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THE EFFECT OF PROBIOTICS ON THE AGEING NERVOUS SYSTEM

Amanda Giannitelli

29/03/21

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Abstract

Probiotics are live bacteria which carry many beneficial effects to the host and have been shown to play a role in preventing or treating diseases, with most being linked to the gastrointestinal tract and dysbiosis. Dysbiosis of the microbiome refers to an imbalance in the gut microbial community and is associated with ageing and disease. In this study the nematode worm *C. elegans* is used to examine whether probiotics have an effect on health and ageing, with a focus on the ageing nervous system. We focus on Alzheimer's disease (AD) and Parkinson's disease (PD) models, whereby human Aß and α-synuclein are expressed in muscle. This study focuses closely on the probiotic strains, *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111. ROO11 and HA-111 significantly delayed the rate of paralysis in Aß transgenic *C. elegans* ($p<0.05$), however, had no effect against motility decline in the α-synuclein transgenic *C. elegans.* This study examined the effects of these probiotic strains on host immunity and found that *L. rhamnosus* ROO11 had the ability to increase activation of the innate immune system via the p38 MAPK pathway. Furthermore, this study also suggests that these probiotic strains have the ability to form biofilms, potentially having many benefits, which were not discussed in this thesis, such as increasing lifespan and pathogen resistance.

List of Abbreviations

C	Celsius
C. elegans	*Caenorhabditis elegans*
L. rhamnosus	*Lactobacillus rhamnosus*
SCFAs	Short-chain fatty acids
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
AD	Alzheimer's disease
PD	Parkinson's disease
Aβ	Amyloid Beta
ATP	Adenosine triphosphate
E. coli	*Escherichia coli*
ICV	Intracerebroventricular
L1/2/3/4	Larval stage
DNA	Deoxyribose nucleic acid
LB	Lysogeny broth
CFU	Colony Forming Units
NGM	Nematode growth medium
Rpm	Round per minute

1. **Introduction**

1.1 The Gut Microbiome

The gut microbiome has been described as the entire population of microorganisms, bacteria, viruses, protozoa, and fungi present in the gastrointestinal tract, as well as their combined genetic material. Both commensal and pathogenic bacteria constitute the gut microbiota (1). The gut microbiota plays a role in many functions such as the absorption of nutrient and minerals , amino acids, enzymes, and vitamin synthesis, as well as the creation of short-chain fatty acids (SCFAs). Acetate, propionate, and butyrate are examples of SCFAs and play a fundamental part in the maintenance and protection of the gut health and in providing energy for epithelial cells. These SCFAs also decrease the permeability of the epithelial barrier and provide immunomodulation and resistance against pathogens (2). The gut microbiota is formed by four main phyla which include Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. The human body consists of as many microbial cells as our somatic cells and the microbial cells that colonise our bodies contain a much greater number of genes than our human genome. Our bodies are hosts to around 500 to 1000 bacterial species, with each of these species having genomes containing thousands of genes, meaning that in addition to being affected by our own genes, our bodies are influenced by millions of microbial genes (3).

1.2 Changes in the Gut Microbiome throughout life

The development of a stable gut microbiota occurs during breast feeding, which results in the gut microbiota being dominated by *Bifidobacterium*. Following this, consumed food and breast milk allows the gut microbiota to develop further with the presence of the phyla *Bacteroidetes* and *Firmicutes* (Figure 1). The early establishment of the gut microbiota is decided by the method of delivery, as infants born with vaginal delivery have different bacteria to those delivered by caesarean section. Infants delivered by caesarean section have similar microbiomes to skin microbes and consist mainly of taxa such as *staphylococcus ssp*. Some studies have suggested that delivery by caesarean section results in the infant having a lower bacterial cell count in faecal samples and a greater number of antibody-secreting cells. Other factors such as breast milk feeding, and antibiotic use can also influence microbiome establishment. Colonisation is essential in order for the immune system to function. Interferences during the critical window of the gut microbiota formation may result in immunological diseases such as food allergies and asthma (4). During adulthood, the microbiome remains mostly stable until we age. During ageing the microbiota diversity decreases and gut function becomes defective as the immune system function is suppressed, and the microbiome becomes imbalanced (5). This results in a flawed gut function and increased gut permeability, allowing microbes to travel through the intestinal barrier, leading to inflammatory reactions, including neuroinflammation and chronic inflammation, and other processes such as mitochondrial dysfunction can be affected (6).

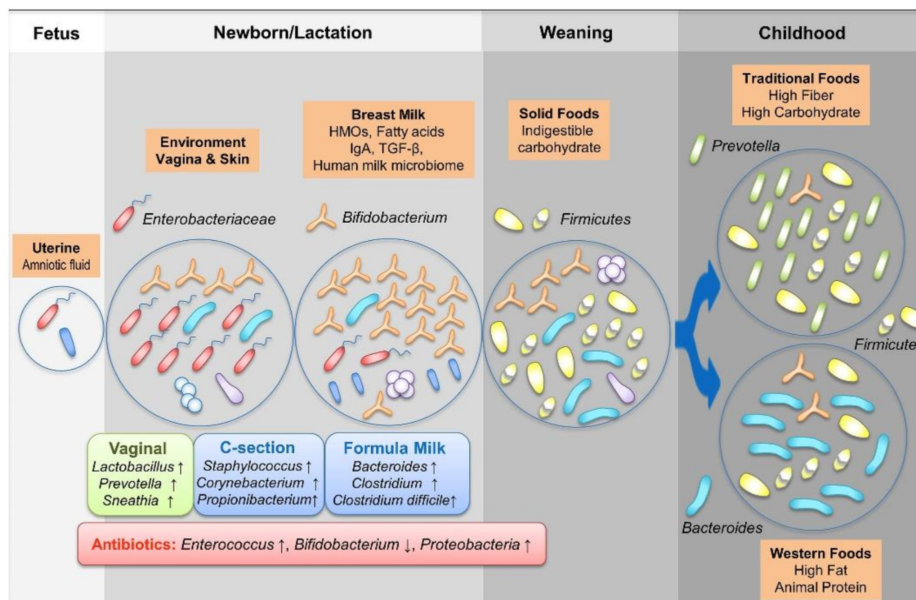


Figure 1 extracted from Allergology International (Tanaka., M), showing the gut microbiota colonisation from a new-born to childhood and elements influencing this. Gut microbiota establishment begins in utero and diversifies with age, becoming stable during adulthood (4).

Furthermore, SCFAs are important for host health as they allow for the maintenance of a low luminal pH, and increase microbial biomass, promoting the production of mucus and the growth of beneficial bacteria. It also supports gut barrier integrity and gut homeostasis. They also contribute to 5-10% of the total energy needed by a healthy human body (7). One example of an SCFA is butyrate, which is produced by the gut microbiota, and research has implied its possible use against colorectal cancer through the inhibition of histone deacetylase, which would usually promote the proliferation of colorectal cancer cells. Levels of SCFAs have been shown to be lowered with age, possibly due to a change in diet with age such as decreased appetite (8). This can result in intestinal bacteria being capable of disrupting mucin, increasing the chance of pathogens and opportunistic bacteria entering the intestinal mucosa. Overall, this results in increased susceptibility to inflammatory diseases such as inflammatory bowel disease (IBD) and may cause gut-related disorders (2).

1.3 The link between the Microbiome and Immunity

As we age there is a loss of microbiota diversity. Age-related changes to the gut microbiome are believed to be as a result of antibiotic use, a high intake of fat and sugar in a diet, and declines in nutrient intakes, resulting in a loss of beneficial gut bacteria, and in turn the chronic activation of the immune system (6).

Dysbiosis is an important factor that contributes to age-related inflammation, explaining how the microbiota might affect ageing. Age-related inflammation is characteristic amongst the elderly and differs from the pathogen-induced acute inflammation as it is a chronic inflammatory response which consists of continuous activation of the immune system, even when there is no presence of infection (9). This is because it is known that aging results in the dysregulation of the immune system, resulting in the acute inflammatory response

failing to resolve. For example, interleukin (IL)-6 and tumour necrosis factor (TNF) have been shown to be upregulated in aged tissues and cells and studies have shown that IL)-6 is associated to chronic inflammatory changes in ageing (10)(11).

When beneficial bacteria populations decrease in the intestine, gram-negative bacteria take control and the death of these bacteria results in the release of endotoxins, known for their pro-inflammatory action through the activation of plasma membranes such as toll-like receptor 4 (TLR4). Furthermore, the increased gut permeability that usually occurs during ageing, causes an increased number of undigested protein molecules, toxins, and pathogens to enter our circulation, triggering an immune response and inflammation (12).

1.3.1 Acute and chronic inflammation in ageing

With age, chronic inflammation becomes very common and dysbiosis is often a factor that causes this age-related inflammation (13)(9).

Inflammation usually commences with acute inflammation involving tissue-resident macrophages, neutrophils, and mast cells, as these take part in the first line of defence. Transcription factors such as Nuclear Factor Kappa b (NF-κb) are activated, which can go on to activate genes responsible for the production of proinflammatory molecules such as cytokines and chemokines. If this does not clear the cause of inflammation, chronic inflammation follows (13).

There have been two suggested hypotheses of chronic inflammation: molecular inflammation and inflamm-aging (13).

Molecular inflammation is characterised by molecular changes in transcription factors that are responsible for inflammation as well as changes in the levels of expression of their target genes and the hypothesis suggests that this is a result of the ageing process and age-related diseases. This could be due to the significant sensitivity of NF- κB to oxidative stress and to changes in redox balance. Reduced antioxidant defence systems and continuous oxidative stress during ageing are said to lead to increased levels of reactive species (RS). Therefore, the inability to maintain redox homeostasis results in the activation of many proinflammatory signalling pathways, further increasing levels of transcription factors (14). Cellular redox signalling usually activates tyrosine kinases, which further activate serine/threonine kinases. This results in downstream kinases such as NF- κB to be activated, which then further activates the age-related NF- κB. This overall results in an upregulation in the expression of pro-inflammatory cytokines such as TNF-α (8). NF- κB is a protein transcription factor responsible for DNA transcription, cell survival and cytokine production, which is activated by molecules such as TNF-α binding to TNF receptors, resulting in an interaction with the IκB Kinase (IKK) complex, leading to IκB phosphorylation and then degradation. This ultimately leads to NF- Kb translocating to the nucleus, where it activates target genes such as cytokines e.g. IL-1 (14)(15).

On the other hand, inflamm-aging is characterised by a gradual increase in proinflammatory cytokines that are usually present during ageing. It suggests that activation of an aged innate immune system results in a disruption in inflammation that weakens the ability to

initiate an effective immune response to antigens or environmental stimuli. This hypothesis has been suggested to link to development of neurodegenerative disorders such as AD (8). As seen in figure 2, ageing is associated with inflammed adipose tissue, leading to an increase in pro-inflammatory cytokines. Ageing is also associated with a decrease in lean mass, mainly skeletal muscle, which is a huge source of anti-inflammatory cytokines. This may result in an imbalance between pro-inflammatory and anti-inflammatory cytokines. This overall results in inflammation (16).

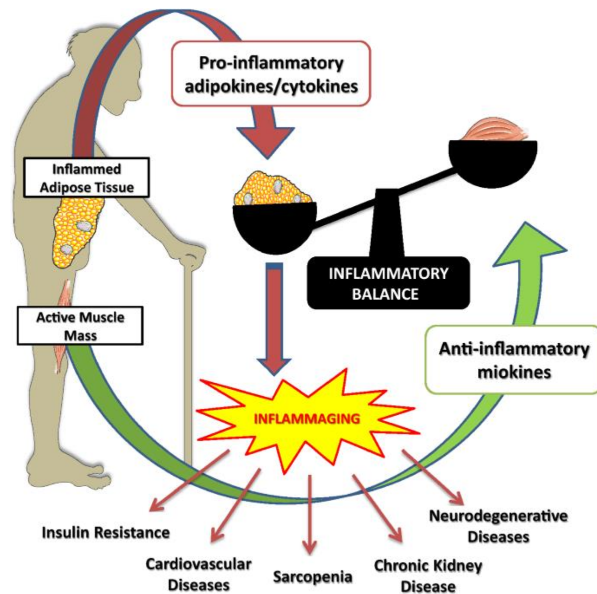


Figure 2 showing the process of inflamm-aging. There is an increase in pro-inflammatory cytokines with age due to the expansion of inflamed adipose tissue. Loss of lean mass associated with aging leads to an imbalance between pro-inflammatory and anti-inflammatory cytokines (16).

1.4 The 'Gut-Microbiota-Brain' axis

The 'gut-microbiota-brain' axis describes the continuous two-way communication between the gastrointestinal tract and the central nervous system (CNS) and refers to the link between the gut microbiota and neural networks of the host, to keep body homeostasis. There has been an increase in research showing that the gut microbiota can balance gut and brain functions, such as behaviour, cognitive functions, and mood. However, the utmost intricacy of this gut-microbiota-brain axis means there is a huge amount not yet understood at this stage (17).

1.5 Link between the gut microbiome and neurodegenerative diseases

There have been many studies suggesting a link between gut microbes and neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD), which result from the aggregation of amyloid fibrils in the central nervous system. Amyloid fibrils are developed by soluble proteins, which accumulate to form insoluble fibres, protected from degradation (18).

An inflammatory response, triggered by immune activation due to a defective gut barrier, results in a damaged blood-brain barrier and leads to the development of neuro-inflammation and degeneration (19).

1.5.1 Neuroinflammation in AD

Microglia are glial cells located throughout the brain and spinal cord and are activated in response to infections or brain damage under normal circumstances. They can phagocytise (engulf) pathogens, including amyloid beta, and damaged neurons, resolving neuroinflammation (20).

Chronic neuroinflammation is observed in AD. The microglia, which are activated by amyloid beta via the receptor for CD36, Fc receptors, TLRs, and complement receptors, continuously secrete cytokines, and can increase amyloid beta production and decrease its clearance, leading to neuronal damage. The binding of amyloid beta to microglia results in an increase in the release of pro-inflammatory cytokines including IL-6, leading to tau hyper-phosphorylation and neuronal loss (21).

Furthermore, inflammation from the periphery nervous system can spread into the central nervous system by crossing the blood-brain barrier. Amyloid beta can also cross the blood-brain barrier into the brain via the receptor for advanced glycation end-products (RAGE), a pattern recognition receptor (PRR), which are proteins with the ability to recognise molecules found in pathogens called Pathogen-Associated Molecular Patterns (PAMPs) as part of the innate immune system (22). The microglia are activated following binding of amyloid beta to RAGE on microglia, resulting in an inflammatory response and a raise in pro-inflammatory cytokines (23).

Studies using knockout mice have suggested that some memory loss in AD could be a result of amyloid beta oligomers activating microglia, causing the microglia to engulf and exclude synapses via the complement factors C1q and C3, which are a part of the innate immune system process and aid in the removal of microbes or damaged cells by phagocytes (24).

1.5.2 Neuroinflammation in PD

Changes in the microbiota could lead to increased permeability of the gut epithelium and blood-brain barriers, resulting in substances being transferred from the gut to the brain, and some of these substances contribute to the formation of the common PD pathology, fibrillar alpha-synuclein. This gives access to lipopolysaccharide (LPS) and metabolites produced by the microbiota to enter the central nervous system via permeable barriers. Studies suggest LPS may play an enormous role in neuroinflammation in neurodegenerative disease. LPS stimulates the release of pro-inflammatory cytokines like TNF-α and promotes the expression of chemokines which contribute to neuroinflammation (25).

1.6 Alzheimer's Disease (AD)

AD is identified by the existence of plaques and neurofibrillary tangles. It is the cause of 60-70% of cases of dementia and is predominantly found in individuals above the age of 65. AD

is characterised by the loss of short and long-term memory, loss of motor skills and language and disorientation (26).

The plaques found in AD consist of insoluble deposits of amyloid beta peptide (Aβ), a fragment of the amyloid precursor protein (APP). Aβ is the product when β-secretase and γ-secretase join to cleave the transmembrane amyloid precursor protein by proteolysis (Figure 3). The Aβ appears to bind together just outside the neurons, forming amyloid plaques (27). There are two major isoforms of Aβ; Aβ42 and Aβ40, Aβ42 has two extra residues at the C-terminus, is toxic to neurons, and forms most of the amyloid plaques seen in AD (28). These plaques can interfere with neuron signalling, resulting in memory impairment and could also result in an immune response leading to inflammation, which could damage surrounding neurons (27)(29).

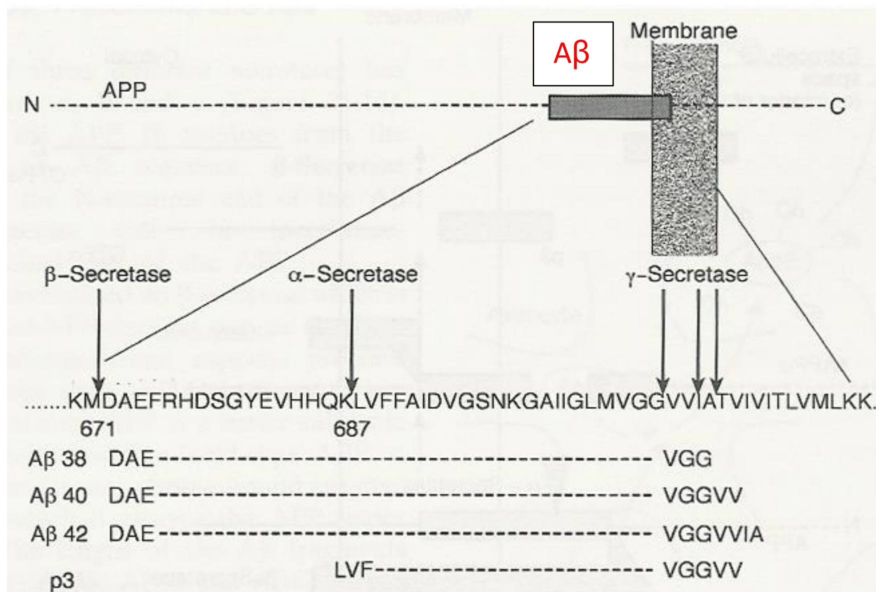


Figure 3 shows the formation of amyloid beta plaques. Aβ is formed by the cleavage of APP by β-secretase and γ-secretase. Different isoforms of Aβ include Aβ38, Aβ40, and Aβ42 (28). Extracted from Elements of Molecular Neurobiology by C. U. M. Smith.

Another characteristic of AD is the accumulation of neurofibrillary tangles in neurons. Neurofibrillary tangles are made up of paired helical filaments containing hyperphosphorylated tau, a microtubule-associated protein responsible for the regulation of functional processes like axonal transport, neurite outgrowth, and synaptic plasticity through stabilising the microtubular cytoskeleton (30).

AD can be divided into two types, sporadic and familial. Sporadic AD is a late onset type, affecting around 50% of people aged 85 or above and is usually caused through both genetic and environmental factors. The risk has been shown to increase significantly with age (31). The inheritance of an apolipoprotein e4 allele raises the risk of developing AD. It has been suggested that apolipoprotein may help with the breakdown of Aβ as the loss of *Abca1,* which is responsible for the transfer of lipids to ApoE, has been shown to inhibit Aβ degradation. However, the E4 allele appears less effective than other alleles like

apolipoprotein e2, suggesting there is an increased likelihood of patients developing Aβ plaques (32).

Familial AD is an early onset form of AD and makes up around 5-10% of cases. This is due to a mutation in presenilin 1 (PSEN-1) or presenilin 2 (PSEN-2), protein subunits of gamma secretase. These mutations can result in gamma secretase cleaving APP at an incorrect location, producing different lengths of beta amyloid molecules and therefore causing more plaques. Furthermore, Down's syndrome is another risk factor for developing this form of AD as the gene responsible for creating APP is located on chromosome 21, meaning there will be an extra APP gene present, leading to increased expression of APP (33).

Studies have implied that amyloid misfolding and amyloid plaque formation may be linked to amyloid secretion by bacteria in the gut microbiota and our diet. This has been supported by amyloid secretion being shown from bacteria such as Pseudomonas and Staphylococcus. This may become a problem with age due to the increased permeability of the epithelial barrier (34).

1.7 Parkinson's Disease (PD)

PD is the second most common neurodegenerative disorder, affecting around 0.2% of the world's population (35). It is characterised by tremor of the limbs, difficulty initiating movements, and jerky movements due to rigidity around joints due to the dopaminergic neurons in the substantia nigra of the brain experiencing degeneration (36).

The substantia nigra is composed of two sub regions, the pars reticula, which receives signals from the striatum, which then sends messages to the thalamus through neurons containing high amounts of the neurotransmitter, gamma amino butyric acid (GABA). The second region is the pars compacta, which is affected in PD. The nigrostriatal pathway, involved in the activation movement, is responsible for sending signals to the striatum through neurons which are highly concentrated in the neurotransmitter dopamine. In PD there is an increased inhibitory input to the thalamus, resulting in decreased activation of the thalamocortical spinal pathway and prevention the initiation of movement from being smooth, coordinated and controlled (36).

Genetic factors which are linked to increased PD risk include mutations in the SNCA gene that encodes the alpha synuclein protein as well as PARK2, a gene that encodes the protein parkin. Parkin is an E3 ubiquitin ligase important for the elimination of damaged or excess proteins, which is inactivated in sporadic PD, resulting in the loss of its ubiquitin E3 ligase function (37).

One of the leading causes of PD is the accumulation of dense deposits called Lewy bodies in nigrostriatal neurons, impairing the function of the neurons. Lewy bodies are round, eosinophilic intracytoplasmic occlusions in the nucleus of neurons, and the major components consist of proteins such as alpha synuclein, tau, parkin, and ubiquitin. Lewy bodies have been shown to be made up of mainly alpha synuclein aggregates which arise from protein misfolding. These aggregates have been said to lead to many implications such

as microtubule, synaptic, and mitochondrial dysfunctions, oxidative stress, and disruptions in calcium signalling (38).

Some studies have shown that abnormal tau protein has been linked to Parkinson's disease. Post-mortem studies have discovered that the striatum of PD patients have shown a rise in tau hyperphosphorylation. This has been supported by other animal studies which have shown that increased alpha-synuclein expression may also trigger tau hyperphosphorylation (39).

Research has suggested that PD and the gut microbiota are linked. Studies using mice to make comparisons between mice with a complete microbiome and germ-free mice have shown that germ-free mice perform better motor skills. Furthermore, studies using short chain fatty acids (SCFAs) were performed, as an indication of whether an imbalance of SCFAs may be involved in alpha synuclein aggregation. Germ-free mice fed SCFAs showed increased activation of microglia and slowed motor skills and alpha synuclein aggregation in the brain. This implies there is indeed a link between the gut microbiome and PD pathology (39)(40).

1.8 Probiotics

Probiotics are live microorganisms which have been shown to provide health benefits ranging from alleviating gastrointestinal symptoms and improving immune function. *Bifidobacteria* and *Lactobacilli* are two examples of microorganisms shown to work well as a probiotic (41). *Bifidobacteria* make around 10% of the bacteria in the adult gut microbiome. One of the main properties of this type of bacteria is to digest fibre and complex carbohydrates that the human body is unable to digest. This has been linked to reduced weight gain, diabetes, and heart disorders (42).

Lactobacillus species are a type of bacteria that produce an enzyme, lactase, which is responsible for the break-down of sugar lactose into lactic acid. Heath benefits have been linked the production of lactic acid as it aids in the prevention of the build-up of harmful bacteria in the digestive tract (43).

Lallemand, a Canadian health company providing microbiological solutions for different industries, has provided us with 11 different probiotic strains isolated from the human gut (table 1), which we will research in this project.

Bacterial Strain	Code
Lactobacillus rhamnosus	HA-111
Lactobacillus brevis	HA-112
Lactobacillus rhamnosus	HA-114
Lactobacillus salivarius	HA-118
Lactobacillus plantarum	HA-119
Bifidobacterium breve	HA-129
Bifidobacterium longum	HA-135
Lactobacillus rhamnosus	R0011
Lactobacillus helveticus	R0052
Bifidobacterium animalis ssp.lactis(B94)	R0421
Lactobacillus paracasei(L26)	R0422

Table 1: Lallemand *Bifidobacterium* and *Lactobacillus* probiotic strains isolated from the human gut that are used in this study. Strains will be tested for effects on health and ageing in *C. elegans.*

Lactobacillus rhamnosus

This study will look at three different strains of *L. rhamnosus;* HA-111, HA-114 and R0011. *L. rhamnosus* is a short, gram-positive heterofermentative, meaning that they produce both lactic and alcohol. It is a facultative anaerobic and non-spore forming rod, allowing the organism to switch to anaerobic respiration in cases where oxygen is absent and when oxygen is present, it is able to produce ATP by aerobic respiration. *L. rhamnosus* is commonly used in yogurt and dairy products. Research has demonstrated that *L. rhamnosus* aids the treatment of infections such as bacterial vaginosis as *L. rhamnosus* inhabits the healthy female genito-urinary tract and may restore vaginal flora (44). This species has also been shown to have benefits such as preventing diarrhoea and irritable bowel syndrome (IBS) symptoms (44)

Research has further demonstrated benefits provided by *L. rhamnosus*, for example a study using human and rodent intestine epithelial cell (IEC) models identified the proteins p75 and p40 in *L. rhamnosus,* which are secreted proteins with anti-apoptotic and cell protective properties, on human intestinal epithelial cells. They have also been shown to have cell wall hydrolase activity. This suggests that this probiotic may improve the digestive system of the host and prevent infection.

Additionally, studies have shown that *L. rhamnosus* may decrease pathogen gut colonisation, stabilises the gut epithelial barrier, and ameliorate antibiotic-associated

diarrhoea symptoms, further suggesting the benefits, such as an improved digestive and immune system in the host as well as reduced gastrointestinal symptoms (45).

Studies have looked into the *L. rhamnosus* R0011 strain and have shown that this strain promotes a healthy gut via the inhibition of pathogen activity in the gut and the prevention of gastrointestinal symptoms. This strain has also been shown to increase mucin production by epithelial cells. Mucin is a protein with the ability to form gels which aid in a variety of functions such as lubricating epithelial surfaces and promoting protection from damage as well as being able to bind to pathogens (44). This is advantageous to host health as it results in the reduction of infections (45).

Another study investigated the immunomodulatory activity of milk fermented with R0011 on macrophages with LPS, a pro-inflammatory stimulus. Human monocytes were used and cultured *in vitro* with R0011 and LPS, leading to the downregulation of LPS-induced Scd54 (ICAM-1), which has been linked to increased cardiovascular disease risk. The study concluded that overall, milk fermented with *L. rhamnosus* R0011 upregulates the production of pro-inflammatory cytokines as well as cytokine production by human monocytes challenged with LPS. Therefore *L. rhamnosus* R0011 influences macrophages and the innate immune system, strengthening the immune function of the host (46).

The *L. rhamnosus* HA-111 strain has demonstrated its ability to stimulate antimicrobial activities against intestinal pathogens such as E.coli (42), also protecting against infection. Research looking into the *L. rhamnosus* HA-114 strain has not been done.

Lactobacillus brevis

Lactobacillus brevis is a gram-positive, heterofermentive and rod-shaped species, especially found in different cheeses. This probiotic can be found in the human intestines, vagina, and faeces (35) and has the ability to attach to the gut epithelium and prevent adhesion of pathogens via steric hindrance (42).

Research has suggested that *L. brevis* can survive in the GI tract in humans and that may improve the immune system function in humans. Research where *L. brevis* has been used in milk, has been shown to play a role in the increase of cellular immunity and this probiotic, taken orally, has been shown to upregulate the natural killer cell activity in the elderly. This probiotic has also been shown to help restore the microbiome (47). Overall, this probiotic could potentially help improve immune function, especially in the elderly and can be useful for treating gastrointestinal infections such as IBS.

Lactobacillus salivarius

L. salivarius is found in the gastrointestinal tract and one of its properties involves the activation of macrophages and monocytes, which play a huge role in the activation of antigen-specific immunity and IgA immunity, which suggests that this probiotic assists the host in fighting pathogens (48) (49).

Similarly, to other *lactobacillus* probiotics, it also produces lactic acid and has been shown to grow very rapidly (49).

Little research has been undertaken to explore this probiotic; however, it has been found to have benefits such as helping with conditions such as irritable bowel syndrome (IBS), ulcerative colitis and inhibiting pathogenic bacteria such as E. coli, protecting the host from pathogenic infection (50). Studies have shown that this species, in addition to other probiotic species such as *L. casei,* may be able to minimise bacterial translocation by inhibiting pro-inflammatory cytokines and preventing further bacterial overgrowth in the small intestine, improving intestinal barrier function, and normalising the gut microbiota balance in the host (51).

Research has not been undertaken for the *L. salivarius* HA-118 strain, which will be used in this project.

Lactobacillus plantarum

Fermented foods and anaerobic plant matter are the most common sources of *Lactobacillus plantarum*. It is also found in salvia, which is where it was first discovered. It is a gram positive, bacilli shaped bacterium. Among the lactic acid bacteria, it has one of the largest genomes known. These bacteria contain both D and L isomers of lactic acid and respire oxygen, however they lack a respiratory chain and cytochromes, meaning that the absorbed oxygen results in hydrogen peroxide, which may help minimise the number of bacteria competing for food (52).

L. plantarum has been shown to enable antioxidant activities and aid in intestinal permeability maintenance. It has also been shown to inhibit the growth of gas-producing bacteria in the intestine as well as alleviate symptoms in those with irritable bowel syndrome. Overall, it is useful in the maintenance of a stable microbe balance. Experiments have shown that this probiotic may increase hippocampal brain derived neurotrophic factor and therefore could possibly play a role in treating depression (53).

This strain obtained from Lallemand for this project is HA-119 and limited research has been done on this strain, however it has been shown to adhere to the gut epithelium, preventing the attachment of pathogens via steric hindrance (42).

Bifidobacterium breve

This is a gram positive, anaerobic, and non-motile organism.

A study using mice and ICV infusion of Aβ to imitate the effects of Alzheimer's disease suggested that the strain A1 of *B. breve* may have an effect on brains suffering from AD (41). It was shown that ICV infusion of Aβ resulted in a change in gene expression in the hippocampus of mice as well as genes involved in the immune system response and their response to external stimuli were shown to be increased. Experiments with mice treated with the A1 strain of *B. breve* suggested that *B. breve* could inhibit toxicity caused by Aβ and that it may be able to normalise the gene expression in the hippocampus. Furthermore, brain-derived neurotrophic factor (BDNF), which is usually low in Alzheimer's disease, was found to be upregulated in the presence of A1 *B. breve* (54).

Finally, it was also seen that the use of *B. breve* A1 increased plasma acetate levels and addition of acetate to drinking water led to some cognitive improvement in AD model mice. This could suggest that the beneficial effects of *B. breve* A1 seen in the model mice could have something to do with the increased production of acetate (54). This is very interesting and relevant to this research project as it shows potential for probiotics to play a role in inhibiting Aβ toxicity.

The *B. breve* species has also been shown to help with the treatment of IBS (55) as well as protecting from infection and inflammation, suggesting improvement in immune function (56). An interesting discovery of the benefits of this probiotic is that it may play a role in helping those suffering from asthma due to its strong anti-inflammatory properties (57).

There has been no research into the strain HA-129 being used for this project.

<u>Bifidobacterium longum</u>

B. longum is a rod-shaped, gram-positive, catalase-negative bacterium that lives in the human gastrointestinal tract. It is an anaerobe that's believed to be one of the first to colonise an infant's gastrointestinal tract. It is though to be part of the gut microbiota, and its production of lactic acid suggests its ability to prevent the growth of pathogenic bacteria (58).

B. longum has the ability to break down proteins and carbohydrates, particularly lactose and protect intestinal walls from harmful bacteria. It has also been shown to protect DNA from carcinogen damage and inhibit rotavirus and diarrhoea (59). Furthermore, *B. longum* BB536 has been shown to promote host human health by restoring gut microbiota balance, improving gastrointestinal health, modulating host immune system function, and providing anti-allergy effects. This is beneficial for the host as it may play a role in reducing infectious conditions and restoring gut microbiota balance can potentially aid in reducing immune, metabolic, and neurological issues (60).

There has been little research on the HA-135 strain being used in this project.

<u>Lactobacillus helveticus</u>

L. helveticus bacteria are rod-shaped bacterium that produce lactic acid and are most commonly used in cheese. The strain being used in this project is R0052.

Studies in humans have suggested that the *Lactobacillus helveticus* species has potential to promote overall gut health by increasing the production of butyrate, promoting gut balance and stability (61). Studies has also shown that this probiotic may improve upper respiratory tract illnesses by reducing the period of these illnesses (62).

Research papers have shown that this strain taken alongside *B. longum* R0175 result in beneficial psychological effects such as decreasing depression and anxiety levels, as well as aiding with problem solving and lowering cortisol in human volunteers. (63).

<u>Bifidobacterium animalis ssp.lactis</u>

Bifidobacterium animalis is a probiotic that is commonly found in Dannon's activia yogurt. These bacteria are anaerobic, gram-positive, and rod-shaped. They can be found in the large intestine. The benefits of this probiotic include the maintenance of a good digestive health and immune system (64). Studies have suggested that this probiotic has the potential to improve gastrointestinal and immune system health. It has also been shown to aid in diarrhoea symptoms and to improve bowel function generally. *Bifidobacterium animalis* has also shown positive effects such as increasing resistance to respiratory infections (65) (66). No research has been undertaken for the R0421 strain being used in this project.

Lactobacillus paracasei

Lactobacillus paracasei is a gram-positive species of lactic acid bacteria that functions by commensalism. This bacterium is mostly used in dairy product fermentation and probiotics (67). Research has suggested that the *L. paracasei* strain R0422 has the ability to decrease the secretion of pro-inflammatory cytokines, increase immunomodulatory control, increase the production of anti-inflammatory cytokines, and reduce irritable bowel syndrome symptoms (68), therefore improving the immune system of host health and decreasing infection.

1.8.1 Beneficial effects of the probiotic strains on different organisms.

In order to investigate what has been previously researched about the probiotic strains provided by Lallemand, we decided that it would be interesting to determine the effects of the probiotics on different organisms such as human cells, mice, *Drosophila melanogaster*, and *C. elegans*. Although not much research has been performed on the specific strains being looked at in this project, some research has been performed on the general probiotic species. It can be seen that some probiotic species researched in mice, such as *Lactobacillus plantarum, Bifidobacterium breve, Bifidobacterium longum,* and *Lactobacillus rhamnosus,* have beneficial effects on the brain such as ameliorating anxiety and depression symptoms, inhibition of amyloid beta toxicity related to Alzheimer's disease, and upregulation of brain-derived neurotrophic factor (BDNF), a protein of the neurotrophin family of growth factors responsible for promoting nerve cell survive by promoting growth, differentiation, and maintenance of the cells (88, 92, 99, 102). Most probiotics are shown to extend *C. elegans* lifespan and protect against pathogenic infection (83, 86, 90, 100, 105, 110). The majority of research carried out on probiotics has been performed on humans and mice. Table 2 demonstrates a few of the benefits provided by each probiotic species.

Table 2: ***Probiotic species provided by Lallemand have shown many beneficial effects in studies on performed on humans, mice, drosophila flies and C. elegans. However, little or no research has been done on individual strains provided by Lallemand.***

Probiotic species	Human	Mice	*Drosophila melanogaster*	*C. elegans*
Lactobacillus brevis	*Lactobacillus brevis subsp. Coagulans* taken orally increased IFN-alpha production as dose increased (80).	In ageing mice, it was shown to minimise age-related colitis, memory impairments, and NF- κB activation (81).	Produces compounds which inhibit growth of antagonistic fungi and bacteria (82).	Increased longevity related to p38 MAPK *pmk-1* pathway, activating the innate immune system. Feeding with *L.brevis* results in increased *pmk-1* levels (83).
Lactobacillus salivarius	*L. salivarius* LS01 given to 20–35-year-old asthmatic patients, resulting in a decrease in pro-inflammatory cytokines and increases in beneficial immunomodulatory activity (84).	May improve gut microbial balance in patients with diabetes (85).	No research performed on Drosophila flies for this species.	In a calorie-restriction based manner, *L.salivarius* taken from centarian faecal samples increased the lifespan of *C. elegans* significantly(86).
Lactobacillus plantarum	*L.plantarum* 299v administered in combination with antibiotics in a small group of patients found to reduce cases of *Clostridium difficie*-associated diarrhoea (87).	Produces butyrate, strengthening intestinal barrier. Regulates BDNF expression and reduces inflammation in the brain. Has been linked to reduced depressive-like behaviours (88).	Reduces fly death from *Serratia marcescens* and *Pseudomonas aeruginosa* when fed ahead of infection (89).	Increased average lifespan of *C. elegans* compared to life span of those fed OP50 (90).
Bifodobacterium breve	Daily dose of 10^9 cells of *B.breve* M-16V administered as bacterial powder for 6 weeks, which resulted in a significant decrease in abdominal pain and a better quality of life for people suffering with IBS (91).	Strain A1 may inhibit toxicity caused by Aβ and may be able to normalise the gene expression in the hippocampus (92). Anti-influenza virus IgG levels in serum increased following *B.breve* YIT4064 administration in mice, resulting in protection from infection (91).	Not much research performed on drosophila flies.	*luxS* of *Bifodobacterium breve* UCC2003 was discovered to be involved in the development of the interspecies signalling molecule autoinducer-2 (AI-2), which has been shown to be necessary for *Salmonella* infection defence (93).
Bifodobacterium longum	Stimulated an immune response in 45 elderly patients who received an influenza virus (94).	Improves survival in mice infected with *Salmonella* Typhimurium (95). Oral administration protects mice against gut-derived sepsis linked to *P. aeruginosa.* (96).	Not much research performed on drosophila flies.	*Bifidobacterium longum* BB68 increased *C. elegans* lifespan by 28% as treatment with this strain resulted in increased expression of the SOD-3 gene, a DAF-16 target gene (99).

		Normalised anxiety behaviour and BDNF in mice infected with colitis (97). Reduced the level of inflammation and promoted longevity (98).		
Lactobacillus rhamnosus	Ability to adhere to epithelial cells and maintain gut epithelium barrier function. Shown to block the adhesion of pathogens (100).	*L.rhamnosus* JB-1 reduced anxiety, depression, and stress-induced corticosterone (101).	*L.rhamnosus GG* fed before infection reduces fly death from *Serratia marcescens* and *Pseudomonas aeruginosa* (102).	*L.rhamnosus* Lcr35 may be effective for preventing *Candida albicans* infection by reducing the virulence of the pathogen. *L.rhamnosus* Lcr35 responsible for activating DAF-16/FOXO transcription factor which is linked to increased longevity (103). *L.rhamnosus* CNCM I-3690 significantly increased average *C. elegans* lifespan (104).
Lactobacillus helveticus	Strengthens the tight junctions between epithelial cells, stabilising the gut barrier and resulting in reduced pathogen translocation (105).	*L.helveticus* SBT2171 prevented the development of arthritis caused by collagen (106).	No research performed on drosophila flies for this species.	No research performed on *C. elegans.*
Bifidobacterium animalis	Modulate the production of cytokines. Helps with abdominal pain and discomfort, such as bloating and constipation from IBS (107).	In chronically inflamed mice, *Bifidobacterium animalis* ssp. *Lactis* CNCM-I2494 improves gut barrier permeability (108).	No research performed on drosophila flies.	*Bifidobacterium animalis* subsp. *Lactis* CECT8145 exhibited high oxidative stress tolerance, partially dependent on the IIS pathway (109). Strain CECT 8145 decreases fat content while also modulating lipid metabolism and antioxidant response (110).
Lactobacillus paracasei	*Lactobacillus paracasei* IMC 502 demonstrated inhibitory properties against *Candida albicans* (111). May aid in the treatment of ulcerative colitis when used in combination with conventional therapies. Evidence of *L.paracasei* LP-33 strains in treating allergic rhinitis (112).	Splenocyte proliferative responses linked to concanavalin A increased in mice fed with *L.paracasei* LAFTI L26 (113). Phagocytic activity of peritoneal macrophages increased in mice fed with *L.paracasei* LAFTI L26 (113).	No research performed on drosophila flies.	*L. paracasei* isolate 28.4 inhibited *C. albicans* filamentation *in vitro* by inhibiting the TEC1 and UME6 genes which are needed for hyphae development (114).

1.9 The use of *C. elegans* as a model organism

Caenorhabditis elegans is a free-living transparent worm which is about 1mm in length with a 3-day lifecycle. They have 4 larval stages and adulthood. The fourth larval stage can be identified by the vulva formation. The worm then develops into an adult hermaphrodite (69) (figure 4).

It has been a challenge to understand the human host-microbiota communication as well as interaction within microbial communities in the gut, mainly due to its complexity and the cost and time required to use germ-free and monoxenic murine models for experiments (70). *C.elegans* will be used as a model organism in this project. This is because *C. elegans* are a relatively simple organism that serve as a beneficial *in vivo* model for studying ageing as well as gut microbiota-host interactions, how probiotics interact with the host and the mechanisms involved in pro-longevity (71). They have a short lifespan of 2-3 weeks at 20℃, which makes it easier to study ageing (70). They can be grown cheaply and in large quantities on bacterial plates and cultures of *C. elegans* can be frozen and stored until needed. Furthermore, c. elegans have a structurally simple nervous system with molecular and cellular functions like the mammalian nervous system, making them useful models for human diseases such as neurodegenerative disorders. They also host certain behaviours and simple forms of learning and memory which deteriorate with age, allowing simple experiments to be undertaken to measure effects on the ageing nervous system. They are also transparent, making it possible to image live intact animals (72).

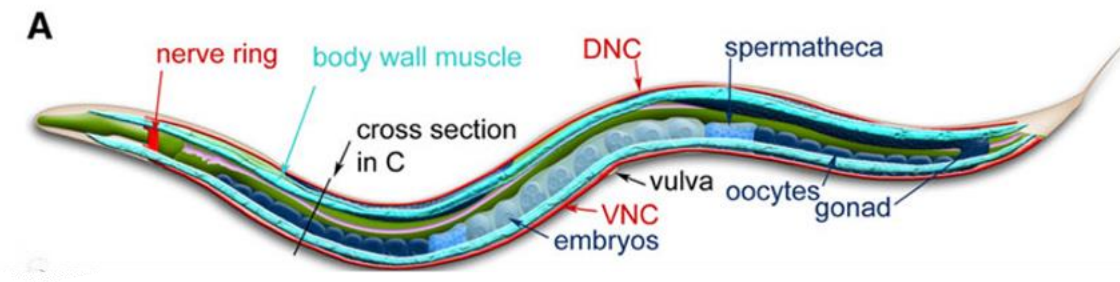


Figure 4: The structure of C. elegans. A: features of a hermaphrodite. Adult Hermaphrodites are used as model organisms as they are the simplest organisms with a nervous system. Extracted from Corsi et al (73).

1.10 The life cycle of C. elegans

The embryonic stage, four larval stages, and adulthood are all part of the life cycle of *C. elegans.* The four larval stages are comprised of L1 larva, L2 larva, L3 larva, and L4 larva. This entire life cycle usually takes three days to be completed and grow at a temperature of 20℃ (74) (69).

The cycle begins with the hatching of the egg after 12 hours, into the L1 larva stage. The next stage is L2 lasting 7 hours. This stage involves the continuation of germ cell divisions

and the elongation of the gonad begins. However, in cases of unfavourable conditions such as starvation or change in temperature, the L2 larva is converted into dauer state and is able to exit this stage once favourable conditions return and the dauer larva is converted into the L4 larva stage. Following the L2 stage, the somatic gonad precursors give rise to a total of 143 cells, which form the anterior and posterior gonadal sheaths, the spermathecae, and the uterus. By early L4 stage, committed cells have divided to form the vulval terminal cells, and the two sex myoblasts formed in L3 have grown into 16 sex muscle cells. This stage takes around seven hours to be completed. The L4 stage lasts around 9 hours and includes the termination of gonadogenesis as well as the development of vulval and uterine terminal cells. Final modifications allow the animal to begin adulthood (74) (69).

1.11 The use of C. elegans in probiotic research

Probiotics have been used in studies investigating whether probiotics are able to extend *C. elegans* lifespan. Probiotic strains used in *C. elegans* studies have been shown to act through signalling pathways such as the p38 MAPK pathway, which is a pathway that is essential for the *C. elegans* response to pathogens and plays a role in the extension of lifespan. Another pathway is the insulin/IGF-1 signalling (IIS) pathway, which is involved in the control of lifespan in *C. elegans* (75).

Bifidobacterium longum BR-108 is an example of a probiotic that has been shown to prolong the lifespan of *C. elegans.* The experiment involved worms being fed killed *Bifidobacterium longum* in addition to *E. coli,* which displayed a decrease in body length in a *B. longum* dose dependent manner. When compared to those fed *E. coli* alone, these worms had a higher survival rate following heat stress at 35 °C and hydrogen peroxide-induced oxidative stress (76).

It was suggested that when *Bifidobacterium longum* BR-108 is fed to *C. elegans,* following hydrogen peroxide-induced oxidative stress, the IIS pathway is activated. DAF-16, in the nucleus, has been suggested to co-localise with the heat shock transcription factor (HSF)-1, a family of DNA-binding proteins, following a series of phosphorylation events. This results in the transcription of the chaperones *hsp-16.2* and *hsp-70,* which play a role in stress response and longevity (75)(76).

1.11.1 A brief overview of the IIS pathway

The IIS pathway plays a role in *C. elegans* lifespan. The process begins with the Dauer formation (DAF-2), an insulin-like growth factor-1 receptor ortholog, being activated (figure 4). This leads to a series of phosphorylation events, resulting in the activation of kinases and downstream enzymes such as the phosphatidylinositol 3-kinase AGE-1 and phosphoinositide-dependent kinase (PDK)-1, which results in the phosphorylation of AKT1/2 and SGK-1 (figure 5). AKT-1 phosphorylates the transcription factors DAF-16, the ortholog of the FOXO family of transcription factors, and SKN-1, which is an important part of *C. elegans'* response to oxidative stress and adds to life span extension in animals with reduced IIS (76). This pathway may be downregulated by factors such as infections, heat and oxidative stress, and absence of oxygen (77). This results in DAF-16 travelling to the nucleus so that it may activate the expression of target genes responsible for processes such as

apoptosis to pro-longevity and anti-ageing. The IIS pathway regulates multiple genes which play a role in the immune system responses, associated with longevity in *C. elegans* (75)(78).

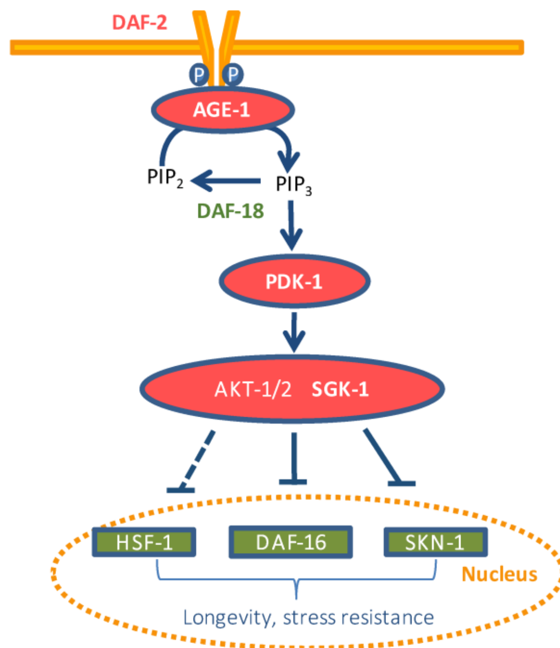


Figure 5: Overview of the Insulin/IGF-1 signalling pathway (IIS) in C. elegans. Insulin-like peptides bind to DAF-2 resulting in a cascade of phosphorylation events through different kinases shown in red (79)

1.11.2 MAPK signalling pathway in C. elegans

Mitogen-activated protein kinase (MAPK) signalling pathways are involved in many biological responses such as stress response, osmotic shock, and cell cycle progression, as well as being involved in processes such as apoptosis, autophagy, cell differentiation and proliferation. MAPK pathway activation starts with protein kinase phosphorylation and activation of MAPKKKs, which then directly phosphorylate MAPKKs, which, as soon as activated, phosphorylate MAPKs. Activated MAPKs are responsible for modulating transcription factors that power gene specific expression. There are three different types of MAPK signalling components responsible for mediating extracellular signals into the nucleus, resulting in the mammalian cells needed being activated. Examples of these types include, JNK, ERK, and p38 kinase (80)(81).

In *C. elegans* the innate immune system is regulated by the *pmk-1* p38 mitogen-activated protein kinase (MAPK) pathway. The mechanism by which this occurs is still unknown. Research has shown that *pmk-1* loss of function mutants has been shown to be linked to a decrease in pathogen resistance by *C. elegans,* suggesting that *pmk-1* plays an important role in immune system function and pathogen resistance. Research has suggested that *pmk-1* p38 MAPK pathway may function by upregulating the expression of genes such as C-type lectins and antimicrobial peptides, which are responsible for infection resistance. Furthermore, *pmk-1* has been shown to regulate the transcription factor ATF-7, resulting in the activation of intestinal expression of genes involved in host pathogen defence (81)(82).

Studies have linked the *C. elegans* p38 MAPK pathway to the transcription factor SKN-1. It has been shown that *pmk-1* phosphorylates SKN-1, leading to SKN-1 activating the transcription of *gcs-1,* a detoxification enzyme gene, which ultimately causes an oxidative stress response (82).

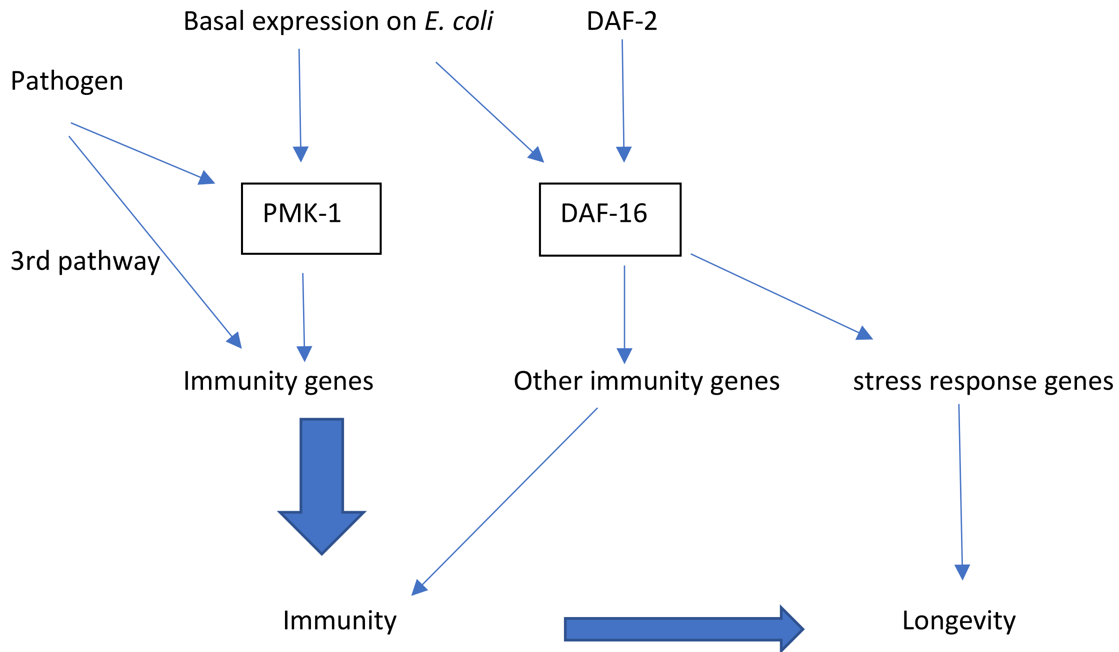


Figure 6 showing the regulation of immunity and longevity via pmk-1 and DAF-16 pathways. Modified from Troemel., E et al (83).

2. Project aims

In collaboration with Lallemand, we will determine the effect of 11 probiotic strains mentioned above, isolated from human guts, and currently marketed by Lallemand as health solutions, and available for purchase and consumption. We will test how these strains affect health and ageing in C. elegans, with a focus on the ageing nervous system.

Aim 1: Determine the effects of the probiotic strains on healthy ageing in wildtype animals. We will compare effects of the probiotic strains with the standard laboratory strain *E. coli* OP50. This will include testing effects on age-related motility, gut function, nervous system function and longevity using well-established behavioural and survival assays. We will also assay if the strains affect development and reproduction, and the extent to which the bacteria colonise the *C. elegans* gut.

Aim 2: Determine the effects of the probiotic strains on neurodegenerative disease models, testing to see whether the probiotic strains improve the age-related decline of neurogenerative disease models, Aß peptide (Alzheimer's disease) and α-synuclein (Parkinson's). We will test if the probiotic strains improve the age-related decline of several human neurodegenerative disease models, including Aß peptide (Alzheimer's disease) and α-synuclein (Parkinson's). We will use models where human Aß and α-synuclein are expressed in muscle, causing motility effects that can be quantified under a dissecting microscope.

Aim 3: Determine the effects of the probiotic strains on host immunity and stress responses. Using transgenic reporters and fluorescence imaging we will measure expression levels of markers of immunity, unfolded protein response and mitochondrial stress to determine possible mechanisms by which the probiotic strains affect host health.

Aim 4: Determine if the effects of the probiotic strains on the host are mediated by secreted metabolites. Using sterile cell-free media from the probiotic bacterial cultures we will test if the effects we identify above are generated from secreted microbial compounds or require live cells. Animals will be assayed on plates with E. coli OP50 supplemented with cell-free media isolated from the probiotic strains and compared with on plates with *E. coli* OP50 supplemented with *E. coli* OP50.

3. Materials and methods

3.1 Strains

Bacterial Strain	Code
Lactobacillus rhamnosus	HA-111
Lactobacillus brevis	HA-112
Lactobacillus rhamnosus	HA-114
Lactobacillus salivarius	HA-118
Lactobacillus plantarum	HA-119
Bifidobacterium breve	HA-129
Bifidobacterium longum	HA-135
Lactobacillus rhamnosus	R0011
Lactobacillus helveticus	R0052
Bifidobacterium animalis ssp.lactis(B94)	R0421
Lactobacillus paracasei(L26)	R0422

Table 3 - list of bacterial strains used. All strains were isolated from human guts and provided by Lallemand. Strains are currently being marketed by Lallemand as health solutions and available for consumption.

Name	Genotype
CGCM	N2 wildtype
NL5901	*Pkls2386 [unc-54p::alphasynuclein::YFP + unc-119(+)]*
GMC101	*dvIs100 [unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP]*
AU78	*T24B8.5p::GFP::unc-54-3' UTR + ttx-3p::GFP::unc-54-3' UTR] III.*
TJ356	*[daf-16p::daf-16a/b::GFP + rol-6(su1006)]*

Table 4 – list of C. elegans strains used acquired from the Caenorhabditis Genetic Center (CGC) at the University of Minnesota.

3.2 List of reagents

Reagents were acquired from Melford Laboratories Ltd, Suffolk, UK, and Sigma-Aldrich, Missouri, US.

1L of LB medium

25g LB Miller broth, dH_2O to 1L

LB plates

25g LB Miller broth, 17g agar, dH_2O to 1L

1.6L NGM agar plates

4.8g NaCl, 27.2g agar, 4g Bactopeptone, dH_2O to 1.6L. Allow to cool down to 55°C. Then add 40mL KH_2PO_4, 1.6mL $MgSO_4$, 1.6mL $CaCl_2$, 1.6mL cholesterol

1L M9 Buffer

3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml 1 M $MgSO_4$, dH_2O to 1L.

M63 minimal media

5X stock:

10g $(NH_4)_2SO_4$, 68g KH_2PO_4, 2.5mg $FeSO_4.7H_2O$, adjust to pH 7 with KOH

1X working solution:

Add 1mL 1M MgSO4.7H2O, 10ml 20% glycerol

Egg prep

1:1 bleach (20% sodium hypochlorite) and 4M NaOH

Tetramisole

1% Titron-X-100 in M9

***Myb*Mix**

A mix of 13 bacterial strains isolated from the native microbiome of the worms.

3.3 Methods

3.3.1 *C. elegans* maintenance

Worms are maintained in maintenance plates which contain 400µl of E. coli OP50 pipetted onto NGM petri dishes. This forms a thick bacterial lawn. 8 adult worms are picked and transferred onto another NGM plate every 3-4 days. Experimental plates contain 200µl of E. coli OP50 and bleached worms are pipetted onto the plate. *C. elegans* were maintained at 20°C

3.3.2 Bacterial cultivation methods

OP50 is a non-pathogenic *Escherichia coli* strain used to maintain *Caenorhabditis elegans* on agar in the laboratory (84). OP50 bacterial liquid cultures are produced by inoculating the LB Miller media broth with a single colony of OP50 from the culture plate and growing overnight at 37°C. The bacterial liquid culture is then stored at 4°C. All probiotic strains are also inoculated overnight at 37°C in sterile LB Miller media.

3.3.3 Seeding plates

C. elegans are cultured on 60mm diameter Petri plates containing 10ml of Nematode Growth Medium (NGM). This is poured aseptically and left to dry for around 24-48 hours. Once dried, the plates can be seeded immediately. The worms can be visualised easily using OP50 as this forms a thin lawn due to its growth being limited on NGM.

To seed plates, 200µl of OP50 is aseptically transferred onto experimental NGM petri plates and 400µl of OP50 for maintenance plates. These seeded plates must be incubated at room temperature overnight. The probiotic plates are seeded using 100µl of OP50 and 100µl of the probiotic strain and incubated at room temperature overnight (85).

3.3.4 Transferring worms

A flame sterilised platinum wire is used to 'pick' worms from one plate to another fresh plate by carefully picking up the worm with the tip of the wire and allowing the worms to crawl off the wire and onto the fresh plate.

3.3.5 Bleach drop cleaning of stocks

In order to remove bacterial or fungal contamination present on the plate, a bleach drop technique is utilised. A 7:8 mix of thin bleach and 4M NaOH is used to kill the infection whilst allowing embryos to survive. 5-10µl of this bleach solution is transferred to the edge of a seeded NGM plate, away from the lawn, and around 10 gravid adult worms from the contaminated plate are transferred into the bleach drop. This plate is left at 20°C for around 2 hours or until offspring develop and these worms are transferred onto new NGM plates.

3.3.6 Bleach prep

Bleach can be used to remove bacterial and yeast contamination from a worm strain as well as to age synchronise the worms. A week before performing an experiment around 40 adult worms should be picked, 10 on each plate. A week before performing the paralysis assay around 40 adult worms must be picked, 10 on each plate and allow worms to grow 2-3 days so that there are lots of eggs and gravid adults on the plate. Once there are plenty of eggs, pour 2 mL of M9 per plate and gently swirl to dislodge the worms. Using a 10mL pipette, transfer the liquid containing the worms to a 15mL falcon tube. The next step is to centrifuge for 1 minute at 3000rpm and then aspirate most of the M9 taking care not to disrupt the worm pellet. Following this, 300µl of egg prep bleach must be added to the tube and the tube must be mixed gently for 6-8 minutes or until decrease in the number of intact worms is seen. Bleaching for longer than this can kill the eggs. Once this step is done, the falcon tube is filled with sterile M9 and centrifuged again. This step should be repeated 2-3 times. The required amount of liquid should be enough to seed 100µl onto each plate. The plates should be placed at 20°C for 2 days until eggs have hatched and worms have reached L4 stage and L4 stage worms can be picked and transferred onto a fresh plate.

3.3.7 Developmental assay

Worms were age-synchronised and transferred to fresh plates seeded with 200µl of each probiotic strain on separate plates. They were left to grow at 20°C and regularly checked to assess their growth ability whilst fed with the probiotic strains.

3.3.8 Optical density

Bacterial strains were grown overnight in 7ml LB at 37°C. Spectrophotometers were blanked using LB broth. A 3ml aliquot of bacterial liquid culture is transferred into a 10mm cuvette, and the optical density is measured at 600nm. Readings are taken at 24 and 48 hours.

3.3.9 Paralysis Assay

NGM plates must be prepared beforehand and allowed to solidify overnight at room temperature. Each plate must then be seeded with OP50 and probiotic strains mixed with OP50 grown in LB media overnight. Following this step, the worms must be age-synchronised to L4 stage via bleaching. At the L4 stage, 100 worms are picked per treatment and are shifted to 25°C. The number of paralysed worms is scored each day at the same time. Nematodes were scored as paralysed if they failed to complete full body movement either spontaneously or when touched with a pick. Experiments were replicated to ensure accurate and reliable results.

3.3.10 Thrashing Assay

Worms were age synchronised by performing a bleach and time points day 1, 7, and 14 were used to execute the ageing experiment. At each time point, 10 worms were tested individually by pipetting 200µl of M9 onto the inside of a dish on a 96 well plate. A single animal was picked and transferred onto an unseeded plate and allowed to crawl to get rid of the food for around 30 seconds. The animal was then placed into the drop and left to settle for 1 minute before counting the number of thrashes for 1 minute. A thrash is characterised by the animal moving their head and tail towards each other.

3.3.11 Biofilm Formation Assay

The Microtiter Dish Biofilm Assay allows for the formation of a biofilm on the wall and bottom of a microtiter dish. This assay was carried out to study biofilm formation by the bacterial strains, *L.rhamnosus* HA-111, *L.rhamnosus* ROO11, *B.breve, L.brevis, L.paracasei, L.plantarum, B…ssp lactis, B.longum,* OP50, and Mybmix strains. *Pseudomonas aeruginosa* was also used as a control as it has been shown to form a biofilm.

The first step of the assay consists of growing liquid cultures of each of the bacterial strains overnight at the required temperatures. The cultures are then diluted 1:100 into M63 minimal medium, a standard biofilm assay medium. 100µl of the dilution is added into each well in a 96 well dish and 8 replicate wells were used per treatment. This was then incubated overnight at 37°C.

Following incubation, the culture supernatants are discarded by inverting the plate. The plate is then submerged in a small tub of water and the water is shaken out. This step is repeated twice to help remove unattached cells and media components that can be stained in the next step, and also dramatically lowers background staining. Subsequently, 125µl of a

0.1% crystal violet solution in water is added into each well and incubated at room temperature for 15 minutes. Plates are then rinsed 4 times with water, shaken out and blotted vigorously on paper towels to get rid of all the excess cells and dye. The microtiter plate is left upside down to dry overnight.

24 hours later, 125µl of 30% acetic acid in water is added into each well in order to solubilise the crystal violet. The microtiter plate is incubated at room temperature for 15 minutes and then 125µl if the solubilised crystal violet is transferred to a new 96 well microtiter dish and absorbance is quantified at 550nm using a plate reader. The blank for this assay is 30% acetic acid in water.

3.3.12 Gut colonisation assay

To assess the ability of *C. elegans* to colonise specific bacteria an accurate count of intestinal bacteria from *C. elegans* can be calculated by measuring the number of Colony Forming Unit (CFU) isolated from the worm intestine. Firstly, an age synchronised nematode population was prepared via bleaching. Exactly 50 worms were picked into a sterile Eppendorf tube containing 500µl of sterile M9. The exact number of worms transferred into the tube must be known. The next step consisted of pipetting 25µl of 100mM tetramisole into each tube and mixing well by inversion for 5 minutes, immobilising the worms. The worms were then spun down at 6,000 *g* for 1 minute to create a worm pellet at the bottom of the tube and the supernatant was carefully pipetted away. Following this, 500µl of 3% lab bleach (egg prep) in M9 is added and mixed for 5 minutes while the worms are surface sterilised. The tubes are spun down at 13,000 *g* for 1 minute and the supernatant carefully removed. The tube is then topped with 1mL of M9 to wash the worms and this washing step is repeated twice. It is then required to add 200µl of 1% Titron-X-100 in M9 and a pestle with a sterile attachment is used to grind the sample until a creamy suspension is achieved and the bodies of the worms cannot be seen. The tubes should be centrifuged at 13,000 *g* for 10 minutes and then the supernatant removed. The bacterial pellet is then resuspended in 500µl M9 and this tube is labelled as 'original sample'. Serial dilutions of the original sample must then be formed by taking 50µl of the suspension, vortex the sample and adding it to 450µl of milliQ water. This tube is labelled 10^{-1}. Vortex tube 10^{-1} and remove 50µl of suspension and add to new tube of 450µl of milliQ water. This tube is labelled $10^{-2.}$ This step is repeated, and the tube is labelled $10^{-3.}$ Following this, 50µl from each tube is plated onto three separate LB agar plates. This should be done under aseptic conditions using a sterile glass spreader and dipping the spreader in ethanol between each plate. Plating 50µl of each increases the dilution factor by a factor of 10. Label each plate with the new dilution factors $10^{-2,}$ $10^{-3,}$ $10^{-4.}$

The final step requires the incubation of the plates for 24 hours at the required temperature. OP50 and the probiotic strains were incubated at 37°C, whereas the experimental microbiome Mybmix strain is incubated at 25°C. (used as a control as it has been shown to colonise the gut of C. elegans). Plates must be incubated upside-down to prevent condensation falling onto the agar.

The formula for calculating the number of CFUs/worm:

#CFUs/worm= (Number of single colonies x Dilution factor)/ Number of worms used

3.3.13 Leica DMR and Quantitative fluorescence imaging

L. rhamnosus HA-111, *L. rhamnosus* ROO11, and OP50 cultures were grown overnight in LB and incubated at 37°C. Plates were seeded with 100µl *L. rhamnosus* HA-111 mixed with 100µl OP50, 100µl *L. rhamnosus* ROO11 mixed with 100µl OP50. OP50 was used a control.

Animals were age-synchronised at day 1 adulthood by performing a bleach prep and picking L4 animals the day before the imaging. Following this, animals were imaged at day 1 adulthood. 50 µl of 2% agarose pads were placed on microscope slides and 15µl of 25mM tetramisole hydrochloride was added. Around 10 worms were picked and transferred to each slide, with 20 worms being used per condition. A Leica DMR was used to visualise GFP fluorescence at 10x magnification, with an exposure of 150ms. Images were analysed using the FIJI distro of ImageJ.

3.3.14 Statistical analysis

All experiments were performed with three independent biological replicates and expressed as a mean with standard deviation. Statistical comparisons between the different groups were analysed by one-way ANOVA and Bonferroni post hoc analysis. A 'statistically significant' result had a value of $P \leq 0.05$, rejecting the null hypothesis. Prism software was used to carry out the analysis.

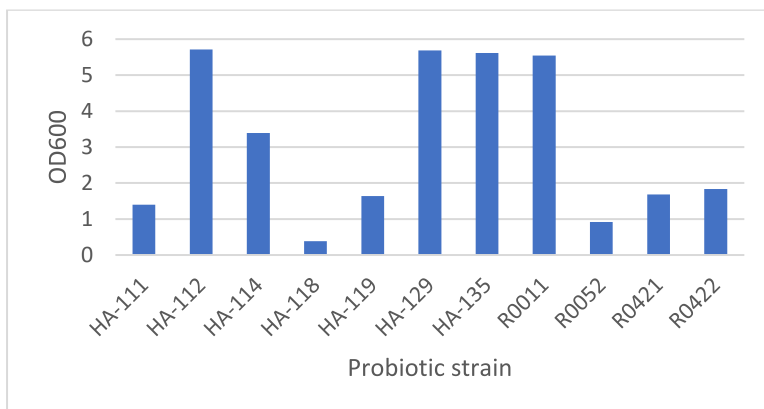
4. Results

4.1 *Lactobacillus salivarius* HA-118 and *Lactobacillus helveticus* R0052 do not grow under our laboratory conditions

In order to assess microbial growth of the probiotic strains and determine their suitability for this research project, the optical density was measured at a wavelength of 600nm in LB media at 24 and 48 hours as shown in figure 7B.

As shown in figure 7A, the optical density was measured at 24 hours in one trial and it is evident that strains *Lactobacillus salivarius* HA-118 and *Lactobacillus helveticus* R0052 have a very low optical density, suggesting that they were not growing in our laboratory conditions. Therefore, it was decided to eliminate these two strains and the optical density of the remaining probiotic strains were measured after 24 and 48 hours to investigate whether the probiotics required more than 24 hours to grow. It was seen that *Lactobacillus Brevis* HA-112 showed the highest optical density compared to other strains and OP50, as shown in figure 7B. Measuring optical density after 48 hours demonstrated that strains *Lactobacillus paracasei* R0422 and *Lactobacillus plantarum* HA-119 require 48 hours to fully grow. Some strains such as *Bifidobacterium breve* HA-129 and *Bifidobacterium animalis ssp.lactis* R0421 show a decrease in optical density at 48 hours and therefore more repeats of this experiment is required.

A



B

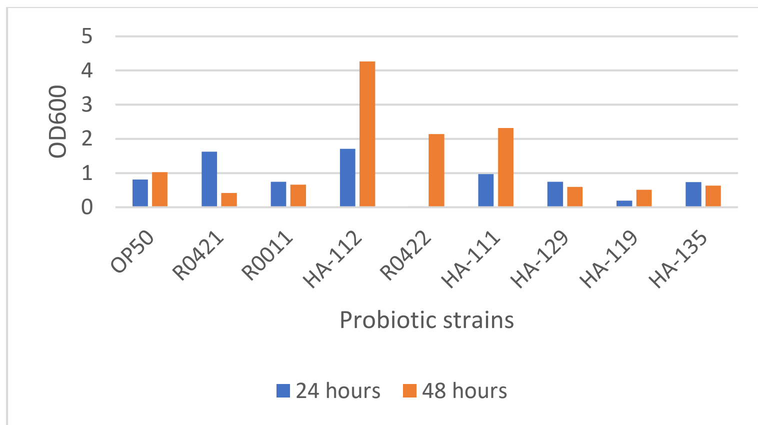


Figure 7 – Showing the optical density of bacterial strains of 1 trial. Optical density of probiotic strains measured after 24 hours. Lactobacillus salivarius HA-118 and Lactobacillus helveticus R0052 shown to not grow under our laboratory conditions (A). 1 Trial showing optical density of remaining probiotics and OP50 measured after 24 and 48 hours, with Lactobacillus brevis HA-112 showing the highest OD600 (B).

4.2 *C. elegans* seem to grow best on OP50 rather than probiotic strains alone.

The next stage was to carry out a developmental assay to test whether the *C. elegans* were able to grow normally on the probiotic strains alone. The result of this showed that the *C. elegans* were not growing on the probiotic strains alone. Therefore, it was decided to grow the worms on plates seeded with 100µl of OP50 and 100µl of the probiotic strain as this allowed the worms to grow.

4.3 It is unclear whether the probiotics colonise the *C. elegans* gut.

In order to determine whether *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 colonise and survive in the *C. elegans* gut, a gut colonisation assay was attempted and the total number of bacterial colony forming units (CFUs) were counted. This experiment was carried out with wildtype N2 worms, using OP50 and *MybMix* as a control. *MybMix* is made up of 13 bacterial strains isolated from the native microbiome of the worms and has been shown to colonise the *C. elegans* gut in previous experiments in our laboratory. Two trials were performed with the four bacterial strains and the total number of CFUs was found to be zero. Therefore, it was decided to repeat the experiment using only OP50 and *MybMix.* This also resulted in zero CFUs. Further experiments would need to be performed to understand what may have caused the experiment to not work. Possible causes for this could be due to the fact there may have been an issue with the egg prep (3% lab bleach) being too strong. This may have potentially killed the worms. Additionally, the *MybMix* requires around 4 days to grow, however the CFUs were counted after 24 and 48 hours. Therefore, next time this experiment is performed the bacteria should be left to grow for longer.

4.4 *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 partially suppress β-amyloid toxicity.

In order to investigate the effect of the probiotic strains against β-amyloid toxicity compared to OP50, a paralysis assay was carried out, using the C. *elegans* strain GMC101 with muscle specific, temperature inducible expression of the β-amyloid peptide (1-42).

Temperature upshift from 20°C to 25°C leads to an accumulation of the β-amyloid, dysfunction of muscle cells and paralysis of worms. This assay measures the time it takes for the transgenic worms to become paralysed following the upshift in temperature.

Figure 8 displays the percentage of paralysed worms on day 1 to 3 of adulthood. Worms treated with the probiotic strains *L. rhamonsus* ROO11 and *L. rhamnosus* HA-111 mixed with OP50 show a statistically significant delay in paralysis compared to worms growing on OP50, however still result in an increase in percentage of paralysed worms with age, similarly to OP50. By day 3, around 88% of worms fed *L. rhamnosus* ROO11+OP50 and 94% of worms fed *L. rhamnosus* HA-111+OP50 were paralysed, compared to 100% of worms growing on OP50 being paralysed. This suggests that the probiotic strains *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 mixed with OP50 partially supresses β-amyloid toxicity.

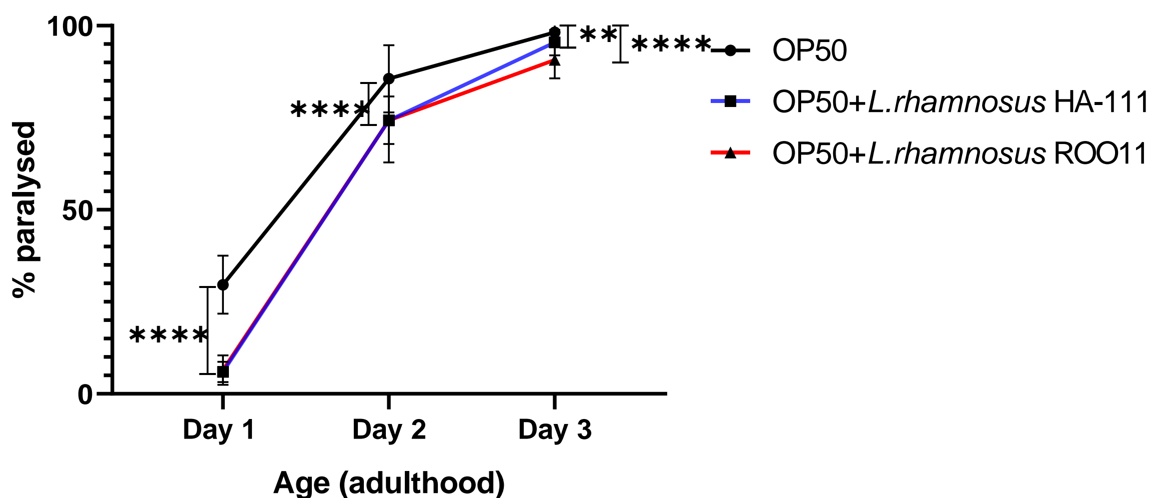


Figure 8 – Effects of L. rhamnosus ROO11 and L. rhamnosus HA-111 on the paralysis of GMC101 worms. Paralysis Assay using GMC101 worms, grown on OP50 and treated with L. rhamnosus ROO11 and L. rhamnosus HA-111 mixed with OP50. Paralysis curve presents the average percentage of paralysed worms scored on day 1, 2, and 3, with data being pooled from three trials. Both probiotic strains show a statistically significant lower percentage of paralysed worms in comparison to OP50 (P≤ 0.0001). L. rhamnosus HA-111+OP50 shows a significantly lower percentage on day 3 compared to OP50 (P ≤ 0.01).

4.5 *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 show no improvement against motility decline in NL5901 *C. elegans.*

In order to investigate whether the partial suppression of β-amyloid toxicity seen in the AD model was specific for that model, or if the probiotic strains improve health in general, thrashing assays were carried out on wildtype worms to test the effect of the probiotic strains *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 on the locomotion of the worms, compared to OP50, by scoring the number of lateral swimming movements performed by the *C. elegans* per 1 minute. Thrashing assays were performed with wildtype worms. As shown in the Figure 9, all strains result in a decrease in the number of thrashes performed

by each worm and by day 14 of adulthood both *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 show a statistically significant decrease in the frequency of thrashing movements of the wildtype strain, compared to OP50, suggesting that the probiotic strains either have no effect on age-related locomotion or may worsen the effects, compared to OP50.

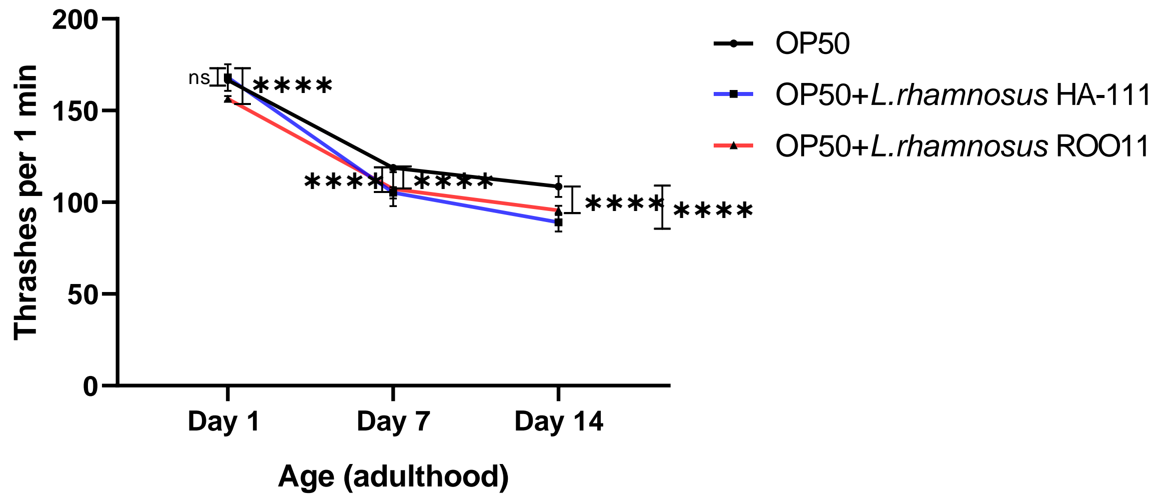


Figure 9 – Effects of L. rhamnosus ROO11 and L. rhamnosus HA-111 on the locomotion of wildtype worms. Date pooled from 3 trials of thrashing assays using wildtype worms on day 1, 7 and 14 of adulthood, growing on OP50 and treated with L. rhamnosus ROO11 and L. rhamnosus HA-111 mixed with OP50. Both probiotic strains mixed with OP50 show a statistically significant lower number of thrashes on day 7 and 14, compared to OP50 ($P \leq 0.0001$).

4.6 *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 show no improvement against motility decline in the NL5901 *C. elegans* PD model.

The aim of this experiment was to investigate if the probiotic strains improve the age-related decline of neurodegenerative disease models such as α-synuclein in Parkinson's disease by using a transgenic strain NL5901 which expresses α-synuclein in muscle, causing motility effects.

Figure 10 demonstrates a thrashing assay for the strain NL5901 grown on *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 mixed with OP50 and OP50 as a control. The worms grown on both *L. rhamnosus* Ha-111 and *L. rhamnosus* ROO11 follow a similar pattern to worms grown on OP50, where there is an increase in motility decline with age. This shows that the probiotics show no improvement against motility decline in *C. elegans.* There is a significant difference between worms grown on OP50 and worms grown on OP50 mixed with *L. rhamnosus* HA-111, with OP50 showing more of a suppression of motility decline compared to *L. rhamnosus* HA-111.

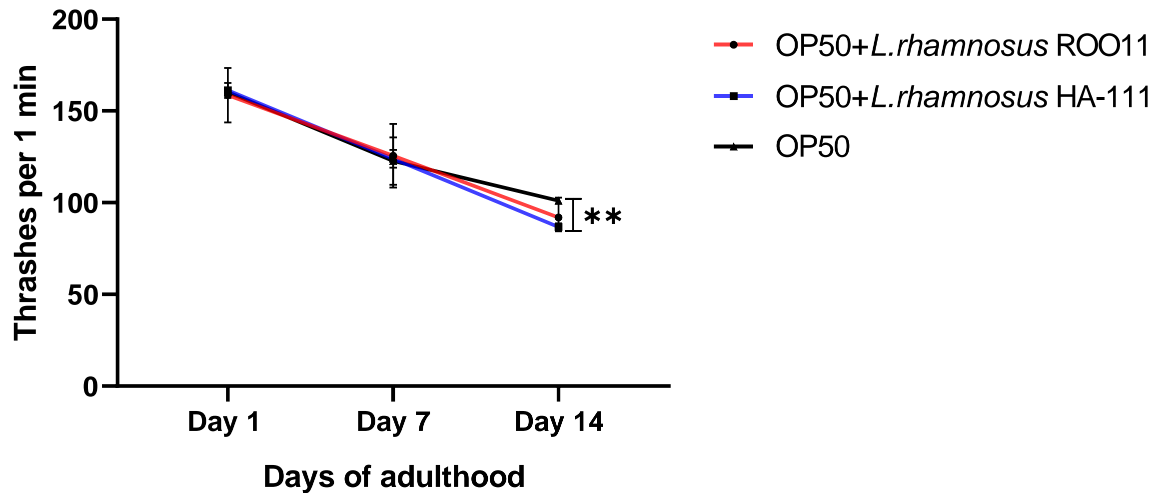


Figure 10 - Effects of L.rhamnosus ROO11 and L.rhamnosus HA-111 on the locomotion of NL5901 C. elegans. Data pooled from 3 trials of thrashing assays for strain NL5901 worms growing on OP50 and treated with L.rhamnosus ROO11 and L.rhamnosus HA-111 mixed with OP50. Day 14 worms grown on OP50+ L.rhamnosus ROO11 show a statistically significant lower number of thrashes, compared to OP50 (P ≤ 0.01).

4.7 Probiotic strains form a biofilm.

In order to investigate whether the probiotic strains have any properties that may benefit health, we looked at a microtiter dish biofilm formation assay to have a look at the effect of several non-pathogenic probiotic strains to see whether they produce a biofilm, so that if they did, we could potentially explore the impact of a biofilm on host physiology and ageing.

P. aeruginosa was used as a positive control, as it has previously been shown to produce biofilms. The experimental microbiome, *MybMix* was also tested as we know that *MybMix* colonises the worm gut. The probiotic strains *P. aeruginosa,* and OP50 liquid cultures were incubated at 37°C. *MybMix* liquid culture was incubated at 25°C. The concentration of the crystal violet dye in each well is proportional to the number of cells in the biofilm.

All strains produced a biofilm; however, it can be seen that *B. animalis ssp. lactis* and *L. rhamnosus* HA-111 were the strains which showed the highest absorbance, therefore produced a higher number of cells in the biofilm. Differences between the probiotic strains and OP50 are statistically non-significant.

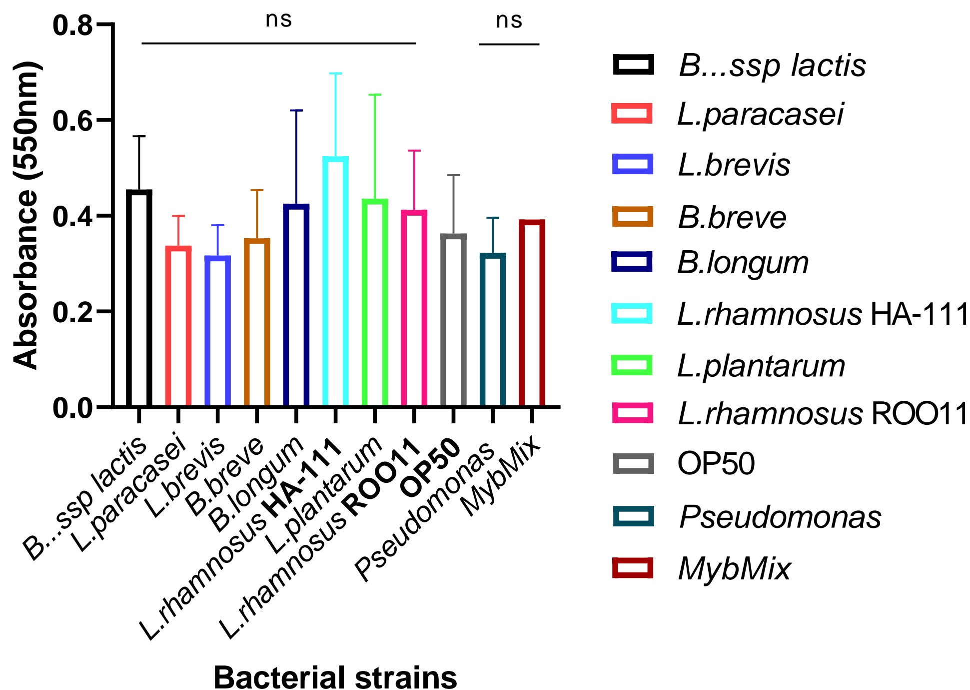


Figure 11 – All probiotic strains have an absorbance measured at 500nm greater than 0.3nm, suggesting the formation of biofilms. Biofilm formation Assay graph where absorbance is proportional to number of cells in biofilm. Graph showing the absorbance at 550nm of each of the bacterial strains with data being pooled from 6 trials, using P. aeruginosa as a positive control. B...ssp lactis, B. longum, L. rhamnosus HA-111, L. plantarum, and L. rhamnosus ROO11 shown to produce a higher number of cells in biofilm, compared to OP50 ($P > 0.05$). All probiotic strains are being compared to OP50.

4.8 *L. rhamnosus* ROO11 may increase immune system activation.

In order to determine in what ways *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 is affecting host health, we decided to focus on immunity and stress, by looking at whether these probiotics induce an innate immune response regulated by the p38 MAPK pathway in *C. elegans* as the immune response in *C. elegans* is regulated by the p38 MAPK pathway. Fluorescence imaging was used with a transgenic reporter called AU78 expressing T24B8.5p:GFP::unc-54-3' UTR + ttx-3p::GFP::unc-54-3' UTR] III. This gene is downstream of *pmk-1* and is attached to a green fluorescent protein (GFP), which is used as a marker protein. Worms are treated with *L. rhamnosus* HA-111+OP50, *L. rhamnosus* ROO11+OP50, and OP50 is used as a control. The aim of this experiment was to investigate the effects of these probiotic strains on host immunity and stress by measuring expression levels of this gene. From our results (figure 12), it can be seen that there is a significantly greater fluorescence intensity in worms fed on *L. rhamnosus ROO11*+OP50, compared to OP50 alone, suggesting that there is an increase in *T24B8.5* gene expression, resulting in increased PMK-1 activation. This suggests that *L. rhamnosus* ROO11 may potentially have the ability to activate the immune system. Furthermore, *L. rhamnosus* HA-111 mixed with OP50

demonstrates a non-significant increase in fluorescence, compared to OP50. Further trials would be useful to produce a more reliable conclusion.

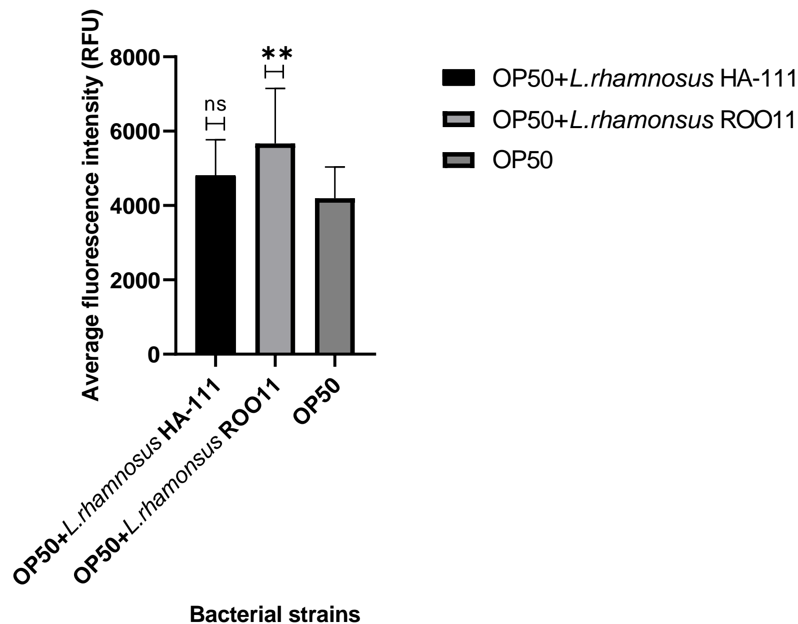


Figure 12 – Effects of probiotic strains on expression levels of markers of immunity, with data pooled from 2 trials. Fluorescence imaging using transgenic reporter AU78 used as a measurement of immune system activation. Worms are grown on OP50 and treated with L. rhamnosus HA-111+OP50 and L. rhamnosus ROO11+OP50. Worms treated with L. rhamnosus ROO11+OP50 show a statistically significant higher mean, compared to worms grown on OP50 (P ≤ 0.01). Worms treated with L. rhamnosus HA-111+OP50 show a statistically non-significantly higher mean, compared to OP50 (P > 0.05). L. rhamnosus ROO11 *and L. rhamnosus* HA-111 *are being compared to OP50.*

5 Discussion

The aim of this study was to investigate the effects of the 11 probiotic strains provided by Lallemand, in order determine whether they improve ageing and if they have any effects on the neurodegenerative disease models, Aß peptide (Alzheimer's disease) and α-synuclein (Parkinson's disease). This study was mainly focused on the probiotic strains *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11.

5.1 Probiotics have been shown to have positive health effects.

Research performed on different animal models such as human, mouse, *Drosophila melanogaster* and *C. elegans* have shown that probiotics provide many positive benefits and may have the ability to restore gut microbiome dysbiosis (120). Some studies have suggested the ability of some probiotic strains such as *Bifodobacterium breve* A1 to have a positive effect on neurodegenerative disorders. *Lactobacillus plantarum, Bifidobacterium breve, Bifidobacterium longum,* and *Lactobacillus rhamnosus* showed beneficial effects against depression and anxiety, further suggesting that there may be a link between probiotics, the gut microbiome, and the brain (121). Therefore, this led us to research the effects of the specific strains.

5.2 It is unclear whether *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 colonise the *C. elegans* gut.

One of the aims of this project was to determine whether the probiotic strains had the ability to colonise the *C. elegans* gut and to what extent. Unfortunately, the experiment had some limitations which may have been due to the lab bleach used, possibly killing the worms, and also due to the possibility of insufficient time being allowed for the bacterial strains to grow. Further trials would allow us to investigate more accurately whether gut colonisation occurs by these probiotic strains. Previous research has demonstrated that the probiotic strain *L. rhamnosus* GG is able to colonise the *C. elegans* gut compared to *E. coli* OP50 (122). Due to these technical problems, it is not clear whether the worms are eating the probiotics.

5.3 *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 partially suppress β-amyloid toxicity.

One of the main characteristics of AD involves the formation of plaques composed mainly of insoluble Aß peptides in the brain. This has been shown to impact neuron signalling, resulting in memory impairment, which is a key symptom of AD, as well as neuroinflammation, damaging the neurons (123).

Research has shown that those with AD have a reduced microbiota diversity and has suggested that probiotics may ameliorate the symptoms and pathologies of Alzheimer's disease. One paper suggested that the A1 strain of *B. breve* prevented cognitive dysfunction, as well as suppressing the immune response and neuronal inflammation caused by Aß (124). Another probiotic formulation called SLAB51, containing lactic acid bacteria and *bifidobacteria*, was shown to have similar effects on mice, by reducing brain damage and Aß aggregation, and delaying AD progression (125). This suggests that some probiotic strains could be beneficial to those with AD, but it is important to determine the specific bacteria

that play a role in the bidirectional communication between the gut and the brain. Our study looked at some strains which have not previously been researched in depth.

The first aim of this project was to investigate whether the probiotic strains have an effect on a transgenic *C. elegans* Aß toxicity model which expresses human Aß, resulting in the formation of amyloid plaques. The model has muscle-specific temperature-inducible expression of the Aß peptide, dysfunction of muscle cells and paralysis of worms (126).

We decided to focus on the probiotic strains *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 as the *C. elegans* fed with these strains mixed with OP50 were able to grow and develop normally. Results from the paralysis assay suggest that these strains do indeed have an effect on the Aß toxicity model, indicating that the probiotic strains mixed with OP50 partially suppress β-amyloid toxicity due to a highly statistically significant delay in paralysis on day 1 and 2 compared to OP50, shown in figure 16. This suggests that the two probiotic strains may have beneficial effects on the Aß toxicity model by potentially decreasing protein aggregation or reducing Aß expression. These strains may also play a role in increasing the ability of the animal to cope with toxicity by perhaps reducing neuroinflammation caused by protein aggregation.

5.4 *L. rhamnosus* HA-111 and *L. rhamnosus* ROO11 do not improve age-associated motility decline of wildtype *C. elegans.*

Following on from these results, we asked whether these probiotic strains were having a general effect on health or whether the effect seen was specific to protein aggregation.

Research on humans has shown that lactobacillus probiotic strains have the potential to increase intestinal barrier integrity, leading to the maintenance of immune tolerance as well as decreased passage of bacteria across the intestinal mucosa and decreased gastrointestinal infections and IBS symptoms. This could potentially decrease innate immune system activation and reduce neuroinflammation (127).

Therefore, using *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111, a thrashing assay was carried out to investigate the effects of these strains on the locomotion of wildtype worms. It was shown that the worms grown on the probiotic strains demonstrated an increase in motility decline with age, like worms grown on OP50. Therefore, it can be said that the probiotics have no effect on age-related motility decline in wildtype *C. elegans* and cannot be said to be having general health effects with this experiment. It is possible that the health benefits of these probiotic strains are specifically related to Aß aggregation.

5.5 The probiotic effect may be specific to Aß.

Similarly, to AD, PD also consists of the accumulation of protein aggregates. A main feature of PD is the accumulation of alpha synuclein aggregates in Lewy bodies (128). Studies have shown that certain bacteria in the gut microbiota may regulate the pathogenesis of PD, for example, PD patients have shown altered microbiota composition compared to healthy patients, including lower numbers of *C. coccoides* and increased numbers of *Lactobacilliaceae* (129). However, the ways in which the gut microbiota influences PD pathogenesis is not yet fully understood (130).

A study in a mouse model of PD over-expressing α-synuclein have shown that reduction of gut bacteria using antibiotics resulted in decreased expression of α-synuclein, supporting the idea that the gut microbiota may regulate PD pathology (131). Furthermore, research looking into the use of probiotics to ameliorate PD pathology has shown that the probiotic *Bacillus subtilis* PXN21 showed an ability to inhibit and reverse α-Syn aggregation in *C. elegans* through potential mechanisms such as biofilm formation and nitric oxide production (132).

Therefore, we decided it would be interesting to determine whether our probiotic strains have an effect on our PD model by performing a thrashing assay. Furthermore, we asked whether the probiotic strains were having an effect in general on protein aggregation or whether it was an effect specific to the Aß aggregation. A thrashing assay was performed to determine the effects of *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 on the locomotion of the NL5901 transgenic strain, expressing α-synuclein in muscle. Our results showed that the worms fed with the probiotic strains mixed with OP50 displayed an increase in motility decline with age, similarly to worms grown on OP50. Therefore, it can be said that the probiotics have no effect on age-related motility decline in *C. elegans,* suggesting that the probiotics are potentially having an effect specific to Aß, rather than protein aggregation in general.

5.6 Probiotics strain produce a biofilm which may provide beneficial effects to the host.

Biofilms are defined as communities of microbes characterised by cells that irreversibly attach to each other and often to a surface. Biofilms formed by probiotic bacteria has been shown to be advantageous as it may aid in promoting colonisation in the mucosa of the host as well as preventing colonisation by pathogenic bacteria (133). However, not all strains possess this ability to form a biofilm and environmental factors play a part in biofilm formation, therefore it was important to test whether our strains had this property (133). *Bacillus subtilis*, a commensal species of the human gastrointestinal tract, is a robust model organism used for biofilm formation research. *B. subtilis* has developed mechanisms to enable survival in harsh conditions such as its ability to form endospores, increasing survival in extreme environmental conditions such as nutrient depletion (134). Furthermore, as mentioned previously, the probiotic *B. subtilis* PXN21 in *C. elegans* has been shown to suppress α-synuclein aggregation and this was suggested to be partially influenced by the ability of *B. subtilis PXN21* to form a biofilm (132). Additionally, previous research has suggested that biofilm formation by probiotics has increased *C. elegans* lifespan (132).

A microtiter dish biofilm formation assay was carried out to assess whether our probiotic strains produce a biofilm as this would be a way to ensure that the probiotics remain in the gut rather than just passing through and would also allow us to investigate the link between biofilm formation and longevity. The biofilm formation assay, using a crystal violet dye, indicated that all strains produced biofilms, with *B. animalis ssp. lactis* and *L. rhamnosus* HA-111 producing the highest number of cells in the biofilm, compared to other strains. However, the data was variable between trials and therefore a conclusion could not be drawn. This variability could be due to reasons such as our laboratory conditions not being the optimal growth conditions as well as the probiotics possibly requiring a longer time

period to form a biofilm. Furthermore, biofilm formation assays are not performed on *C. elegans,* therefore it is not known what happens in worms.

Research studying *Bacillus subtilis, Lactobacillus rhamnosus* and *Pseudomonas fluorescens* suggest that these bacteria may have the ability to increase *C. elegans* stress resistance. Additionally, biofilms produced by these bacteria have been shown to have benefits such as protecting against pathogenic infection and extending lifespan (135). Therefore, future work could involve testing whether biofilms produced by *B…ssp lactis* and *L. rhamnosus* HA-111, protect against pathogenic infection via a slow-killing assay using *Pseudomonas aeruginosa.*

5.7 *L.rhamnosus* ROO11 may increase immune system activation.

The gene *pmk-1* encodes an MAPK, which is orthologous to the human p38 MAPK and is a fundamental constituent of the MAPK pathway. The p38 MAPK pathway is a pathway shown to regulate the innate immune system and inflammatory response in *C. elegans* and is a key regulator of pro-inflammatory cytokines such as TNF- α and IL-6 (136,137). The p38 MAPK pathway in *C. elegans* regulates the transcription factor ATF-7 which plays a role in activating genes involved host defence against pathogens (138).

It has also been shown that *pmk-1* loss-of-function mutants have decreased resistance to pathogens, emphasising the importance of this gene in the immune response. Research with *C. elegans* using probiotics has suggested that probiotics such as *Lactobacillus fermentum* JDFM216 may increase longevity and immune response via enhanced *pmk-1* signalling in *C. elegans*, shown by a significant decline in lifespan in *pmk-1* mutants compared to wildtype worms during a solid killing assay where *pmk-1* deletion in worms exposed to *Lactobacillus fermentum* JDFM216 lowered survival rate compared to wildtype worms, suggesting that *pmk-1* signalling is involved in longevity and immunity, however, the exact mechanism is unknown(139).

One of the aims of this project was to determine the effects of the probiotic strains on host immunity to see if feeding on *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 induces an innate immune response, enabling possible mechanisms by which the probiotics affect host health, to be determined. This was performed by measuring expression levels of markers of immunity using a transgenic reporter, AU78, which carries a promoter of a transcriptional target of the p38 MAPK pathway, *T24B8.5,* attached to a green fluorescent protein (GFP) (140). Therefore, we questioned whether feeding *C. elegans* on the probiotics could potentially induce an innate immune response via p38 MAPK pathway activation. Both probiotic strains showed an increase in fluorescence intensity compared to OP50, with *L. rhamnosus* ROO11 mixed with OP50 showing a significant increase. This implies that there is an increase in expression of the *T24B8.5* gene, reflected by an increase in fluorescence intensity, suggesting increased *pmk-1* activity. Therefore, it can be said that our results suggest *L. rhamnosus* ROO11 may upregulate the immune system activation via the upregulation of *pmk-1.* It would be interesting to investigate the effects the probiotic strains may have on an ageing immune system and to determine whether they can improve pathogenesis of neurogenerative disorders via the p38 MAPK pathway (141).

5.8 Possibility of a Dietary Restriction (DR) effect

Dietary restriction (DR) is characterised as reducing food intake without causing malnutrition and has been shown to play a role in delaying ageing and age-related disorders (142). Research in the 1930s which involved rats being fed restricted diets, proved that DR in animals could increase longevity and reduce age-related pathologies (143). There is a possibility that there may have been DR in my research as the *C. elegans* seemed to have been developing slower than normal, however more research would need to be done to confirm this.

6 Conclusion

This study focused on the use of *C. elegans* to determine the effects of the 11 probiotic strains, which are currently being marketed as health solutions by Lallemand, on host health and ageing. This study investigated the effects of these probiotic strains on the ageing nervous system by using neurodegenerative disease models such as Aß peptide model and α-synuclein model. *C. elegans* has been an excellent model organism for this study due to its short life cycle of 3 days, structurally simple nervous system, and its similarity to human molecular/cellular functions.

This study has closely looked at the probiotic strains *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111 and has shown that these probiotic strains partially suppress Aß aggregation in *GMC101* transgenic worms, in comparison to the control. However, these probiotic strains showed no improvement against motility decline in both wildtype N2 worms and *NL5901* transgenic worms, which led us to believe that this probiotic effect is specific to Aß aggregation, however more research would allow for a clearer conclusion.

Furthermore, it was seen that all probiotics used in this study produced a biofilm and therefore further research could be carried out to investigate the effects of these probiotics on lifespan and pathogen resistance via biofilm formation. Due to the variability in this data, we are unable to say whether these probiotics produce a biofilm for sure, however previous research has suggested that some probiotic strains, such as *B. subtilis,* do produce biofilms.

Lastly, this study demonstrated that *L. rhamnosus* ROO11 may increase innate immune system activation through the use of the transgenic reporter *AU78*, allowing the expression level of a promoter of a transcriptional target of the p38 MAPK pathway, attached to GFP, to be measured. *L. rhamnosus* ROO11 showed a significant increase in fluorescence intensity, in comparison to OP50, suggesting it may upregulate immune system activation. Further research would allow this study to look into the effects of this strain on an ageing immune system and to investigate the role, if any, of the p38 MAPK pathway in the pathogenesis of AD and PD, in order to focus on potential targets for ameliorating or treating these disorders. *L. rhamnosus* HA-111, on the other hand, showed an insignificant increase in fluorescence intensity, however further trials would allow a more reliable result and conclusion.

Overall, this study further supported the idea and research that has been done to show that probiotics are beneficial to host health and the reasons why they should be consumed by individuals. With further study probiotics can potentially be used to prevent, delay, or treat disorders, especially those linked to ageing such as neurodegenerative disorders.

7 Future Work

There are many unanswered questions about the potential health benefits of the 11 probiotic strains provided by Lallemand. Continuing from this research, I would investigate the following areas:

- It would be interesting to revisit the extent to which the probiotic strains colonise the *C. elegans* gut by performing a gut colonisation assay and measuring the number of bacterial Colony Forming Units isolated from the worm intestine. This would require understanding of what went wrong.
- Following on from the biofilm formation assay, many studies have shown that biofilm formation can increase lifespan and increase pathogenic resistance in *C. elegans.* A study looking at biofilm formation by B. subtilis performed a slow killing assay to test whether the biofilm rendered *C. elegans* resistant to *P. aeruginosa* PA14. This would require feeding the *C. elegans* with the probiotic strain mixed with OP50 for 5 days of adulthood before transferring the worms to a plate seeded with *P. aeruginosa* PA14. The number of dead worms is scored as an indication of how much resistance to *P. aeruginosa* PA14 is shown. This could indicate whether the probiotic strains increase resistance to pathogenic infections.
- Experiments have shown that probiotics such as B. subtilis may increase *C. elegans* lifespan. Therefore, it would be interesting to perform a lifespan assay on *C. elegans* fed with the probiotic strains mixed with OP50. The numbers of worms alive, dead, and censored would be scored every other day. If the worms fed with probiotics survive longer than those fed with OP50 alone, it would suggest that the probiotic strain increases lifespan of *C. elegans.*
- This study only focuses on mainly two strains, *L. rhamnosus* ROO11 and *L. rhamnosus* HA-111. Therefore, it would be useful to explore all other strains provided by Lallemand and carry out paralysis and thrashing assays with these strains to investigate their effects and benefits.
- Use of TJ356 strain and fluorescence imaging to determine possible mechanisms by which the probiotic strains affect host health. This strain consists of a green fluorescent protein (GFP) gene attached to the *daf-16* gene. *Daf-16* is transcription factor found in *C. elegans* and is similar to the FOXO transcription factor in humans. It acts as a major regulator of lifespan, heat, and oxidative stress resistance. *Daf-16* has been shown to play a role in increased longevity in *C. elegans* treated with the probiotic *Bifidobacterium longum* BB68. Another study has supported this by using *daf-16* loss-of-function mutants to show that *C. elegans* fed on vegetative B. subtilis display an increase in the number of α-synuclein aggregates on day 3 of adulthood onwards, implying that *daf-16* may play a role in the late life protection of the worms fed on vegetative B. subtilis. Therefore, it would be interesting to see whether there is an increase in *daf-16* expression in *C. elegans* grown on our probiotic strains.

8 Bibliography

1. Corrigan, M., Roberts, K. and Steiger, E., n.d. *Adult Short Bowel Syndrome*. Chapter 4. Gut microbiome
2. den Besten, G., van Eunen, K., Groen, A., et al., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, 54(9), pp.2325-2340.
3. Thursby, E. and Juge, N., 2017. Introduction to the human gut microbiota. *Biochemical Journal*, 474(11), pp.1823-1836.
4. Tanaka, M. and Nakayama, J., 2017. Development of the gut microbiota in infancy and its impact on health in later life. *Allergology International*, 66(4), pp.515-522.
5. Conway, J. and A Duggal, N., 2021. Ageing of the gut microbiome: Potential influences on immune senescence and inflammageing. *Ageing Research Reviews*, 68, p.101323.
6. Nagpal, R., Mainali, R., Ahmadi, S., et al., 2018. Gut microbiome and aging: Physiological and mechanistic insights. *Nutrition and Healthy Aging*, 4(4), pp.267-285.
7. LeBlanc, J., Chain, F., Martín, R., Bermúdez-Humarán, L., et al. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories*, 16(1).
8. Salazar, N., Arboleya, S., Fernández-Navarro, T., de los Reyes-Gavilán, C., et al. Age-Associated Changes in Gut Microbiota and Dietary Components Related with the Immune System in Adulthood and Old Age: A Cross-Sectional Study. *Nutrients*, 11(8), p.1765.
9. Buford, T., 2017. (Dis)Trust your gut: the gut microbiome in age-related inflammation, health, and disease. *Microbiome*, 5(1).
10. Chung, H., Kim, D., Lee, E., Chung, K., et al. 2019. Redefining Chronic Inflammation in Aging and Age-Related Diseases: Proposal of the Senoinflammation Concept. *Aging and disease*, 10(2), p.367.
11. Chung HY, Sung B, Jung KJ, Zou Y, Yu BP (2006). The molecular inflammatory process in aging. *Antioxid Redox Signal*, 8: 572-581.
12. Bio-K+ Community. 2020. *What Is The Gut-Brain Connection? | Bio-K+*. [online] Available at: <https://www.biokplus.com/blog/en_US/bacteria--you/what-is-the-gut-brain-connection_1> [Accessed 26 May 2020].
13. Chung, H., Kim, D., Lee, E., et al., 2019. Redefining Chronic Inflammation in Aging and Age-Related Diseases: Proposal of the Senoinflammation Concept. *Aging and disease*, 10(2), p.367.
14. Liu, Ting., Zhang, Lingyn., Joo, Donghyun., et al., 2017. NF-kB signalling in inflammation. *Signal Transduction and Targeted Therapy,* 17023 (2017).
15. Albensi, B., 2019. What Is Nuclear Factor Kappa B (NF-κB) Doing in and to the Mitochondrion?. *Frontiers in Cell and Developmental Biology*, 7.
16. de Lemos Muller, C., de Matos, J., Grigolo, G., et al., 2019. Exercise Training for the Elderly: Inflammaging and the Central Role for HSP70. *Journal of Science in Sport and Exercise*, 1(2), pp.97-115.
17. Martin, C., Osadchiy, V., Kalani, A. and Mayer, E., 2018. The Brain-Gut-Microbiome Axis. *Cellular and Molecular Gastroenterology and Hepatology*, 6(2), pp.133-148.
18. Rambaran, R. N., & Serpell, L. C. (2008). Amyloid fibrils: abnormal protein assembly. *Prion*, *2*(3), 112–117. https://doi.org/10.4161/pri.2.3.7488
19. Oluwatobi, P., Ese, I., Oluwatumininu, P. and Eghosa, M., 2020. Microbiota Gut-Brain Axis and Neurodegenerative Disease. *Romanian Neurosurgery*, pp.116-122.

20. Cerovic, M., Forloni, G. and Balducci, C., 2019. Neuroinflammation and the Gut Microbiota: Possible Alternative Therapeutic Targets to Counteract Alzheimer’s Disease?. *Frontiers in Aging Neuroscience*, 11.
21. Smith, J., Das, A., Ray, S. and Banik, N., 2012. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Research Bulletin*, 87(1), pp.10-20.
22. Amarante-Mendes, G., Adjemian, S., Branco, L., Zanetti, L., et al., 2018. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. *Frontiers in Immunology*, 9.
23. Zhang, F. and Jiang, L., 2015. Neuroinflammation in Alzheimer’s disease. *Neuropsychiatric Disease and Treatment*, p.243.
24. Jin, L., 2012. Microglia in Alzheimer's Disease. *International Journal of Alzheimer's Disease*, 2012, pp.1-2.
25. Fitzgerald, E., Murphy, S. and Martinson, H., 2019. Alpha-Synuclein Pathology and the Role of the Microbiota in Parkinson’s Disease. *Frontiers in Neuroscience*, 13.
26. Who.int. 2020. *Dementia*. [online] Available at: <https://www.who.int/news-room/fact-sheets/detail/dementia> [Accessed 1 June 2020].
27. O'Brien, R. and Wong, P., 2011. Amyloid Precursor Protein Processing and Alzheimer's Disease. *Annual Review of Neuroscience*, 34(1), pp.185-204.
28. Gu L, Guo Z. Alzheimer's Aβ42 and Aβ40 peptides form interlaced amyloid fibrils. J Neurochem. 2013 Aug;126(3):305-11. doi: 10.1111/jnc.12202. Epub 2013 Mar 12. PMID: 23406382; PMCID: PMC3716832.
29. Chen, G., Xu, T., Yan, Y., Zhou, Y., et al., 2017. Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacologica Sinica*, 38(9), pp.1205-1235.
30. Smith, C., 2003. *Elements Of Molecular Neurobiology, Third Edition*. Chichester: John Wiley & Sons, Ltd.
31. Weller, J. and Budson, A., 2018. Current understanding of Alzheimer’s disease diagnosis and treatment. *F1000Research*, 7, p.1161.
32. Jiang, Q., Lee, C., Mandrekar, S., Wilkinson, B., et al., 2008. ApoE Promotes the Proteolytic Degradation of Aβ. *Neuron*, 58(5), pp.681-693.
33. Bekris, L., Yu, C., Bird, T. and Tsuang, D., 2010. Review Article: Genetics of Alzheimer Disease. *Journal of Geriatric Psychiatry and Neurology*, 23(4), pp.213-227.
34. Friedland, Robert P. ‘Mechanisms of Molecular Mimicry Involving the Microbiota in Neurodegeneration’. 1 Jan. 2015 : 349 – 362.
35. Khandhar, S. and Marks, W., 2007. Epidemiology of Parkinson’s Disease. *Disease-a-Month*, 53(4), pp.200-205.
36. DeMaagd, G., Philip, A., 2015. Parkinson’s Disease and Its Management, Part 1: Disease Entity, Risk Factors, Pathophysiology, Clinical presentation, and Diagnosis. *Pharmacy and Therapeutics,* 40(8), pp. 504-510, 532
37. Oczkowska, A., Kozubski, W., Lianeri, M. and Dorszewska, J., 2014. Mutations in PRKN and SNCA Genes Important for the Progress of Parkinson’s Disease. *Current Genomics*, 14(8), pp.502-517.
38. LIU, H., 2007. Alpha-synuclein and Parkinson disease. *Neural Regeneration Research*, 2(4), pp.239-243.
39. Haggerty, T., Credle, J., Rodriguez, O., Wills, J., Oaks, A., Masliah, E. and Sidhu, A., 2011. Hyperphosphorylated Tau in an α-synuclein-overexpressing transgenic model of Parkinson’s disease. *European Journal of Neuroscience*, 33(9), pp.1598-1610.
40. *British Dental Journal*, 2016. Gut microbiota dysbiosis motor deficits and neuroinflammation in a model of Parkinson's Disease. 221(12), pp.772-772.
41. Nutraceuticalbusinessreview.com. 2020. *Probiotic 2.0: A Revolution About To Begin*. [online] Available at:

<https://www.nutraceuticalbusinessreview.com/news/article_page/Probiotic_20_a_revolution_about_to_begin/116363> [Accessed 7 April 2020].

42. Healthline. 2021. *Why Bifidobacteria Are So Good for You*. [online] Available at: <https://www.healthline.com/nutrition/why-bifidobacteria-are-good#TOC_TITLE_HDR_3> [Accessed 16 March 2021].
43. Healthline. 2021. *Lactobacillus rhamnosus: Benefits, Side Effects, and Dosage*. [online] Available at: <https://www.healthline.com/nutrition/lactobacillus-rhamnosus> [Accessed 16 March 2021].
44. Jeffrey, M., Strap, J., Jones Taggart, H. and Green-Johnson, J., 2018. Suppression of Intestinal Epithelial Cell Chemokine Production by Lactobacillus rhamnosus R0011 and Lactobacillus helveticus R0389 Is Mediated by Secreted Bioactive Molecules. *Frontiers in Immunology*, 9.
45. *Reactions Weekly*, 2020. Lactobacillus rhamnosus. 1785(1), pp.306-306.
46. Jeffrey, M., Jones Taggart, H., Strap, J., Edun, G. and Green-Johnson, J., 2020. Milk fermented with Lactobacillus rhamnosus R0011 induces a regulatory cytokine profile in LPS-challenged U937 and THP-1 macrophages. *Current Research in Food Science*,.
47. Kishi, A., Uno, K., Matsubara, Y., Okuda, C. and Kishida, T., 1996. Effect of the oral administration of Lactobacillus brevis subsp. coagulans on interferon-alpha producing capacity in humans. *Journal of the American College of Nutrition*, 15(4), pp.408-412.
48. Drago, L., Nicola, L., Iemoli, E., Banfi, G. and De Vecchi, E., 2010. Strain-dependent release of cytokines modulated by Lactobacillus salivarius human isolates in an in vitro model. *BMC Research Notes*, 3(1), p.44.
49. Ren, D., Wang, D., Liu, H., et al., 2019. Two strains of probiotic Lactobacillus enhance immune response and promote naive T cell polarization to Th1. *Food and Agricultural Immunology*, 30(1), pp.281-295.
50. Pandey, V., Solanki, N., Berwal, V. and Malik, N., 2015. Probiotics: Healthy bugs and nourishing elements of diet. *Journal of International Society of Preventive and Community Dentistry*, 5(2), p.81.
51. Dailyvita.com. 2020. *How Do I Choose A Probiotic?*. [online] Available at: <https://www.dailyvita.com/default/blog/post/what-probiotic-do-i-pick> [Accessed 7 April 2020].
52. *Neville, BA.; O'Toole, PW. (May 2010). "Probiotic properties of Lactobacillus salivarius and closely related Lactobacillus species". Future Microbiol.* ***5*** *(5): 759–74.*
53. Brooijmans, R., de Vos, W. and Hugenholtz, J., 2009. Lactobacillus plantarum WCFS1 Electron Transport Chains. *Applied and Environmental Microbiology*, 75(11), pp.3580-3585.
54. Bested, A., Logan, A. and Selhub, E., 2013. Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: part III – convergence toward clinical trials. *Gut Pathogens*, 5(1), p.4.
55. Bozzi Cionci, N., Baffoni, L., Gaggìa, F. and Di Gioia, D., 2018. Therapeutic Microbiology: The Role of Bifidobacterium breve as Food Supplement for the Prevention/Treatment of Paediatric Diseases. *Nutrients*, 10(11), p.1723.
56. Kobayashi, Y., Sugahara, H., Shimada, K., Mitsuyama, E., Kuhara, T., Yasuoka, A., Kondo, T., Abe, K. and Xiao, J., 2017. Therapeutic potential of Bifidobacterium breve strain A1 for preventing cognitive impairment in Alzheimer's disease. *Scientific Reports*, 7(1).
57. *Proceedings of the National Academy of Sciences*, 2005. Correction for Schell et al., The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract, PNAS 2002 99:14422-14427. 102(26), pp.9430-9430.
58. Natividad, J., Hayes, et al., 2013. Differential Induction of Antimicrobial REGIII by the Intestinal Microbiota and Bifidobacterium breve NCC2950. *Applied and Environmental Microbiology*, 79(24), pp.7745-7754.

59. Sagar, Seil et al. 16 Apr. 2014 "Bifidobacterium breve and Lactobacillus rhamnosus treatment is as effective as budesonide at reducing inflammation in a murine model for chronic asthma." *Respiratory research* vol. 15,1 46.
60. O'Callaghan, A. and van Sinderen, D., 2016. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in Microbiology*, 7.
61. Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., Bisson, J., Rougeot, C., Pichelin, M., Cazaubiel, M. and Cazaubiel, J., 2010. Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *British Journal of Nutrition*, 105(5), pp.755-764.
62. Wong, Odamaki et al. 2019 "Beneficial effects of Bifidobacterium longum subsp. Longum BB536 on human health: Modulation of gut microbiome as the principal action" *Journal of Functional Foods* vol. 54, pp 506-519.
63. Taverniti, Valentina, and Simone Guglielmetti. "Health-Promoting Properties of Lactobacillus helveticus." *Frontiers in microbiology* vol. 3 392. 19 Nov. 2012, doi:10.3389/fmicb.2012.00392
64. Michalickova D, Minic R, et al. July 2016. Lactobacillus helveticus Lafti L10 supplementation reduces respiratory infection duration in a cohort of elite athletes: a randomized, double-blind, placebo-controlled trial. Appl Physiol Nutr Metab.
65. Tuomilehto, J., Lindström, J., Hyyrynen, J., *et al* 2004. Effect of ingesting sour milk fermented using Lactobacillus helveticus bacteria producing tripeptides on blood pressure in subjects with mild hypertension. *Journal of Human Hypertension*, 18(11), pp.795-802.
66. Jungersen, Mikkel et al. 2014 "The Science behind the Probiotic Strain Bifidobacterium animalis subsp. lactis BB-12(®)." *Microorganisms* vol. 2,2 92-110.
67. Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., Vlieg, J. and Siezen, R., 2013. Lactobacillus paracasei Comparative Genomics: Towards Species Pan-Genome Definition and Exploitation of Diversity. *PLoS ONE*, 8(7), p.e68731.
68. Choi, J., Moon, C., Shin, T., Kim, E., McDowell, A., Jo, M., Joo, Y., Kim, S., Jung, H., Shim, K., Jung, S. and Kim, Y., 2020. Lactobacillus paracasei-derived extracellular vesicles attenuate the intestinal inflammatory response by augmenting the endoplasmic reticulum stress pathway. *Experimental & Molecular Medicine*,.
69. Brenner, S., 1974. THE GENETICS OF CAENORHABDITIS ELEGANS. *Genetics*, 77(1), pp.71-94.
70. Tissenbaum, H., 2014. Using C. *elegans* for ageing research. Invertebr Reprod Dev, 59(sup1), p59-63
71. Roselli, Schifano, Guantario, Zinno, Uccelletti and Devirgiliis, 2019. Caenorhabditis Elegans and Probiotics Interactions from a Prolongevity Perspective. *International Journal of Molecular Sciences*, 20(20), p.5020.
72. Yourgenome.org. 2020. *Why Use The Worm In Research?*. [online] Available at: <https://www.yourgenome.org/facts/why-use-the-worm-in-research#:~:text=elegans%2C%20where%20specific%20genes%20are,useful%20model%20for%20human%20diseases.> [Accessed 5 June 2020].
73. Oxford Instruments. 2020. *Advantages Of Using Caenorhabditis Elegans As A Model Organism - Andor Learning Centre- Oxford Instruments*. [online] Available at: <https://andor.oxinst.com/learning/view/article/advantages-of-using-caenorhabditis-elegans-as-a-model-organism> [Accessed 5 June 2020].
74. Hall, D., Herndon, L. and Altun, Z., 2017. WormAtlas Embryo Handbook - Introduction to C. elegans Embryo Anatomy. *WormAtlas*,.
75. Roselli, Schifano, Guantario, Zinno, Uccelletti and Devirgiliis, 2019. Caenorhabditis Elegans and Probiotics Interactions from a Prolongevity Perspective. *International Journal of Molecular Sciences*, 20(20), p.5020.

76. Ogg, S., Paradis, S., Gottlieb, S., Patterson, G., et al., 1997. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature*, 389(6654), pp.994-999.
77. Murphy, C., 2013. Insulin/insulin-like growth factor signaling in C. elegans. *WormBook*, pp.1-43.
78. Sugawara, T. and Sakamoto, K., 2018. Killed Bifidobacterium longum enhanced stress tolerance and prolonged life span of Caenorhabditis elegans via DAF-16. *British Journal of Nutrition*, 120(8), pp.872-880.
79. Ayuda-Durán, B., González-Manzano, S., Miranda-Vizuete, et al., 2019. Exploring Target Genes Involved in the Effect of Quercetin on the Response to Oxidative Stress in Caenorhabditis elegans. *Antioxidants*, 8(12), p.585.
80. Sciences, L., Analysis, C., Pathways, C. and Pathway, M., 2021. *MAPK Cell Signaling Pathway | Thermo Fisher Scientific - UK*. [online] Thermofisher.com. Available at: <https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/signaling-pathways/mitogen-activated-protein-kinase-mapk.html [Accessed 17 March 2021].
81. Troemel ER, Chu SW, et al., 2006 Nov., p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans. PLoS Genet.10;2(11) pp 183.
82. Inoue, H. et al. (2005) 'The C . elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response', pp. 2278–2283. doi: 10.1101/gad.1324805.2278.
83. Troemel, E., Chu, S., Reinke, V., Lee, S., Ausubel, F. and Kim, D., 2005. p38 MAPK Regulates Expression of Immune Response Genes and Contributes to Longevity in C. elegans. *PLoS Genetics*, preprint(2006), p.e183.
84. Arata, Yukinobu et al., 5 Feb 2020., "OP50, a bacterial strain conventionally used as food for laboratory maintenance of *C. elegans*, is a biofilm formation defective mutant." *microPublication biology* vol. 10
85. Chaudhuri, Jyotiska et al. 11 Jan. 2011., "An introduction to worm lab: from culturing worms to mutagenesis." *Journal of visualized experiments : JoVE* ,47 2293.
86. Kishi A, Uno K, Matsubara Y, Okuda C, Kishida T. Effect of the oral administration of Lactobacillus brevis subsp. coagulans on interferon-alpha producing capacity in humans. J Am Coll Nutr. 1996 Aug;15(4):408-12.
87. Jeong, J., Kim, K., Hwang, Y., Han, M. and Kim, D., 2016. Anti-inflammaging effects of Lactobacillus brevis OW38 in aged mice. *Beneficial Microbes*, 7(5), pp.707-718.
88. Venu, I., Durisko, Z., Xu, J. and Dukas, R., 2014. Social attraction mediated by fruit flies' microbiome. *Journal of Experimental Biology*, 217(8), pp.1346-1352.
89. Hesselager M, Harders R, Morch M. C. elegans reveals probiotic secrets L.brevis increases lifespan via the p38 MAP kinase PMK-1. AALBORG UNIVERSITET.
90. Drago, L., De Vecchi, E., Gabrieli, A., De Grandi, R. and Toscano, M., 2015. Immunomodulatory Effects ofLactobacillus salivariusLS01 andBifidobacterium breveBR03, Alone and in Combination, on Peripheral Blood Mononuclear Cells of Allergic Asthmatics. *Allergy, Asthma & Immunology Research*, 7(4), p.409.
91. Chung PH, Wu YY, Chen PH, Fung CP, Hsu CM, Chen LW. Lactobacillus salivarius reverse diabetes-induced intestinal defense impairment in mice through non-defensin protein. J Nutr Biochem. 2016 Sep;35:48-57
92. Zhao Y, Zhao L, Zheng X, Fu T, Guo H, Ren F. Lactobacillus salivarius strain FDB89 induced longevity in Caenorhabditis elegans by dietary restriction. J Microbiol. 2013 Apr;51(2):183-8.
93. Huebner, E. S., & Surawicz, C. M. (2006). Treatment of Recurrent *Clostridium difficile* Diarrhea. *Gastroenterology & hepatology*, *2*(3), 203–208.

94. Yong, Shin Jie et al. “Antidepressive Mechanisms of Probiotics and Their Therapeutic Potential.” *Frontiers in neuroscience* vol. 13 1361. 14 Jan. 2020,
95. Fast, D., Duggal A., Foley E. (2018). Monoassociation with *Lactobacillus plantarum* Disrupts intestinal homeostasis in adult *Drosophila melanogaster.* mBio, 9(4)e01114-18.
96. Ikeda, T., Yasui, C., Hoshino, K., Arikawa, K. and Nishikawa, Y., 2007. Influence of Lactic Acid Bacteria on Longevity of Caenorhabditis elegans and Host Defense against Salmonella enterica Serovar Enteritidis. *Applied and Environmental Microbiology*, 73(20), pp.6404-6409.
97. Bozzi Cionci, N., Baffoni, L., Gaggìa, F., & Di Gioia, D. (2018). Therapeutic Microbiology: The Role of *Bifidobacterium breve* as Food Supplement for the Prevention/Treatment of Paediatric Diseases. *Nutrients*, *10*(11), 1723.
98. Kobayashi, Y., Sugahara, H., *et al.* 2017., Therapeutic potential of *Bifidobacterium breve* strain A1 for preventing cognitive impairment in Alzheimer’s disease. *Sci Rep* **7,** 13510.
99. Bozzi Cionci, N., Baffoni, L., Gaggìa, F., & Di Gioia, D. (2018). Therapeutic Microbiology: The Role of *Bifidobacterium breve* as Food Supplement for the Prevention/Treatment of Paediatric Diseases. *Nutrients*, *10*(11), 1723.
100. Christiaen, S., O'Connell Motherway, et al., 2014. Autoinducer-2 Plays a Crucial Role in Gut Colonization and Probiotic Functionality of Bifidobacterium breve UCC2003. *PLoS ONE*, 9(5), p.e98111.
101. Akatsu H, Iwabuchi N, et al. sep 2013., Clinical effects of probiotic Bifidobacterium longum BB536 on immune function and intestinal microbiota in elderly patients receiving enteral tube feeding. JPEN J Parenter Enteral Nutr, 37(5):631-40.
102. Silva, A., Barbosa, F., Duarte, R., Vieira, L., Arantes, R. and Nicoli, J., 2004. Effect of Bifidobacterium longum ingestion on experimental salmonellosis in mice. *Journal of Applied Microbiology*, 97(1), pp.29-37.
103. Matsumoto T, Ishikawa H, et al. 2008 Mar., Oral administration of Bifidobacterium longum prevents gut-derived Pseudomonas aeruginosa sepsis in mice. J Appl Microbiol. 104(3):672-80.
104. Bercik P, Park AJ, Sinclair D, et al. 2011 Dec., The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil. 23(12):1132-9.
105. Badal, V. D., Vaccariello, E. D., et al., (2020). The Gut Microbiome, Aging, and Longevity: A Systematic Review. *Nutrients*, *12*(12), 3759.
106. Zhao L, Zhao Y, Liu R, et al. 2017 Aug., The Transcription Factor DAF-16 is Essential for Increased Longevity in C. elegans Exposed to Bifidobacterium longum BB68. Sci Rep. 7;7(1):7408.
107. Sherman, P. M., Johnson-Henry, K. C., et al. (2005). Probiotics reduce enterohemorrhagic Escherichia coli O157:H7- and enteropathogenic E. coli O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. *Infection and immunity*, *73*(8), 5183–5188.
108. Bravo, J., Forsythe, P., Chew, M., Escaravage, E., Savignac, H., Dinan, T., Bienenstock, J. and Cryan, J., 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences*, 108(38), pp.16050-16055.
109. Blum, J. E., Fischer, C. N., et al. (2013). Frequent replenishment sustains the beneficial microbiome of Drosophila melanogaster. *mBio*, *4*(6), e00860-13.
110. Poupet, C., Saraoui, T., Veisseire, P., et al (2019). Lactobacillus rhamnosus Lcr35 as an effective treatment for preventing Candida albicans infection in the invertebrate model Caenorhabditis elegans: First mechanistic insights. *PloS one*, *14*(11), e0216184.

111. Grompone, G., Martorell, P., Llopis, S., González, N., Genovés, S., Mulet, A., Fernández-Calero, T., Tiscornia, I., Bollati-Fogolín, M., Chambaud, I., Foligné, B., Montserrat, A. and Ramón, D., 2012. Anti-Inflammatory Lactobacillus rhamnosus CNCM I-3690 Strain Protects against Oxidative Stress and Increases Lifespan in Caenorhabditis elegans. *PLoS ONE*, 7(12), p.e52493.
112. Rao, R. K., & Samak, G. (2013). Protection and Restitution of Gut Barrier by Probiotics: Nutritional and Clinical Implications. *Current nutrition and food science*, *9*(2), 99–107.
113. Yamashita, M., Matsumoto, K., Endo, T., Ukibe, K., Hosoya, T., Matsubara, Y., Nakagawa, H., Sakai, F., & Miyazaki, T. (2017). Preventive Effect of *Lactobacillus helveticus* SBT2171 on Collagen-Induced Arthritis in Mice. *Frontiers in microbiology*, *8*, 1159.
114. Lallemand Health Solutions. 2021. *Bifidobacterium animalis ssp. lactis LAFTI® B94 recognized for its benefits in children with IBS by Health Canada | Lallemand Health Solutions*. [online] Available at: <https://lallemand-health-solutions.com/en/bifidobacterium-animalis-ssp-lactis-lafti-b94-recognized-for-its-benefits-in-children-with-ibs-by-health> [Accessed 3 February 2021].
115. Martín, R., Laval, L., et al., 2016. Bifidobacterium animalis ssp. lactis CNCM-I2494 Restores Gut Barrier Permeability in Chronically Low-Grade Inflamed Mice. *Frontiers in Microbiology*, 7.
116. Martorell P, Llopis S, González N, et al. Probiotic Strain Bifidobacterium animalis subsp. lactis CECT 8145 Reduces Fat Content and Modulates Lipid Metabolism and Antioxidant Response in Caenorhabditis elegans. J Agric Food Chem. 2016 May 4;64(17):3462-72.
117. Verdenelli MC, Ghelfi F, Silvi S, et al. Probiotic properties of Lactobacillus rhamnosus and Lactobacillus paracasei isolated from human faeces. Eur J Nutr. 2009 Sep;48(6):355-63.
118. Costa, D., Marteau, P., Amouyal, M. *et al.* Efficacy and safety of the probiotic *Lactobacillus paracasei* LP-33 in allergic rhinitis: a double-blind, randomized, placebo-controlled trial (GA2LEN Study). *Eur J Clin Nutr* **68,** 602–607 (2014).
119. Paturi G, Phillips M, Kailasapathy K. Effect of probiotic strains Lactobacillus acidophilus LAFTI L10 and Lactobacillus paracasei LAFTI L26 on systemic immune functions and bacterial translocation in mice. J Food Prot. 2008 Apr;71(4):796-801.
120. de Barros, P., Scorzoni, L., et al., 2018. Lactobacillus paracasei 28.4 reduces in vitro hyphae formation of Candida albicans and prevents the filamentation in an experimental model of Caenorhabditis elegans. *Microbial Pathogenesis*, 117, pp.80-87.
121. Hemarajata, P., & Versalovic, J. (2013). Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therapeutic advances in gastroenterology*, *6*(1), 39–51.
122. Kobayashi, Y., Sugahara, H., Shimada, K. *et al.* Therapeutic potential of *Bifidobacterium breve* strain A1 for preventing cognitive impairment in Alzheimer’s disease. *Sci Rep* **7,** 13510 (2017).
123. Sangnam, O., Park, M.R., *et al. Lactobacillus rhamnosus* Strain GG Influences on the Longevity and Immune Response of *Caenorhabditis elegans* to Foodborne Pathogens with Stimulating MicroRNAs. The FASEB journal. 2015 April 01.
124. Murphy, M. and LeVine, H., 2010. Alzheimer's Disease and the Amyloid-β Peptide. *Journal of Alzheimer's Disease*, 19(1), pp.311-323.
125. Kobayashi, Y., Sugahara, H., Shimada, K. *et al.* Therapeutic potential of *Bifidobacterium breve* strain A1 for preventing cognitive impairment in Alzheimer’s disease. *Sci Rep* **7,** 13510 (2017).

126. Bonfili L., Cecarini V., Cuccioloni M., et al. (2018). SLAB51 probiotic formulation activates SIRT1 pathway promoting antioxidant and neuroprotective effects in an AD mouse model. *Mol. Neurobiol.* 55 7987–8000. 10.1007/s12035-018-0973-4
127. Dostal V., Link C. D. Assaying beta-amyloid toxicity using a transgenic *C. elegans* model. *Journal of Visualized Experiments*. 2010;44 doi: 10.3791/2252
128. Hemarajata, Peera, and James Versalovic. "Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation." *Therapeutic advances in gastroenterology* vol. 6,1 (2013): 39-51. doi:10.1177/1756283X12459294
129. L. Stefanis, *α-Synuclein in Parkinson's disease.*Cold Spring Harb. Perspect. Med. 2012. **2**(2), 009399 (2012)
130. Hasegawa, Satoru et al. "Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease." *PloS one* vol. 10,11 e0142164. 5 Nov. 2015.
131. Yang D, Zhao D, Ali Shah SZ, et al. The role of the gut microbiota in the pathogenesis of Parkinson's disease. Front Neurol. (2019) 10:1155 10.3389/fneur.2019.01155
132. Sampson, Timothy R et al. "Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease." *Cell* vol. 167,6 (2016): 1469-1480.e12. doi:10.1016/j.cell.2016.11.018
133. María Eugenia Goya, Feng Xue, Cristina Sampedro-Torres-Quevedo, et al. Probiotic Bacillus subtilis Protects against α-Synuclein Aggregation in C. elegans. Cell Reports. (2020), 30(2), pp.367-380.e7.
134. Kelly, S.M., Lanigan, N., O'Neill, I.J. *et al.* Bifidobacterial biofilm formation is a multifactorial adaptive phenomenon in response to bile exposure. *Sci Rep* **10,** 11598 (2020).
135. Tabbers, M M et al. "Is Bifidobacterium breve effective in the treatment of childhood constipation? Results from a pilot study." *Nutrition journal* vol. 10 19. 23 Feb. 2011, doi:10.1186/1475-2891-10-19)
136. Gingichashvili, Duanis-Assaf, et al. "Bacillus subtilis Biofilm Development – A Computerized Study of Morthology and Kinetics" *Frontiers in Microbiology* VOL. 8. 2017, DOI=10.3389/fmicb.2017.02072
137. Smolentseva, O., Gusarov, I., Gautier, L. *et al.* Mechanism of biofilm-mediated stress resistance and lifespan extension in *C. elegans* . *Sci Rep* **7,** 7137 (2017).
138. Shivers RP, Kooistra T, Chu SW, Pagano DJ, Kim DH. Tissue-specific activities of an immune signaling module regulate physiological responses to pathogenic and nutritional bacteria in C. elegans. *Cell Host Microbe*. 2009;6(4):321-330. doi:10.1016/j.chom.2009.09.001
139. Troemel, E., Chu Stephanie., Reinke V., *et al*. (2006) p38 MAPK Regulates Expression of Immune Response Genes and Contributes to Longevity in C. elegans. Plos Genetics 2(11):e183
140. Park, M.R., Ryu, S., Maburutse, B.E. *et al.* Probiotic *Lactobacillus fermentum* strain JDFM216 stimulates the longevity and immune response of *Caenorhabditis elegans* through a nuclear hormone receptor. *Sci Rep* **8,** 7441 (2018).
141. Shivers RP, Pagano DJ, Kooistra T, Richardson CE, et al., Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in Caenorhabditis elegans. PLoS Genet. 2010 Apr 1;6(4):e1000892.
142. Houthoofd, K. and Vanfleteren, J., 2006. The longevity effect of dietary restriction in Caenorhabditis elegans. *Experimental Gerontology*, 41(10), pp.1026-1031.
143. McCay, C., Crowell, M. and Maynard, L., 1935. The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size. *The Journal of Nutrition*, 10(1), pp.63-79.