteria, such as *Stenotrophomonas*, to have evolved to produce a compound that is able to inhibit the aggregation of amyloid beta. Moreover, the CNS amyloids produced in humans (and also in our GMC101 AD model) are very similar in biological structure to the those produced by bacteria [67]. This could be the reason the compounds produced by bacteria are also able to prevent or breakdown the aggregation of human $\mathrm{A}\beta_{1-42}$, having the protective effects that have been observed in our data. Unfortunately, since the protection is genus specific, we were unable to use a comparative genomics to narrow down the functioning gene and so new approaches will be used to continue this research, and hopefully, get closer to the goal. This could lead to a whole range of natural products – made by gut microbes – that could be used to improve all aspects of human health, due to all the areas of the body that the microbiota have been shown to influence.

Future research

In the next steps for these investigations, the Ezcurra lab will be using transposon insertion to create mutants of *Stenotrophomonas*. This technique has been successfully applied to different bacterial strains to effectively create knock-down mutants [121]. The *Stenotrophomonas* strain that is chosen will have to be one of the strains that shows protection from its CFM, but is also resistant to the antibiotic that is used in the protocol. These mutants can then be screened for paralysis to see if any part of the genome being deleted leads to a lack of protection. Those mutants can then be sequenced and the specific genes within it can help us narrow down which gene is protective. However, this technique will make 100's of mutants, therefore, the screening process needs to be optimized for speed and efficiency.

As another way to take these results further, similar experiments could be designed and executed using other model organisms to see if the results are reproducible in different species. Using *Stenotrophomas* as a food source for the *Drosophila melanogaster* Alzheimer's disease model and comparing to a control food could be one option. But even further, the research could be proceeded on mammalian models, such a mice. Most mouse models that are used to model AD are made to overexpress Aβ, however, these have drawbacks as they also overproduce other proteins [122]. Recently, a new mouse model has been created that produces normal levels of β-amyloid, which can be monitored through the development of behavioural changes and cognitive impairment are they age [122]. This provides a novel way of researching effects of these bacterial strains on a model that resembles many symptomatic patterns seen in humans with Alzheimer's disease. These mice, for example, could be introduced to *Stenotrophomonas* as a probiotic source, and compared to germ-free mice.

Furthermore, to remove any possible experimental bias, it may be interesting if the results could be repeated in a 'blinded experiment' [123]. For this, a colleague – who is ideally unaware of the experimental objective – could re-label the plates so that the experimenter would not know which condition is which. The only difficulty with this is that the bacterial lawn of each of the *Stenotrophomas* species is slightly distinct (in colour and thickness). However, they are all quite different, rather than just one with a unique visible difference, so shouldn't influence the experimenter too much.

Strengths and weaknesses

Overall, the paralysis assay was a very effective way of quantifying the effects of the different bacterial strains on Aβ-toxicity. The protocol had been used and refined in the Ezcurra lab by the senior students and was ideal for the equipment and reagents that were available. Furthermore, this is a protocol that has been used for different application by the scientific community as a reliable and robust experiment [123, 124]. It was simple to set up, however, it was also time consuming as worms were counted every day for 4 days and transferred for 2 days. This was not a drawback in this particular set of experiments because there were not too many conditions. However, this would not be as efficient for an experiment that required screening of many different conditions (such as more bacterial species).

Furthermore, the majority of the paralysis scoring was done by the same individual, as the analysis of a paralysed or non-paralysed worm could be subjective. It is important to note that on some occasions the scoring was done by another member of the lab, however, we tried to be as consistent as possible by having a clear definition of paralysis (as shown in figure 3.1). To further promote consistency, we tried to avoid any variables that may affect the rate of paralysis during the assay. The same NGM was used for all experiments, as well as the same

volumes of bacteria (250µl) and CFM (250µl) used for the conditions. It is also important to note that throughout the assay, there was some contamination on the plates (mostly fungi but also bacteria). This may have been consumed by the *C. elegans*, possibly effecting the results. To control this, sterile techniques were used throughout the preparation and execution of the assay. Notes were made in the lab book if any contamination occurred and in the event of a small amount of contamination, worms were transferred to clean plate with the same bacterial condition, and the experiment continued. When large amounts of contamination occurred, that condition was discarded from that repeat in order to prevent skewing any of the results.

Overall, many repeats were done for each of the conditions and the results were generally consistent. Another member of the Ezcurra lab also found similar results when repeating the same experiment themselves. Therefore, it can be concluded that the data is robust and suggestions made are reliable.

Chapter 4: Investigating the effects of the microbiome on the aging muscle function and strength in *C. elegans*

Introduction

Previous research

In the GMC101 strain, β-Amyloid forms within the body wall muscles causing a progressive paralysis phenotype. Since the experimental microbiome shows protection against this paralysis, one suggested reason may be that the microbiome diet has an effect on the muscle cells, making them less prone to proteotoxicity effects of the Aβ strands. Previously in the Ezcurra lab, a thrashing assay quantified the number of thrashes that worms did in liquid on different food conditions as the worms aged. Figure 4.1 shows that worms fed *E. coli* OP50 started at a high thrashing rate as day 1 adults ($\sim$100 thrashes $30s^{-1}$) which declined sharply as they aged to day 11 of adulthood ($\sim$65 thrashes $30s^{-1}$). On the other hand, worms fed the experimental microbiome started at a lower thrashing rate on day 1 of adulthood ($\sim$78 thrashes $30s^{-1}$), however, showed practically no decline in rate of thrashing as the worms aged showing higher thrashing rate on day 11 of adulthood ($\sim$75 thrashes $30s^{-1}$) compared to the control. It is also important to note that the cell free media of the experimental microbiome had no effect on the thrashing rate when compared to the control. These results would suggest that there may be an effect that the experimental microbiome is having on the thrashing rate, and therefore possibly muscle strength, as the *C. elegans* age.

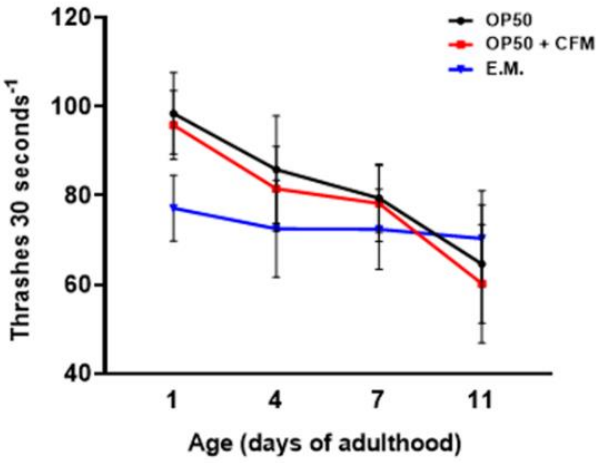

Figure 4.1 - Thrashing rate of C. elegans on an experimental microbiome diet compared to control. *Data gathered by Antonis Karamalegkos in the Ezcurra lab, comparing the thrashing rate of C. elegans (n~100 per condition) in liquid, of day 1, 4, 7 and 11 adults. OP50 was used as a control, the food conditions were the experimental microbiome and the cell free media of the experimental microbiome on OP50. Data were pooled from 10 independent trials and represent means ± SD.*

In this chapter, the aim is to further test whether an experimental microbiome diet has any effect on the muscle health of the worms throughout aging. The focus was on the muscle cell shape and size and also the muscle function of the *C. elegans*.

Results

The experimental microbiome diet has no effect on morphology

The RW1596 strain was used to image the body wall muscle cells of the *C. elegans* using epifluorescence. The roller worms can be identified as worms on the plate that roll sideways as they claw on the plate, and are often found in a C-shape moving around in a circle. The roller worms have a myosin promoter expressing a GFP reporter. Under green fluorescence, this strain fluoresce the myosin fibres within the muscle cells, showing distinct lines forming the muscle cells. The cells that were used for analysis where those that were clear and easy to distinguish, and any worms that had rolled or twisted positions – as they paralysed on the slide prepared for imaging (see methods section) - were not used. The analysis was done using ImageJ, in which the cells were individually outlined and relative area was measured.

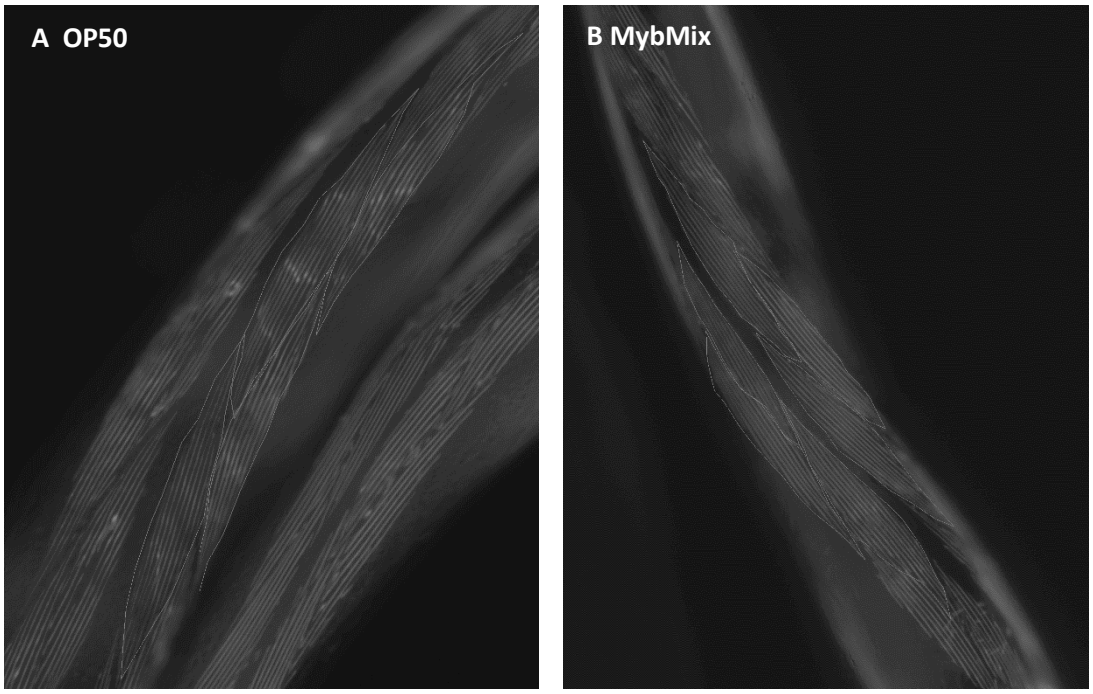

Figure 4.2 - Worm body wall muscle cells. *Images showing RW1596 day 1 adults fed (a) OP50 and (b) MybMix under fluorescence, presenting the myosin fibres of the body wall muscles. Examples of the cells used in the analysis are outlined. Images were taken at 40x oil immersion using a DSMR microscope. Image has been edited (+40% brightness, -40% contrast) for better clarity of myosin strands – See figure 4.3 for original image.*

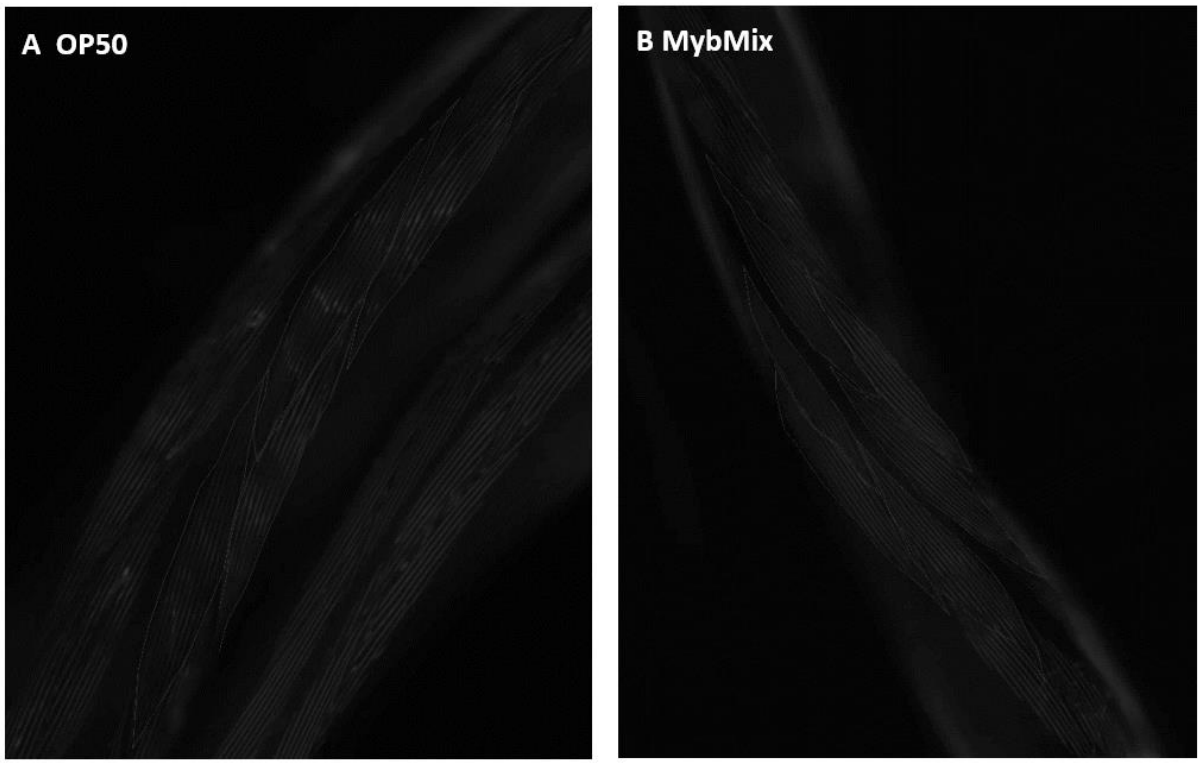

Figure 4.3 - Worm body wall muscle cells. *Images showing RW1596 day 1 adults fed (a) OP50 and (b) MybMix under fluorescence, presenting the myosin fibres of the body wall muscles. Examples of the cells used in the analysis are outlined. Images were taken at 40x oil immersion using a DSMR microscope. Original images.*

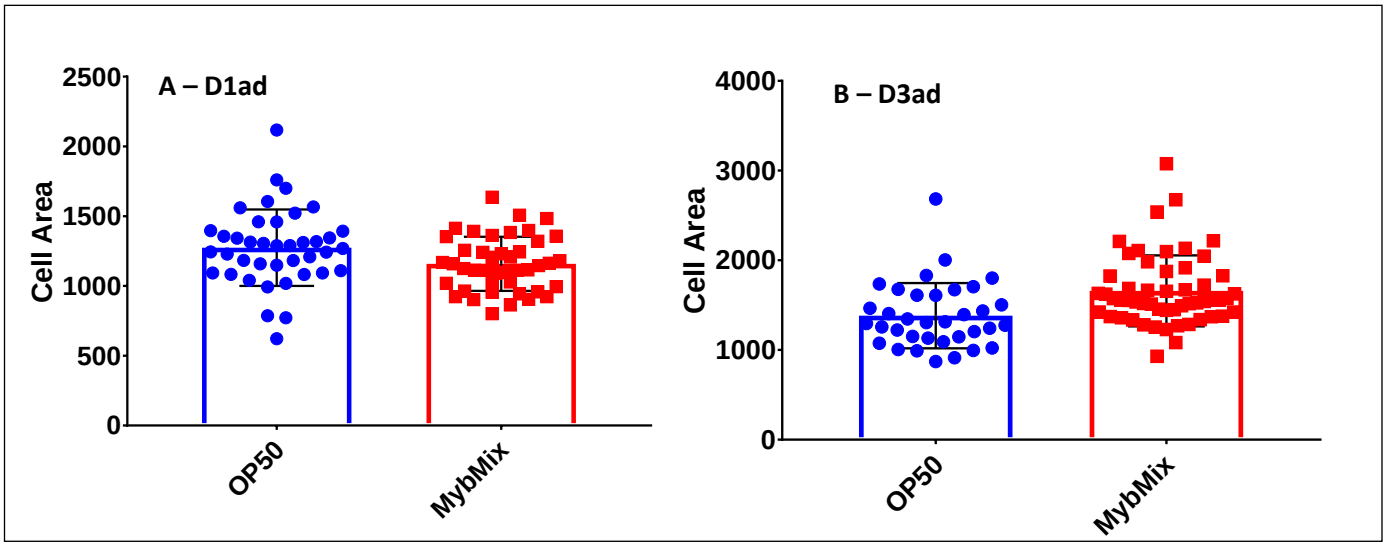

Figure 4.4 - Comparison of average cell sizes. *The average cell area of worms fed OP50 and MybMix on (a) day 1 and (b) day 3 of adulthood. Data was found using measures from GraphPad Prism, averaging from around 40-50 cells per condition and one repeat per condition. Data represent mean cell area (as measured using ImageJ (no units) ± SD and pooled from a single trial. No statistical significance was found between food sources or age.*

From figure 4.2 it is clear that all of the cells generally have a similar diamond shape, with no big differences in the spaces between fibres. The cells can be easily distinguished from one another and are lined up neatly in two rows of overlapping cells along each side of the body. As shown in figure 4.4, there is no significant difference between the average size of Day 1 adults fed on OP50 (1274, measurements made using ImageJ, there are no units, figures are relative to one another) vs. MybMix (1158). Although there seems to be a bigger difference in average size in day 3 adults fed OP50 (1382) compared to MybMix (1658), this is a small difference that is most likely due to chance. It can therefore be concluded that the experimental microbiome diet does not have an effect on the size or shape of the body wall muscle cells of *C. elegans* in early adulthood.

The experimental microbiome has no effect on gaps within the muscle cells

Throughout this analysis, any gaps between the filaments within the cells were also assessed and measured. It has been suggested that the deterioration of filaments that lead to large gaps may contribute to defects in muscle function [125].

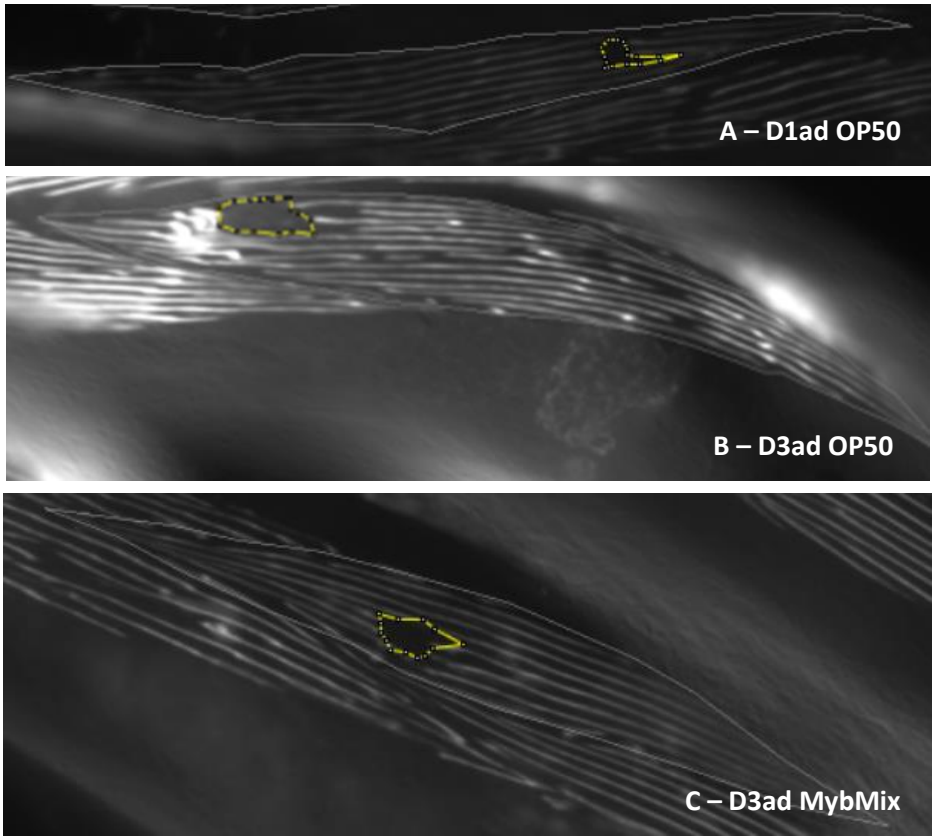

Figure 4.5 - Gaps found in C. elegans body wall muscles. *These images show some examples of the gaps found in each of the conditions analysed (a) Day 1 adults on OP50 diet, (b) Day 3 adults on OP50 diet and (c) Day 3 adults on MybMix diet - there were no gaps found in D1ad MybMix fed worms. The cell is outlines with a fine grey line and the gap is outlines with a yellow dotted line. These were measured for area within the analysis. All images were taken of RW1596 at 40x oil immersion using a DSMR microscope under a green fluorescence filter.*

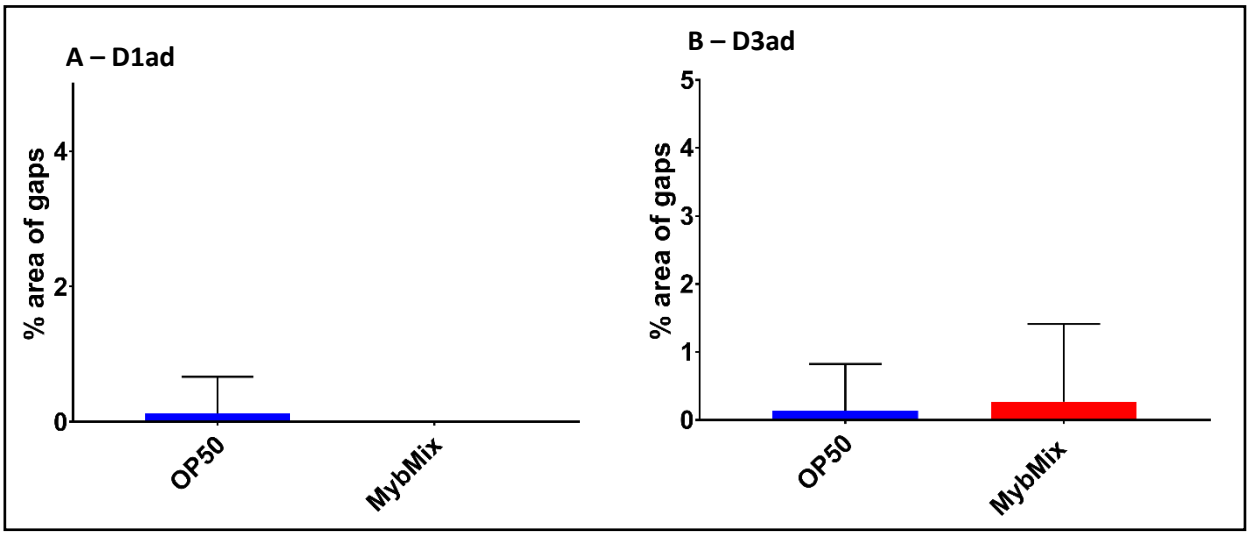

Figure 4.6 - Comparison of percentage area of gaps of OP50 and MybMix fed worms. *Graphs representing the % area of gaps of (a) day 1 and (b) day 3 adults fed OP50 and the experimental microbiome (MybMix). The majority of cell had no gaps (0) making the average area very low (40-50 cells were analysed per condition). No gaps were found in D1ad MybMix fed worms. Data represents average % area of gaps* ($\frac{area\ of\ gap}{area\ of\ whole\ cell} \times 100$) *± SD. All figures were measured using ImageJ (no units) and were pooled from a single trial. No significance found between food sources or age.*

Overall, both in day 1 and day 3 adults, not many gaps were found on either condition. As seen in figure 4.5 the gaps that were found were quite obvious but small. There were no gaps found in the MybMix fed day 1 adults, but since there were only an average of 2 cells found to have gaps in all other conditions, this is most likely due to chance. There is no significant difference between the percentage area of gaps in OP50 fed worms compared to MybMix fed worms in both day 1 and day 3 adults (Fig. 4.6). There seems to be slightly more gaps in the day 3 adults on a MybMix diet compared to day 1 adults, however, this difference is very minimal. These results would suggest that an experimental microbiome diet has no effect on the amount or size of any gaps found in the body wall muscle cells. This further indicates that the experimental microbiome diet does not have any influence on the muscle cell structure and integrity in the *C. elegans* first days of adulthood.

The experimental microbiome may maintain muscle function as worms age

To test the muscle function - measuring strength and motility - of the *C. elegans*, a burrowing assay was performed, in which the worms had to burrow through a layer of gel towards a chemoattractant. It has been suggested that muscle function that is assessed by muscle strength and power could be a better indicator of frailty than muscle size [126]. As in the thrashing assay, the burrowing rate of day 1, 4 and 7 adult worms was assessed to indicate muscle strength. The aim was to also test day 11 adults, however, within this experiment there were not enough worms alive on day 11 for the tests required.

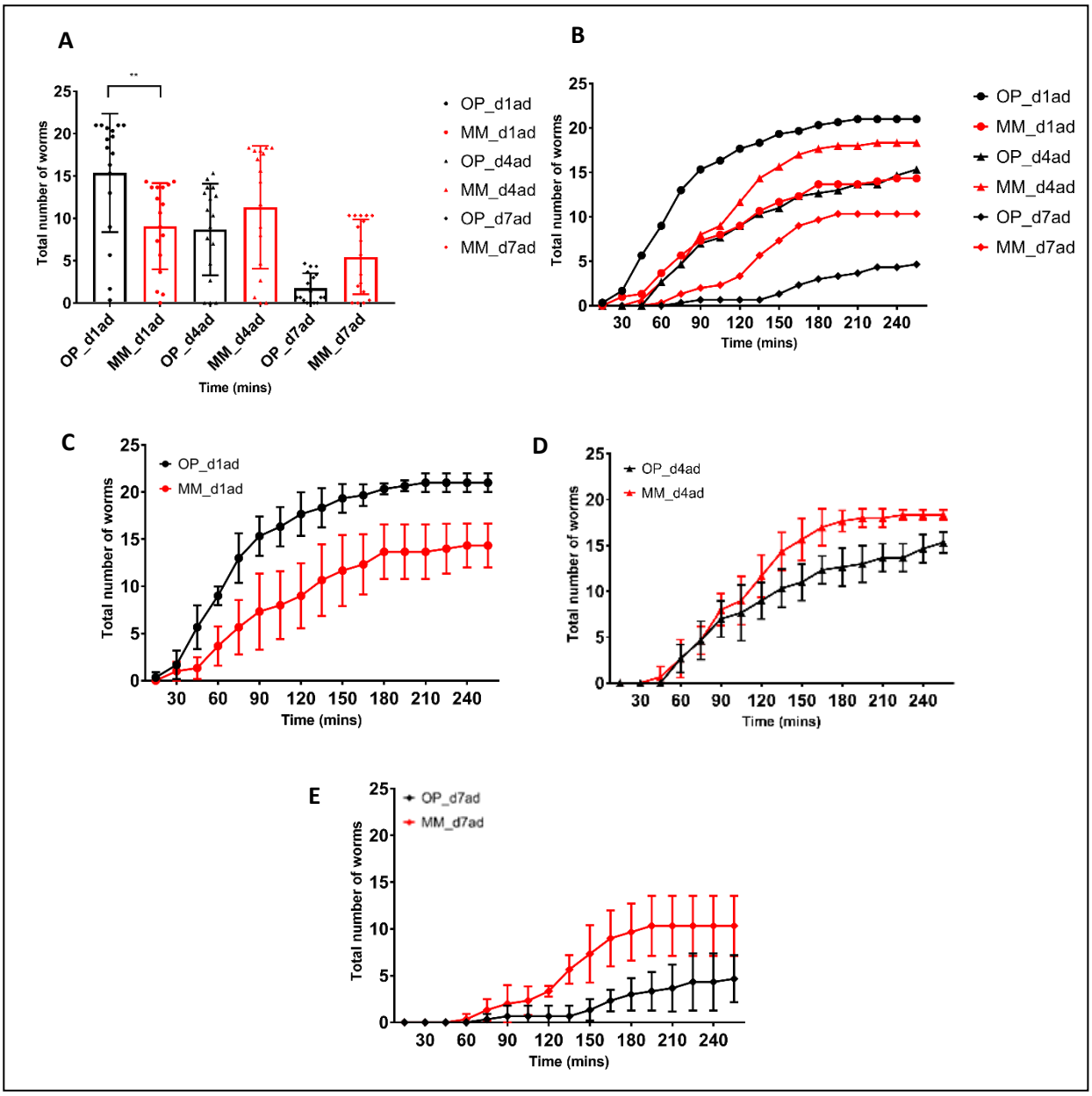

Figure 4.7 - Burrowing rate of C. elegans throughout aging fed the experimental microbiome compared to the control. *(a) Burrowing rate of the day 1, 4 and 7 adults (n=20 per condition per repeat) fed the experimental microbiome compared to OP50 as the control. Numbers are based on the amount of worms that burrowed to the surface towards a chemoattractant every 30 minutes for a time period of 240 minutes in total (b) The same data represented as a line graph and the separate comparisons for each day of adulthood (c) day 1, (d) day 4 and (e) day 7 for increased clarity. The data was collected from 3 technical repeats representing mean ± SD. **= p<0.0021, statistical analysis was done using Turkey multiple comparisons test on GraphPad Prism.*

Figure 4.7 Presents the burrowing assay results pooled from three technical repeats within one biological repeat. The data indicates that worms fed OP50 have a very clear and rapid decline in burrowing rate with aging (Fig. 4.7a). However, worms fed the experimental

microbiome diet had a more consistent burrowing rate throughout aging with a very steady decline in day 7 adults. Figure 4.7c shows that on day one of adulthood, the burrowing rate of worms on an OP50 diet was significantly higher compared to those on an experimental microbiome diet (OP50: ~ 15 vs. MM: ~ 8 average number of worms burrowed to surface over 240 minute period). However, by day 4 and 7 of adulthood (Fig. 4.7d and 4.7e), the burrowing rate of the MybMix fed worms showed higher borrowing rates compared to the control (Day 4 – OP50: ~ 8 vs. MM: ~ 13, Day 7 – OP50: ~ 2 vs. MM: ~ 5 – based on average number worms burrowed to surface over 240 minute period). This is consistent with the thrashing assay results and, therefore, would suggest that although young adult worms fed the experimental microbiome diet may have lower muscle strength compared to OP50 fed worms, they maintain their muscle strength well throughout aging, unlike the control. The data is further confirmed by the results from a collegue in the Ezcurra lab that also performed the same experiments (see figure 4.8)

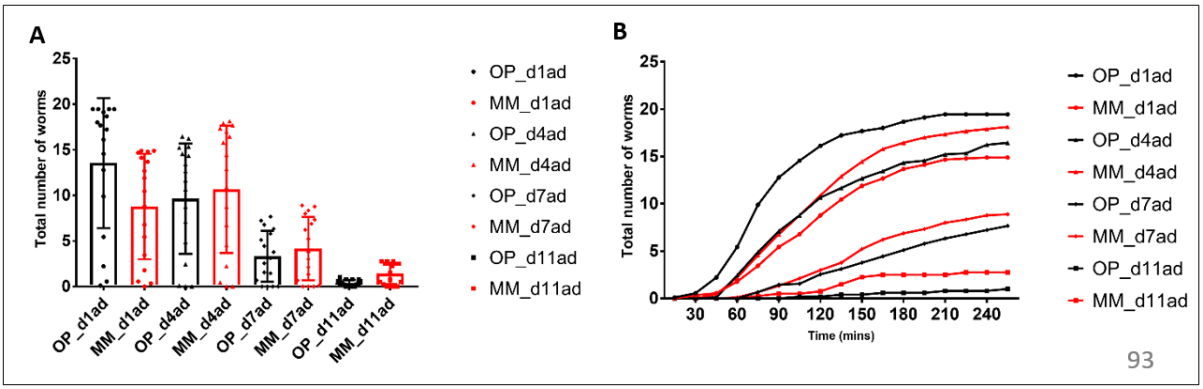

Figure 4.8 - Burrowing assay data pooled from 3 biological repeats. *Shows the burrowing rate of C. elegans throughout aging fed the experimental microbiome compared to the control. (a) Burrowing rate of the day 1, 4, 7 and 11 adults (n=20 per condition per repeat) fed the experimental microbiome compared to OP50 as the control. (b) The same data represented as a line graph. The data was pooled from one biological repeat collected individually and 2 biological repeats collected in collaboration with Feng Xue, a post-doc from the Ezcurra lab.*

The experimental microbiome shows no effect on worm contractability

To further investigate the muscle function of the *C. elegans*, the ability for the muscles to contract was tested and quantified, again comparing an experimental microbiome diet to the control. Levamisole was used to immobilise the worms and cause contraction of the muscles as it is effective but does not kill the nematode at low concentrations [127]. Ten worms were imaged on an empty NGM plate then those same worms were moved onto an NMG plate with 75µM levamisole soaked into the agar. This would cause the worms to stop moving and contract at which point they were imaged again. The length of the worms before and after the contraction was measured to quantify the percentage reduction in length of worms.

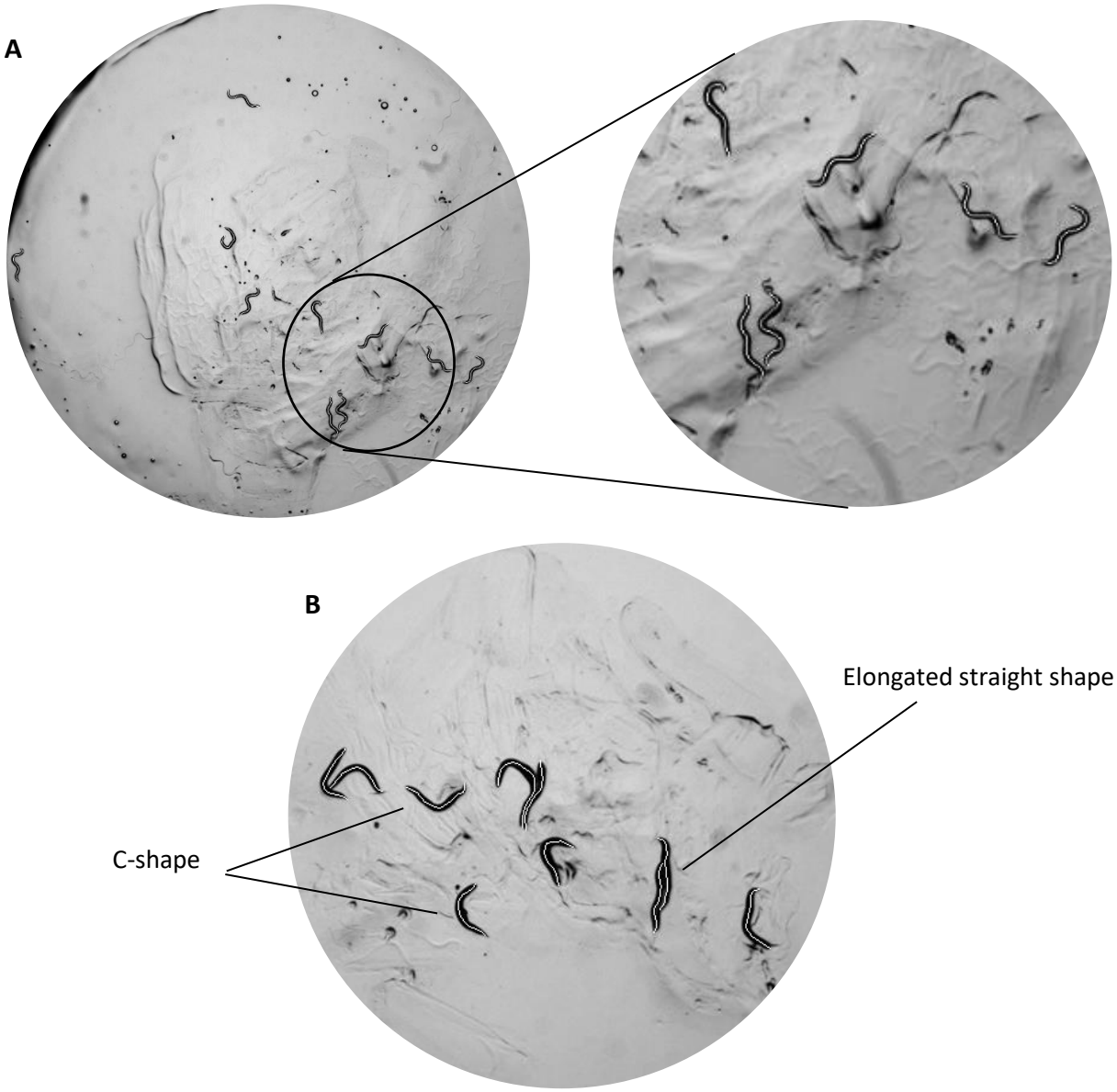

***Figure 4.9 – Images from contractability assay analysis**. (a) showing an image of Day 1 adults fed OP50 on the clear plate, pre-levamisole and (b) showing those same worms post-levamisole once they had immobilised and muscles had contacted. The white line through the middle of the worms from head to tail is what was measured. Measurements were done using ImageJ, there are no units as the measurements are relative to pixels on the line, images were taken with the camera at exactly the same distance away each time to ensure consistency in measurements. This is just one example for demonstration purposes.*

Figure 4.9 represents an example of the images that were taken, showing day 1 adults fed an OP50 diet. All images in the analysis looked very similar. On the plate with no levamisole, the worms were roaming around forming the S-shape as they crawled around the plate (fig. 4.9a). However, once the worms were transferred onto the plate soaked with levamisole they

initially crawl around, but by around 5-6 minutes they were all usually immobile. Their muscles contract and they form a C-shape or a straight elongated shape, shown in figure 4.9b.

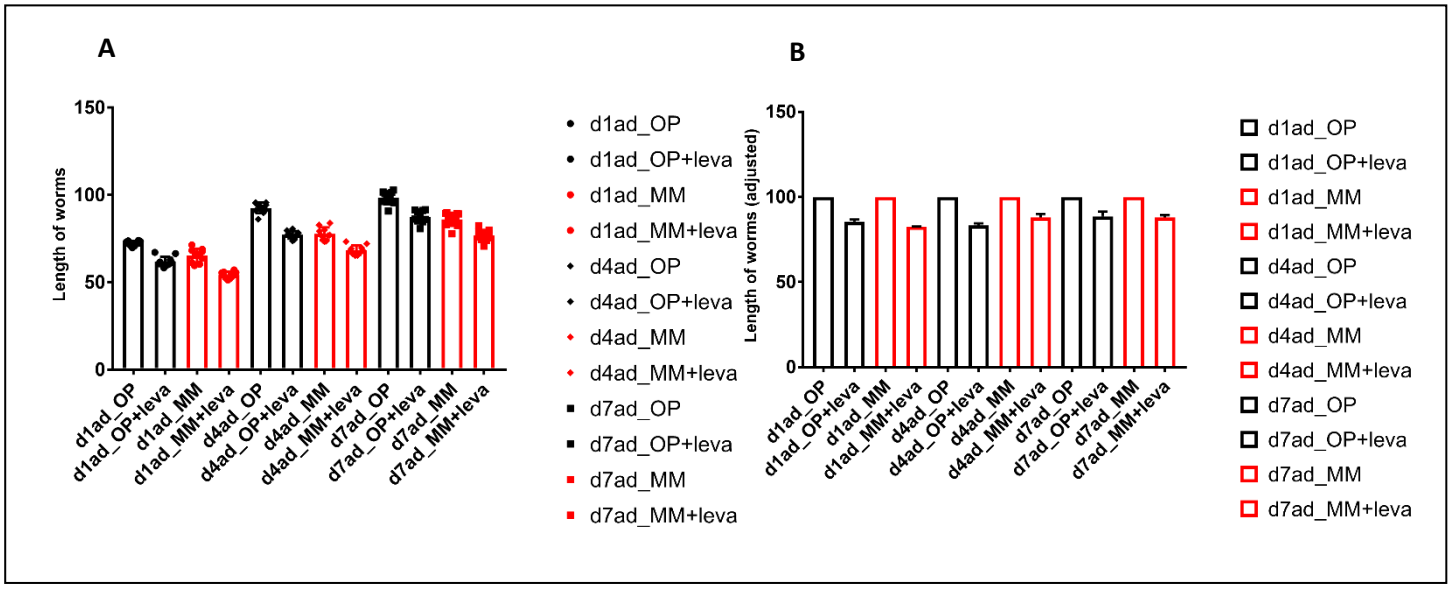

Figure 4.10 - Comparing the contractability of worms fed the experimental microbiome (MM) vs. the control, OP50*. (a) Graph showing the original lengths of worms (n~10) measured using ImageJ before and after levamisole (leva) is added, comparing the OP50 and MybMix (MM or experimental microbiome) diet for Day 1, 4 and 7 adults. (b) The same data but adjusted or normalised so that pre-levamisole is 100 and post-levamisole is the percentage decrease. Results represent mean ± SD and were pooled from 3 technical repeats within a single biological repeat.*

Figure 4.10a shows the quantified lengths of the of the worms before and after being immobilised by levamisole. For clarity and easy comparisons, the average lengths were adjusted so that pre-levamisole would be 100(%), to show a percentage decrease in length (fig. 4.10b). From figure 4.10a, there is a clear increase in worm body length as they age to day 7 adults. There is a steady increase in length for both OP50 and experimental microbiome fed worms. At each stage of adulthood, worms fed the microbiome diet seem to have slightly shorter bodies compared to control.

On day 1 of adulthood, worms fed the experimental microbiome show a slightly bigger reduction in average percentage reduction in length compared to the control (EM: 17.38% vs. OP: 14.36%). On day 4 of adulthood, however, the OP50 (control) fed worms seem to have a higher reduction in body length (EM: 11.90% vs, OP: 16.62%). By day 7, the difference was

shown to be the same in both experimental microbiome fed and OP50 fed worms (EM: 11.87% vs. OP: 11.23%). Overall, with the difference in length, or the amount of contractability, there seems to be no clear trend as the worms age. From these results, it could be concluded that an experimental microbiome diet has no effect on the ability for *C. elegans* muscle to contract. It must be considered, however, that this is data from only 3 technical repeats with a sample size of only 10 worms per repeat. More replicates could lead a more solid and precise conclusion.

Discussion

This chapter centred around the body wall muscles of *C. elegans*, investigating how an experimental microbiome diet affects the morphology of body wall muscle cells, as well as, muscle function and strength as the worms age. The objective was to explore whether a microbiome diet influences sarcopenia in *C. elegans*, which has been shown to be an important biomarker in aging [88].

The effects of a gut microbiota on the muscle cell morphology of *C. elegans* in early adulthood

To study muscle cell morphology, the translational reporter strain RW1596 was used, which expressed GFP-tagged myosin heavy chain A in the body wall muscles of the *C. elegans* [128]. This strain produces clear images of myosin fibres which form distinct diamond shaped cells of the body wall muscle. An example image can be seen in figure 4.2. When comparing the muscle cells of worms fed the experimental microbiome versus the control (*E. coli*, OP50), there were no differences of the shape or relative size of the cell in early adulthood (day 1 and 3 adults). The myosin fibres were all arranged the same way and were organised in the same overlapping manner. In most living organisms, the possession and maintenance of shape is essential for the proper function of cells [129]. In many cases, the morphology of cells is important for interactions involved in cell-to-cell signalling and formation of functioning structures [129]. *C. elegans* has shown to be an ideal model organism for studying muscle structure and function, especially since the sarcomere - the fundamental functioning unit of the muscle cells - is largely conserved from *C. elegans* to humans [88, 130] Furthermore, their transparent bodies along with various microscopic techniques allows easy visualisation of muscle structure [131]. While effective thrashing and crawling assays have been developed to assess muscle function [131].

Further investigation into the muscle cells showed that there were no differences in the number or size of gaps found within the myosin fibres of the body wall muscles. The gaps were investigated because it could assumed that a distorted myosin structure may lead to lack of muscle health. Research has shown that large gaps that are a result of deterioration of myosin filament lattices contribute to a high proportion of defects [125]. This study found that worm mutants with the highest level of defects - *lin-41 (ma104),* a gene that enables mRNA binding activity and translation repressor activity - had a gap area ratio that was ten times higher than wild type (N2) [125, 132]. Their research further suggests that the body wall muscle cells and their structure have an important role in worm locomotion [125].

Not much research has been done in other model organisms concerning the effects of a microbiome diet on muscle cell morphology. However, one mouse study found that germ-free (GF) mice – with no microbiota – had significantly lower muscle mass comparted to pathogen-free (PF) mice that do have a microbiota [133]. They further found that transplanting the gut microbiota from PF mice to GF mice actually restored the muscle mass, with the biggest differences found in the Quadriceps and Gastrocnemius muscles [133]. Although this study does not directly focus on muscle cell morphology, considering there is no exercise intervention involved, it could be assumed that having a gut microbiota directly influenced the muscle cells, possibly increasing them in size. This data is further confirmed in another study which investigated the importance of a microbiota in piglets. Their data showed that germ-free piglets had a lean mass that was ∼40% lower when compared to normal piglets [134]. It was further shown that a lack of a gut microbiota led to weakened muscle function and reduced amounts of myogenic regulatory proteins, important for cell development [134]. This research may contradict our results, showing that there is a clear link between the gut microbiota and muscle mass. However, it could be debated whether muscle

mass can be a direct comparison to muscle cell morphology, and thus, more studies on cell morphology in different animal models could help clarify our findings.

The influence of the gut microbiota on muscle function as *C. elegans* age

Another important element of this chapter was testing the effects of a microbiome diet on the muscle aging. Sarcopenia is a loss of skeletal muscle function and mass due to the accumulation of misfolded proteins, which is common in aging humans [88]. In *C. elegans*, studies have shown that muscles of aging worms also have a progressive loss of strength and motility, that can be detected through changes in locomotion [88]. Sarcopenia could be a valuable indicator of healthspan, with a delayed onset suggesting an extended healthspan [135]. This, along with the ease off visualising muscle structure in vivo make *C. elegans* a great model for studying how the experimental microbiome diet affects muscles as the worms age [88, 135] .

This was done through burrowing and contractability assays. Burrowing ability was used as a way to evaluate neuromuscular health of the *C. elegans*, with higher burrowing rate indicated better muscle strength [95]. The results showed that control (*E. coli*, OP50) fed worms had a very rapid decline in muscle strength (burrowing rate) as they aged from day 1 to day 7 adults. Consistent with previous thrashing assay results (Fig. 4.1), worms fed the experimental microbiome had a lower muscle strength (burrowing rate) at day 1 of adulthood, however, this strength was much more maintained as the worms ages, showing higher burrowing rate on day 4 and 7 adulthood compared to control (Fig. 4.7). This would suggest that a microbiome diet maintains muscle health and strength throughout aging in the *C. elegans* model organism. These results were further confirmed with other members of the lab, who also did repeats of the same assay (See figure 4.8). A mouse study further showed an

association between intestinal bacteria and exercise performance [136]. They showed that *Bacteroides fragilis* gnotobiotic mice had longer endurance swimming time compared to germ free mice [136]. Another study further showed that *Lactobacillus plantarum TWK10* – a probiotic strain isolated from Taiwanese pickled cabbage – enhanced muscle strength in young mice, improved exercise performance and prevented age-related loss of muscle strength[137]. For example, after six weeks of supplementation of *Lactobacillus plantarum TWK10* increased muscle mass and enhanced mouse grip strength by 1.40 fold compared to control [137]. It has been suggested that *Lactobacillus* bacteria may affect exercise performance because they produce lactic acid, which in turn could facilitate lactate-utilising bacteria in the production of butyrate [138]. Butyrate is a short-chain-fatty-acid that has been found to benefit the skeletal muscle [139]. Skeletal muscle cells are known to possess receptors for SCFAs that lead to favourable alterations in phenotype and physiology, that may promote anti-diabetic and anti-obesogenic effects [139]. One mouse study showed mice supplemented with sodium butyrate had lower body fat percentage and a higher muscle content [140]. Furthermore, butyrate has been directly linked to an increase in oxidation in the muscle and an increase lean body mass [139, 141].

Linking back to the experimental microbiome used to feed the *C. elegans*, Leuconostoc – represented as Myb 83 within our microbiome mix - is another lactic acid producing bacterial genus that could be having a similar effect leading to better maintenance of muscle strength found in the burrowing assay [142]. These studies may suggest that probiotic bacteria could be a potential intervention for slowing down onset of sarcopenia in adults.

In regards to human trials, there is evidence that targeting the microbiome, through -pro and -prebiotics, SCFAs and dietary supplements, has successfully enhanced muscle mass and

physical performance [117, 143, 144]. One study showed male runners that took a multi-strain probiotic – including *Lactobacillus, Bifidobacterium,* and *Streptococcus* (45 billion CFUs) – showed an increased running time to fatigue when compared to a placebo group [145]. Their data showed compelling evidence that probiotics increase athletic performance, however, the sample size was small (n=10) and included only men, so a more larger and more varied participant sample would increase accuracy. Furthermore, young teenage female endurance swimmers were also found to benefit from probiotic supplementation [146]. Data showed that intake of a daily probiotic yogurt – including *Lactobacillus Acidophilus spp.*, *Lactobacillus Delbrueckii Bulgaricus, Bifidobacterium Bifidum,* and *Streptococcus Salivarus Thermnophilus* (400ml yogurt with 4×10^{10}CFUs/ml) – resulted in a significant improvement in $VO_{2\,max}$ when compared to control [146]. They further found that those who were taking the probiotics stayed healthier overall with a reduction of episodes of respiratory infections [146]. Overall this would suggest that there must be a direct link between the gut microbiome and muscle health.

The effects of an experimental microbiome on muscle contractability

We further tested how the microbiota effects the contractability of the worm muscles using levamisole as a paralysis agent. This was done because tight regulations of contractions and relaxations of the body wall muscles are important for the sinusoidal locomotion exhibited by *C. elegans* [147]. Furthermore, in humans, contraction of the skeletal muscles is their main function in order to facilitate movement [134]. The results showed that the ability to contract got slightly less as the worms aged. However, overall, the length of the worms after contraction (post-levamisole) reduced by roughly 10-15% on all conditions. When comparing the experimental microbiome with *E.coli*, there were no significant differences in reduction

in length – and thus contractability. There was slightly higher contractability of worms fed the experimental microbiome on day 1 of adulthood, but lower on day 4 of adulthood.

In contrast to our findings, other research has suggested that there is a bigger link between the gut microbiota and skeletal muscles that our data suggests [148]. One study suggests that the gut microbiota have a role in the intrinsic muscle contractile properties [149]. Furthermore, research found that germ-free mice that were transplanted a microbiota from lean pigs, showed and increased proportion of slow-contracting fibres, which are important for slow and even use of energy that can be used for a long time [150]. However, they also found that the introduction of a microbiota decreased fibre size [150]. Another study, using guinea pigs, showed that butyrate – produced by various gut bacteria – increased the colonic contractibility. However, it is important to consider that colonic muscle is made up of smooth muscle, rather than skeletal muscle, and therefore, may behave differently in effect of the microbiota [151]. Overall these studies showed the influence that the gut microbiota can have on the skeletal muscles of animal models but whether their actual ability to contract is greatly influenced could still be argued.

Future work

There are many possibilities when testing the influence of the microbiota on muscles using *C. elegans* as a model organisms. Other assays could be used to test the muscles showing different perspectives of muscle function, which could lead to more solid evidence of the affects of the microbiota. One recent development has been a microfluidics-based system called 'NemaFlex' for strength measurement [131, 152]. These measurements are based on the nematode deflection of pillars as they crawl through a forest of micropillars designed to identify robust measurements of strength that are independent of behaviour and gait [152].

This system is able to define maximum exertable force, which is a quantitative and reproducible measurement of muscle strength amongst nematodes [152]. This could show some interesting results in the context of our work, and may confirm the burrowing assay results, strengthening our evidence.

When imaging the muscle cells of the *C. elegans*, only day 1 and 3 adult RW1596 worms were tested. To investigate these results further, it would be interesting to test how a microbiome diet affects the muscle cell morphology as the worms aged, possibly using the same time points – day 1, 4, 7 and 11 adults – for comparisons to the muscle function assays. One human study suggests that older people (aged 78-84) had a lower level of protein synthesis in the muscles compared to younger participants (aged 23-32) [153]. These proteins are essential for maintaining muscle strength and the integrity and structure of muscle cells [154]. This might suggest that individuals are likely to gain morphological defects in their muscle cells as they age, possibly leading to the sarcopenia that is seen in many older adults. Therefore, testing this in a model organism could lead to a better insight into muscle cell morphology throughout aging. Further, testing the effects of the microbiota on aging muscle morphology may possibly lead to possible microbiome based interventions.

In regards to muscle function, one possible hypothesis discussed was that lactic acid producing bacteria in the gut microbiota facilitating in increase in butyrate production. Butyrate has been shown to benefit the skeletal muscle in various ways [139, 141). To test this hypothesis, a burrowing assay could be performed including a condition in which isolated butyrate is added on top of *E. coli* OP50 compared to the control (OP50). This may reveal whether butyrate has an effect on the *C. elegans* aging body wall muscles. This could lead to a potential SCFA based approach towards an intervention against Sarcopenia, and also give

us a better idea whether the microbiota have influences through multiple compounds and pathways.

Overall, these and many more tests could be done to further investigate these results and show further evidence of how the microbiota can influence muscle health, in order to find novel therapeutic interventions against sarcopenia and other common muscle defects that occur as individuals age.

Strengths and weaknesses

Overall, all the assays were done accurately and to a good standard. The RW1596 strain of C. elegans showed very clear images of the body wall muscles of the worms. Using ImageJ to measure the area of the cells and any gaps was very useful for making interesting comparison between experimental microbiome fed worms and the control from my own work. However, since the measurements do not have units, and different studies use other techniques for measurements, the data could not be compared with literature on a quantitative basis. However on a more qualitative note, comparisons could still be made if other labs were to do similar experiments that studied the size and morphology of muscle cells. Another weakness of this assay was that only 40-50 cells were measured for each conditions. Possibly more time could have allowed for more cells to be analysed, giving a better idea of the bigger picture and leading to a more firm conclusion.

Likewise, the burrowing assay was well designed, using literature for the most effective methodology. However, when setting up the assay, the goal was to have enough viable worms for assays for day 1, 4, 7 and 11 adults. By day 11, there were not enough worms to complete even one technical repeat of each conditions. Another lab member was able to do all 4 days and found that day 11 followed the same trends. Apart from that, when transferring the

worms from their food plates to the drop of Pluronic gel, an intermediate plate was used to allow the worms to crawl out of the food as much as possible. Even so, when transferring with an eyelash pick, very small amount of food were also transferred along with the worms, mostly residue that was stuck on their bodies. This may have affected the results slightly because the chemoattractant used was concentrated *E. coli*, as it is one of their main food sources. Residues of food in the drop may have encouraged them to not burrow up towards the *E.coli* drop above. However, especially for the younger worms, they seem to still burrow up, implying that the small amounts of food had no real impact on their willingness to burrow.

The contractability also produced some good results, however, the small sample size of 10 per repeat may not show the most precise data. Slightly higher sample size and more repeats may have strengthened the conclusions made from the data.

Chapter 5: Thesis Summary

Overall, the microbiota is a huge untapped source of therapeutics against a multitude of human diseases and health markers. From this research, and much more literature out there, it seems that the gut microbiota have an impact on practically all the systems in our bodies. This further solidifies the importance of a health gut diversity and composition, to lead to increased healthspan and an overall better quality of life. This study barely scratches the surface of the potential for a microbiome based approach to lead to treatments against Alzheimer's disease, especially using the compounds produced by the *Stenotrophomonas* species that has shown protection against Aβ aggregation in our *C. elegans* model for AD. Furthermore, a diverse microbiota has also shown to have slight impacts on the muscle health of a wild-type *C. elegans* model, showing maintenance of muscle strength throughout aging. This could lead to natural interventions becoming more mainstream, as a way of preventing frailty and loss of muscle integrity in older adults. All this research is essential, especially now since advances in medicine have increase lifespan and increased our aging population. Healthspan is now a bigger focus in many studies, as it will allow older adults to live a more fulfilling life in their later years.

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