Assessment of the Potential of Probiotics to Impact Upon Dementia Onset and Progression

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**Project summary**

Alzheimer’s Disease (AD) is a major neurodegenerative condition affecting over 44 million people worldwide. Cases are projected to continue rising due to the increase in life expectancy of the global population. Current treatments that are available are not very effective and are often targeted at treating symptoms rather than preventing initial disease onset and progression. Therefore, finding an alternative therapeutic strategy to ease the socio-economic burden of the disease is vital.

Gut function is critical for maintaining our overall health. The intestines contain trillions of bacteria that exist in a well-balanced ratio. Any upset of this ratio due to poor diet or stress can result in dysbiosis which has been closely linked to AD. Dysbiosis may result in the induction of the inflammatory response and the expression of pro-inflammatory cytokines. This increase in systemic inflammation has also been shown to contribute to the onset and severity of AD. In order to counteract dysbiosis, probiotics may be used as a means of replenishing the microbiome to give rise to a more desirable gut environment, which in turn may improve inflammatory status and potentially slow the progression of AD.

Previous work has suggested that bacterial based probiotics are beneficial to the 3xTg AD mouse model by showing improved neuronal cell survival, behaviour and inflammation status after supplementation. Based on these findings, this thesis aims to assess the impact of the Lab4 consortia of probiotics on key immune cells in the brain, known as microglia, in order to investigate the mechanisms behind these previous beneficial results and to determine if probiotics have the potential to impact upon microglial function using in vitro and in vivo techniques.
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List of abbreviations

AD – Alzheimer’s Disease
AICD – APP Intracellular Domain
APOE – Apolipoprotein
APP – Amyloid Precursor Protein
ATP – Adenosine Triphosphate
Aβ – Amyloid Beta
BBB – Blood Brain Barrier
BIOSV – Biological Services
CANTAB – Cambridge Neuropsychological Test Automated Battery
CD68 – Cluster of Differentiation 68
cDNA – Complementary DNA
CFU – Colony Forming Units
cm – centimetre
CM – Conditioned Media
cm² – Centimetre squared
CNS – Central Nervous System
CO₂ – Carbon Dioxide
CSF – Cerebrospinal Fluid
CV – Crystal Violet
DAM – Disease Associated Microglia
DAMP – Danger Associated Molecular Pattern
DMEM – Dulbecco’s Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
dNTP – Deoxynucleoside Triphosphate
EAE – Experimental Autoimmune Encephalitis
ECs – Enterochromaffin Cells
FACS – Fluorescence Activated Cell Sorting
FB – FACs Buffer
FBS – Foetal Bovine Serum
FFAR – Free Fatty Acid Receptor
GABA - Gamma Aminobutyric Acid
GAD – Glutamic Acid Decarboxylase
GALT – Gut Associated Lymphoid Tissue
GI – Gastrointestinal
GOI – Gene of Interest
GPCRs - G-protein Coupled Receptors
GWAS – Genome Wide Association Study
H₂O – Water
HCl – Hydrochloric Acid
HDACs – Histone Deacetylases
Iba-1 - Ionised Calcium Binding Adapter Molecule
IBS – Irritable Bowel Syndrome
ICC – Immunocytochemistry
IDE – Insulin Degrading Enzyme
IgA – Immunoglobulin A
IHC – Immunohistochemistry
IL-10 - Interleukin 10
IL-17 – Interleukin 17
IL-1β – Interleukin 1 Beta
IL-6 – Interleukin 6
ILF - Isolated Lymphoid Follicles
iNos – Inducible Nitric Oxide Synthase
iPSCs – Induced Pluripotent Stem Cells
JKAP – JNK Pathway Associated Phosphate
LDH – Lactate Dehydrogenase
LOAD – Late Onset Alzheimer’s Disease
LPS – Lipopolysaccharide
LTA – Lipoteichoic Acid
M – Molar
MAPK – Mitogen Activated Protein Kinase
MAPT – Microtubule Associated Protein Tau
MCI – Mild Cognitive Impairment
MD20 – Maltodextrin 20
MFI – Median Fluorescence Intensity
mg – milligrams
MUC2 – Mucin 2
NaOH – Sodium Hydroxide
NFTs – Neurofibrillary Tangles
ng – Nanogram
NGS – Next Generation Sequencing
nm – Nanometres
NMDA – N-methyl-D-aspartate
TACE - Tumour Necrosis Factor-α Converting Enzyme
TEER – Transendothelial electrical resistance
T$_{h}$1 – T-helper 1
T$_{h}$17 – T-helper 17
TLR – Toll Like Receptor
TMEM119 – Transmembrane Protein 119
WHO – World Health Organisation
NMR – Nuclear Magnetic Resonance
NOR – Novel Object Recognition
P/S – Penicillin/Streptomycin
PAMPs – Pathogen Associated Molecular Pattern
PBS – Phosphate Buffered Saline
PD – Parkinson’s Disease
PET - positron emission tomography
PFA – Paraformaldehyde
PPs – Peyer’s Patches
PSEN1 – Presenilin 1
PSEN2 – Presenilin 2
RNA – Ribose Nucleic Acid
ROS – Reactive oxygen species
RT – Reverse Transcription
RT-qPCR – Reverse Transcriptase Quantitative Polymerase Chain Reaction
SCFA – Short Chain Fatty Acids
SD – Standard Deviation
SDS-PAGE - Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM – Standard Error of Mean
sIL-6R – Soluble interleukin 6 receptor
SOD - Superoxide Dismutase
TNF-α – Tumour Necrosis Factor Alpha
T-reg – T-regulatory
U/mL – Units per millilitre
UK – United Kingdom
v/v – Volume per volume
w/v – Weight per volume
WT – Wild Type
°C – Degrees Celsius
µL – Microlitre
µM – Micromolar
µm – Micrometre
Chapter 1

Introduction
1.1 Alzheimer’s Disease

1.1.1 - Prevalence of AD

With the life expectancies of the global population continuing to rise, it is not surprising that there has been a subsequent increase of age-related maladies in parallel. Alzheimer’s disease (AD) is neurodegenerative in nature and accounts for over a third of dementia cases (Alzheimer’s Association 2019). Globally around 44 million people currently suffer from AD (Alzheimer’s Association 2015). Since the millennium, the number of deaths related to AD have increased by almost 150% (Alzheimer’s Association 2015). It has been predicted that by 2050 over 100 million people may be suffering with AD if there is no advancement in current available treatment options (Brookmeyer et al. 2007). It is a disease that not only creates huge socio-economic burden due to care and treatment costs, but it is also devasting for families and for the sufferers themselves (Hurd et al. 2013).

1.1.2 - AD symptoms, pathology and mechanisms

AD symptoms initially include the decline of memory, motor skills and co-ordination due to the settling of AD pathology (Nabeshima and Nitta 1994). The diagnosis of AD is typically given later in life and can only be truly confirmed post-mortem. This is because the disease symptoms do not typically manifest until old age in the case of late onset Alzheimer’s disease (LOAD), however the development of underlying pathology and the start of neurodegeneration may begin decades before the onset of symptoms (Amieva et al. 2008).

The key hallmarks of AD are well documented and include the development of intracellular neurofibrillary tau tangles and the deposition of extracellular amyloid beta (Aβ) plaques which arise due to aggregation of Aβ monomers (Arnold et al. 1991, Tseng et al. 1999). Subsequently, this pathogenesis results in increased levels of neuroinflammation and oxidative stress which are also thought to contribute to the progression of AD (Kinney et al. 2018, Buccellato et al. 2021). The key trigger to AD development is still a topic of heated debate.

**Amyloid plaques**

A widely accepted hypothesis is that AD development is governed by the amyloid cascade (Hardy and Higgins 1992). This is the suggestion that AD begins with either the
deposition of Aβ plaques, the lack of clearance of Aβ plaques or a combination of both which creates a knock-on effect causing other pathologies such as tau tangles and the induction of inflammation. The production of amyloid peptide aggregates derives from the cleavage of the amyloid precursor protein (APP) via the amyloidogenic pathway (Figure 1.1). A more common non-amyloidogenic pathway of APP processing also exists and results in small, soluble fragments of amyloid known as p3, that typically do not contribute to Aβ plaque formation (Naslund et al. 1994).

![Figure 1.1 Comparison of the non-amyloidogenic and amyloidogenic pathways initiated by processing of amyloid precursor protein (APP).](image)

APP is an intrinsic membrane protein found in neuronal and glial cells. This is produced in many isoforms due to alternative splicing of the gene, however the most abundant form in the brain is isoform 695 which is expressed in neurons (Belyaev et al. 2010). The amyloidogenic pathway begins with the proteolytic cleavage of APP via secretases, namely β-secretase and subsequently the γ-secretase complex which results in the formation of insoluble Aβ 40/42
monomers, the ratio of which may determine AD severity (Citron et al. 1996, Selkoe and Wolf 2007, Thinakaran and Koo 2008). These monomers are then able to aggregate if not adequately degraded and form Aβ fibrils which can establish themselves to form the characteristic and problematic Aβ plaques which spread over different brain regions as disease severity increases (Figure 1.2 A, B and C).

Figure 1.2 Diagram showing the deposition of amyloid-β (Aβ) in a brain affected by Alzheimer’s disease. (A) After APP processing, amyloid monomers begin to aggregate together, eventually forming fibrils which begin to assemble in a β sheet structure. These plaques cause damage to neuronal cells, resulting in neuronal death and brain atrophy. (B) Immunohistochemical staining of mature Aβ plaques in the brain of a patient with early onset Alzheimer’s disease. (C) The development of Aβ plaques over time in the AD brain begins in the hippocampus region and surrounding areas before moving into the frontal lobe and occipital lobes. Finally, in severe AD the Aβ plaque is present across the cortex and covers most of the brain regions. (B) Image adapted from Querol-Vilaseca et al. 2019, Scientific Reports. (C) Figure part adapted from Masters et al. 2015, Nature.

These extracellular deposits of Aβ plaque contribute to synaptic impairment and neuronal cell damage and death (Masliah et al. 1994, Scheff et al. 2007). However, the amyloid cascade hypothesis has been disputed (Herrup 2015). Many therapies targeting Aβ plaque specifically have proved ineffective, suggesting that while the deposition of Aβ plaque may be a contributor
to AD development, this process may not be the sole cause of the disease (Seubert et al. 2008, Salloway et al. 2009, Golde et al. 2011).

**Neurofibrillary tau tangles**

The development of aberrantly phosphorylated tau protein also contributes to AD pathology (Figure 1.3 A, B and C). Tau proteins are produced from the alternative splicing of the microtubule associated protein tau (MAPT) gene (Goedert et al. 1989). Tau proteins play a major role in cellular structure as they support the formation of microtubules via interactions with tubulin proteins (Weingarten et al. 1975). The six Braak stages can be used to define AD progression and severity based on the development of neurofibrillary tau tangles (NFTs) in key brain regions (Table 1.1).
Figure 1.3 The development of tau neurofibrillary tangles in the brain during Alzheimer’s disease. One of the main elements of Alzheimer’s disease (AD) pathology is the development of tau tangles in the brain. (A) In healthy neurons tau proteins allow for stability of the intracellular cytoskeleton. However, in AD neuronal microtubules become comprised due to the development of tau protein tangles which results in a comprised microtubule structure and loss of integrity of neuronal cell structure. (B) Immunohistochemical staining of neurofibrillary tau tangles in the brain. (C) Typically, the tau tangles begin in the hippocampal region of the brain (Braak stages I and II) before moving into the surrounding areas and transentorhinal region (Braak stages III and IV) before finally moving into the cortex region (Braak stages V and VI). (B) Image adapted from LaFerla and Oddo 2005, Trends in Molecular Medicine. (C) Figure part adapted from Masters et al. 2015, Nature.
Table 1.1 Summary of the six Braak stages for defining the development of Alzheimer’s disease in the human brain. NFTs = Neurofibrillary Tangles (Braak and Braak 1991)

<table>
<thead>
<tr>
<th>Braak Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>• No NFTs present in any brain region.</td>
</tr>
<tr>
<td>I</td>
<td>• NFTs present in transentorhinal region of the brain.</td>
</tr>
<tr>
<td>II</td>
<td>• Possibly some NFTs present in CA1 region of hippocampus.</td>
</tr>
<tr>
<td></td>
<td>• Little to no change in cortex.</td>
</tr>
<tr>
<td>III</td>
<td>• NFTs present in the transentorhinal region and limbic region, particularly the hippocampus and amygdala.</td>
</tr>
<tr>
<td>IV</td>
<td>• Possibly some NFTs in cortex.</td>
</tr>
<tr>
<td>V</td>
<td>• NFTs present in transentorhinal, limbic and cortex regions.</td>
</tr>
<tr>
<td>VI</td>
<td></td>
</tr>
</tbody>
</table>

The effect of both extracellular Aβ plaques and intracellular tau NFT pathology results in neuronal cell damage and death. In AD, neuronal cell death usually originates in the CA1 region of the hippocampus due to NFTs and plaque deposition impacting heavily on learning and memory capabilities which is often the first noticeable symptom of AD (Braak and Braak 1991, Lace et al. 2009). Significant neuronal cell death results in atrophy of the brain causing this impairment of memory and cognitive function (Gosche et al. 2002) (Figure 1.4).
1.1.3 Factors determining AD onset

**Genetics**

There are a range of factors that contribute to the onset of AD pathology. Apart from old age, one of the major factors that determines predisposition for AD development is genetic in nature and can be categorised as familial or sporadic, as determined by the presence of different AD-related risk genes. In familial AD there are 3 key genes that when mutated give rise to autosomal dominant AD manifestation. Mutations in the APP gene located on chromosome 21, Presenilin 1 (PSEN1) gene located on chromosome 14 and Presenilin 2 (PSEN2) gene located on chromosome 1 give rise to early onset AD (Goate et al. 1991, Sherrington et al. 1995, Rogaev et al. 1995).

It is estimated that 1 in 10 people over the age of 65 develop AD and that this increases to 4 in 10 people after the age of 80 (Schultz et al. 2004, Kumar et al. 2015). This sub-type of AD is known as sporadic LOAD and is the most common AD type (Alzheimer's Association...
2018). One of the main genetic components that determines LOAD is the apolipoprotein (APOE) gene which has 3 alleles: ε2, ε3 and ε4 (Ashford 2004). The combination of these alleles that an individual possesses predisposes them to the likelihood of developing AD and how severely the disease could present (Coon et al. 2007). Carriers of APOE ε2 allele are less common, making up around 1 in 10 of AD sufferers (Corder et al. 1993). It has been suggested that the ε2 allele acts in a more protective manner compared to the other APOE alleles. Studies have shown that patients with the APOE ε2 allele show fewer depositions of Aβ plaques in the post-mortem brain and clinically display lower rates of cognitive deterioration (Martins et al. 2005). However, the mechanisms behind why this is the case are currently unclear. Typically, those who possess the APOE4 allele are most likely to experience earlier onset AD pathology in comparison with those who have the other alleles APOE2 and APOE3 (Coon et al. 2007).

Genome wide association studies (GWAS) have uncovered a host of genes that may play a role in LOAD development. It is important to note the importance of polygenicity when discussing the genetics of AD and GWAS have highlighted the complexity of AD at a genetic level and allowed the consideration of a range of new potential gene targets (Kamboh et al. 2012, Wightman et al. 2021).

This can be illustrated by looking at the case of ADAM17, a noted AD risk gene (Hartl et al. 2020). ADAM17 is a metalloprotease that plays a role in the mediation of receptor shedding at the cell membrane allowing for the release of cytokines and growth factors, contributing to normal signalling pathway functionality (Peschon et al. 1998). To add to the complexity ADAM17 has over 80 different substrates and is involved in immunity, inflammation, cell growth and cell adhesion (Scheller et al. 2011). ADAM17 is also involved in APP metabolism and plays a role in the α-secretase processing of the APP protein as part of the non-amyloidogenic pathway which is why it is of interest in the context of AD (Buxbaum et al. 1998). However, this finding is interesting as it suggests that dysregulation of the non-amyloidogenic pathway may also be implicated in AD onset as well as the amyloidogenic pathway.

Other studies have also implicated novel AD risk genes involved in microglial activation including GRN. The GRN gene is responsible for the production of growth factor granulin which has been previously implicated in neurodegenerative disease such as frontotemporal dementia (Mackenzie et al. 2006). Additionally, microglial progranulin has also been noted to
increase in AD patient cerebrospinal fluid (CSF) highlighting the association of microglial activation in worsening AD (Suárez-Calvet et al. 2018).

Another meta-analysis that specifically looked at AD risk genes in LOAD confirmed that along with APOE, risk genes for LOAD also include ADAM10, ACE and IQCK (Damotte et al. 2019). Previous work suggests that ADAM10, like ADAM17, is heavily involved in the non-amyloidogenic pathway (Haass et al. 2012). ADAM10 is also involved in microglial function by mediating the shedding of triggering receptor expressed on myeloid cells 2 (TREM2) which is expressed by microglia (Kleinberger et al. 2014, Forabosco et al. 2013). One study showed that the overexpression of ADAM10 in AD mouse models reduced the cleavage of APP and consequently the aggregation of amyloid plaque, suggesting a possible opportunity for therapeutic intervention (Postina et al. 2004).

TREM2 itself is another well-known AD-related risk gene expressed by microglia in the brain (Kiialainen et al. 2005). It is a cell surface receptor that mediates signalling pathways involved in inflammation and allows for the regulation of microglial activation and survival (Kleinberger et al. 2014). TREM2 gene variants have been closely linked to an increase in the risk of AD development which has generated interest in how microglial cells contribute to AD progression (Guerreiro et al. 2013).

Despite our increasing knowledge of the genetic complexity and potential risk loci involved in AD onset and progression, a full understanding is still yet to be reached. However, GWAS analyses such as these are useful for determining genes of interest in AD pathology and help to offer paths for further exploration.

**Biological sex**

An additional determinant of how likely a person is to develop AD may be based on biological sex. Prevalence of AD has been reported to be more common in women as evidenced by a study which showed that around two thirds of AD patients are women in the United States (Hebert et al. 2013). It is thought that this may be due to increased variations in oestrogen hormone production, specifically the steroid hormone oestrogen 17β-estradiol, in females in comparison with males due to the menstrual cycle and the menopause (Rosario et al. 2009). Oestrogens confer protective benefits to the brain such as providing neuroplasticity in the hippocampal region (McClure et al. 2013, Yan et al. 2017). Therefore, a decline in oestrogen during the menopause may contribute to cognitive decline. Additionally, a previous
study has shown that there are sex differences in the aging immune response where genes associated with B-cell activity were significantly activated in women and not activated in men (Márquez et al. 2020).

Numerous accounts of sex differences between male and female AD mouse models have also been acknowledged affecting lifespan, tau phosphorylation and levels of Aβ (Rae and Brown 2015, Koppel et al. 2014, Hirata-Fukae et al. 2008). One study in particular found that in the 3xTg AD mouse model female mice displayed significantly increased levels of Aβ, particularly in the frontal cortex in comparison with male mice, thought to be caused by differing levels of sex hormones (Carroll et al. 2010). Another study found that after selectively blocking the glutamate receptor mGluR5 which is required for memory that male mice showed an improvement in memory whereas female mice did not (Abd-Elrahman et al. 2020).

In terms of translating and elaborating on findings from these works, it is very important not to exclude female cohorts, especially when women more commonly develop the disease (Hebert et al. 2013). While this is something that has been acknowledged in recent years, it seems that there are still discrepancies in the use of male cohorts over female cohorts in scientific research (Woitowich et al. 2020). Therefore, developing our understanding of the hormonal fluctuations and the differences in how males and females age that appear to contribute to the AD would be beneficial in terms of developing therapeutic strategies suitable for both sexes.

**Environmental factors**

While genetic determinants of AD development are beyond our control, environmental factors may also play a role in determining the likelihood of AD development. For example, exposure to dangerous chemicals, lack of cognitive stimulation and smoking have all been linked to the development of AD (Campdelacreu 2014). Lifestyle may also play a part in this, with obesity and related diseases such as diabetes and hypertension possibly creating a predisposition for a person to develop AD (Whitmer et al. 2008, Crane et al. 2013, Moonga et al. 2017).

The term “type 3 diabetes” has been coined as a means to describe to the role of insulin as a factor related to AD onset (Steen et al. 2005). Previous work has shown that there is a clear link between diabetes and AD via the insulin degrading enzyme (IDE). Typically, the protease IDE plays a key role in the degrading insulin and is dysregulated in Type II diabetes.
leading to uncontrolled insulin production (Duckworth et al. 1998). It also been found that Aβ is a substrate of IDE and that IDE mediates clearance of Aβ through proteolytic action (Kurochkin and Goto 1994, Farris et al. 2004). Evidence suggests that levels of IDE decline during aging and that somatostatin mediates IDE expression in microglia (Stargardt et al. 2013). Decreases in levels of somatostatin cause reduced IDE activity resulting in reduced Aβ clearance (Tundo et al. 2012). This has led to the idea that IDE could potentially be a therapeutic target for AD treatment (Akter et al. 2011).

High levels of inflammation have been associated with obese patients and studies have shown that obese patients over the age of 50 have a higher risk of developing AD (Ma et al. 2020). Obesity has also been linked with contributing to brain atrophy and lesion development (Raji et al. 2010). Adipose tissue secretes a range of molecules including pro-inflammatory cytokines such as TNF-α and IL-6 which can contribute to low grade chronic inflammation (Ouchi et al. 2011). One study also found a correlation between high fat diets among middle aged people and an increase in risk of mild cognitive impairment (MCI) (Eskelinen et al. 2008). Obesity and bad dietary habits can drive gut microbiome dysbiosis, which has also been linked to AD onset and progression (Cattaneo et al. 2016). A lack of key nutrients such as antioxidants and vitamins from poor diets can also contribute to oxidative stress within the body, however treatment with antioxidants such as Astaxanthin have shown to have a positive effect in AD by protecting neurons in the hippocampus from damage via Aβ (Lobos et al. 2016). Collectively, this previous work suggests that a balanced diet, the maintenance of a healthy gut microbiome and healthy lifestyle choices are factors that are within our control and could contribute to the minimisation of AD development and severity.

Whether or not there is a single driving cause for the development of LOAD in an individual is a matter of debate. From the evidence, it appears that AD onset and development is complex and it is perhaps more likely that a combination of a range of factors increases the risk and severity of AD. The complexity of disease onset and progression must therefore be carefully considered when developing therapeutic interventions.

1.1.4 Current AD therapeutics

There are multiple treatments available to AD patients at present. However, these typically treat the symptoms of AD rather than directly intervening on disease development and progression. Available treatments that are used mainly include pharmaceutical therapies such
as Donepezil and Rivastigmine which are Acetylcholinesterase inhibitors and more recently antibody-based therapies such as Aducanumab.

**Acetylcholinesterase inhibitors**

Acetylcholinesterase inhibitors including Donepezil, Rivastigmine and Galantamine are widely used to treat the effects of AD (Wilkinson et al. 2004). They are often used as a treatment in the earlier stages of AD, however Donepezil can also be prescribed for severe AD. They work by binding to the cholinesterase enzyme which breaks down the acetylcholine neurotransmitter that crosses between the synaptic cleft to the post synaptic neuron to potentiate the action potential that allows transmission of a nervous signal (Davidsson et al. 2001). It has been shown that acetylcholine helps to mediate learning and memory, with a decrease in cholinergic activity known to correlate with the development of AD in the early stages (Francis et al. 1993). By inhibiting the breakdown of acetylcholine it is possible to account for its decrease during the onset of AD providing therapeutic benefits and helping to lessen the progress of the disease (Watanabe et al. 2008).

**Memantine**

The molecule 1-amino-3,5-dimethyladamantane, also known as Memantine, is widely used as a treatment for moderate to severe AD (Reisberg et al. 2003). It works by blocking the action of the neurotransmitter glutamate from binding with N-methyl-D-aspartate (NMDA) receptors which mediates the passage of action potentials across the synaptic cleft (Chen et al. 1992). Glutamate is very abundant within human brain tissue and essential for mediating synaptic transmission that co-ordinates synaptic plasticity and neuronal survival (Schousboe et al. 1981). During neurodegenerative diseases such as AD levels of glutamate can be higher than normal and it is thought that these high levels can interrupt normal nervous signalling, therefore, blocking the glutamate receptors acts as a means of preventing such aberrant signalling and lessening symptoms (Wilcock et al. 2008).

**Aducanumab**

A newer addition to AD treatments is Aducanumab; a human monoclonal antibody that works by binding to the aggregated Aβ plaques in both their soluble oligomeric form and
aggregated form (Sevigny et al. 2016). One study suggested the mechanism of clearance relies on Aducanumab recruiting microglia to the amyloid plaques to mediate phagocytosis, and effectively reduces amyloid load over a period of a year shown via positron emission tomography (PET) scans (Sevigny et al. 2016). Another study has shown that use of Aducanumab can lead to a decrease in Aβ oligomer accumulation resulting in a reduction of tau pathology downstream (Haeberlein et al. 2020). However, this method of treatment is typically administered after up to 20 years of Aβ plaque accumulation, rather than tackling the developing pathology before it progresses. Additionally, much controversy has surrounded the use of Aducanumab to date, putting into question its efficacy and potential as an AD therapeutic intervention (Alexander et al. 2021, Knopman et al. 2021).

While these treatment options are often given individually as with most treatments a combination of drugs can provide better efficacy. For example, studies have shown that patients treated with a combination of Donepezil and Memantine showed better cognitive performance and improved AD behaviours compared to a group treated with Donepezil alone (Tariot et al. 2004). However, it is important to note that combining drugs can lead to an increase in undesirable side effects in patients such as nausea. These treatments also introduce problems such as a lack of efficacy due to low level penetrance across the blood brain barrier (BBB). This makes the administration of therapeutics such as antibody-based therapies particularly problematic since the size of molecules that can pass through are limited to around 400 Da or less and must be lipid soluble (Fischer et al. 1998). The BBB acts as a filter which can regulate the passage of blood solutes through into the extracellular fluid of the central nervous system (CNS). The BBB is highly important for maintaining the homeostasis of the CNS, creating an appropriate milieu for neurons to function and is largely made up of endothelial cells, tight junctions and mural cells (Daneman and Prat 2015).

Despite the problem of the BBB in drug development, some studies have shown that it is possible to open up the BBB using targeted ultrasound treatment in conjunction with AD therapeutics (Lipsman et al. 2018, Janowicz et al. 2019). This method temporarily allows the passage of drugs through and helps to increase their effectiveness. However, this does add extra time and cost onto an already expensive process. Therefore, developing an effective treatment option with fewer side effects for patients and that can circumvent the need to pass through the BBB would be highly desirable and currently is not available.
1.2 Inflammation in AD

Inflammaging has become a popular term over the last two decades, used to describe the dysregulation of the production of cytokines in correlation with aging resulting in the development of low-grade chronic inflammation (Franceschi et al. 2000). In elderly individuals, pro-inflammatory cytokines such as TNF-α and IL-6 have been found to be upregulated (Ershler et al. 1993, Ferrucci et al. 2005). Increases in levels of oxidative stress have also been found to be correlated with inflammation and old age (Gorni and Finco 2020). Inflammaging has been linked to the development of a range of diseases such as Type II diabetes, cancer and AD (Prattichizzo et al. 2016, Landskron et al. 2014, Büttner et al. 2018).

There are many factors that have been linked to inflammaging including smoking and obesity (Arnson et al. 2009, Pou et al. 2007). Studies suggest that visceral adipose tissue is a serious contributor to high TNF-α and IL-6 levels, thought to be due to the presence of macrophages in adipose tissue (Kern et al. 2001, Xu et al. 2003). Additionally, gut dysbiosis has also been linked to contributing to inflammation in old age. One study used young germ-free (GF) mice and transplanted the gut microbiota of aged mice (17-months), which gave rise to signs of inflammaging in the young mice such as an upregulation of TNF-α and an increase in T-helper cell activation in the spleen (Fransen et al. 2017).

It is therefore unsurprising that the implications of age-related inflammation in neurodegenerative diseases such as AD have received a great deal of interest. Inflammation is widely seen in AD patients and is thought to be the result of the settling AD pathology which triggers the activation of microglial cells which help to co-ordinate an immune response (Cagnin et al. 2001). However, some have questioned whether AD associated inflammation is a result of the deposition of disease pathology or whether inflammation precedes disease development. Evidence suggests that pre-existing inflammation can lead to the development of AD pathology, for example the upregulation of IL-1β and IL-6 due may lead to the development of tau pathology (Quintanilla et al. 2004). There is also evidence to suggest that people who experience high levels of inflammation due to head injury are more at risk of developing AD (Nemetz et al. 1999, Johnson et al. 2012).

It remains unclear whether inflammation in AD is a cause or effect of disease pathology, or most likely, a combination of both. Either way, one of the key mediators of inflammation in AD are the microglial cells, which play an important central role in orchestrating an AD dependent inflammatory response.
1.2.1 The role of microglia

Microglia are innate immune cells and the resident macrophages of the CNS (Kreutzberg 1996). During the early stages of development microglia are derived from the embryonic yolk sac and migrate into the CNS before the closing of the caudal neuropore, making them brain specific (Alliot et al. 1999, Stiles and Jernigan 2010). They are able to self-renew in the brain to maintain population levels during adulthood (Rossi et al. 2007). Their lineage makes them distinct from other circulating macrophages which derive from haematopoietic lineages in the bone marrow (Fogg et al. 2006).

One important role microglia play is the maintenance of neural networks where they are able to maintain normal signalling and synaptic plasticity via degradation of abnormal neurons and synapses (Schafer et al. 2012). CNS microglia are also found to be upregulated in a range of neurodegenerative conditions and are a key player in coordinating the inflammatory response in AD. The deposition of Aβ plaques and tau pathology in and around neurons in the brain induces low grade inflammation mediated by microglial cells (Smith et al. 2011). ATP is a vital communication element between microglia and neurons. Microglia are programmed to have chemoattraction towards ATP which allows them to respond to disturbances in homeostasis. Neurons that require microglial assistance will release ATP via pannexin hemichannel activation (Li et al. 2012). Newer research also suggests that microglia and neurons can communicate more directly through junctions detected where microglial processes directly interact with neuronal cell bodies (Cserép et al. 2020).

Microglial phenotypes

Microglia survey their surroundings primed and ready to respond to pathogens or detrimental changes to their environment in a ramified or relaxed state (Nimmerjahn et al. 2005). It is widely accepted that in response to infection, damaged neurons or an otherwise potentially detrimental entity, microglia become activated and amoeboid in shape and take on one of two conformations: the M1 state or the M2 state (Kettenmann and Hanisch 2007, Hu et al. 2015, Tang and Le 2016) (Figure 1.5). M1 microglia supposedly co-ordinate a pro-inflammatory response and is the “classical activation” state, acting as first responders to invading pathogens. In this state the microglia release a range of cytokines which trigger a pro-inflammatory cascade such as IL-1β, IL-6 and TNF-α (Ajmone-Cat et al. 2013). M2 microglia supposedly co-ordinate an anti-inflammatory or immunosuppressive response. This response is known as “alternative activation” and is more closely associated with the
production of protective cytokines such IL-4, IL-10 and IL-13 (Ponomarev et al. 2007). Therefore, M2 is recognised as a protective activation state.

Figure 1.5 Phenotypic changes and cytokine production of innate immune central nervous system (CNS) microglial cells. In the CNS microglia exist in a relaxed or ramified phenotype while surveying their surroundings. Upon recognition of stimuli via pattern recognition receptors the microglia become activated and morph into an amoeboid phenotype. Depending on their environment microglia either become “M1” microglia and produce anti-inflammatory cytokines and reactive oxygen species (ROS) or they become “M2” microglia which produce anti-inflammatory cytokines and play a more protective role. Figure partly created using Biorender.com.

The notion of the “M1” and “M2” microglial states has been debated and there is an increasing amount of evidence to suggest that perhaps these polarising states are not as distinct as once thought (Ransohoff 2016). One of the underlying problems with the theory of M1/M2 states is due to the fact that microglia derive from myeloid lineages rather than bone marrow like other circulating macrophages which may give rise to different properties (Gautiar et al. 2012). It is very possible that by oversimplifying the range of conformations in which CNS microglia exist we may be diminishing their ability to play a complex role in a range of diseases.
**Microglia as inflammatory mediators in AD**

In the AD brain upon recognition of Aβ deposits around neurons, microglia will release a range of pro-inflammatory mediators such as complement proteins and cytokines including IL-1β, IL-6 and TNF-α (Morimoto et al. 2011). There is evidence to suggest that supressing the inflammatory response of microglia in AD may be beneficial and could potentially be used as an option for therapeutic intervention (Ou et al. 2018, Kantarci et al. 2018).

Microglia are equipped with a plethora of receptors allowing for pattern recognition, including a subset of pattern recognition receptors known as Toll Like Receptors (TLRs) which allow microglia to respond to external threats such as cellular debris, pathogens and neurotoxins (Medzhitov and Janeway 1997). Microglia are also major contributors to the inflammatory response via production of pro-inflammatory cytokines co-ordinated by the TLRs and NF-κB (Kawai and Akira 2007). For example, TLR4 induces production of IL-1β and TNF-α (Jin et al. 2008). A study by Keren-Shaul et al. (2017) showed that certain AD risk genes are upregulated when microglia are activated or in a “disease state”. These activated disease state microglia also release inflammatory mediators which go on to trigger the inflammasome, a key part of the innate immune system (Heneka et al. 2013).

**Disease associated microglia**

Populations of specific disease associated microglia (DAM) have been defined (Keren-Shaul et al. 2017). These DAM populations are thought to provide protection to neurons and are found to be exclusively and closely associated with areas of AD pathology. The same study also found incidence of microglial gene Tyrobp in the DAM populations and that DAM activation will not fully occur without TREM2. TREM2 is a well-recognised AD risk gene, where mutations have been linked to AD development as mentioned previously (Song et al. 2017). TREM2 and Tyrobp have also previously been shown to work together as a signalling complex which results in the phagocytosis and clearance of Aβ (Ma et al. 2014). This therefore further suggests that DAM populations act in a protective manner and that mutations in TREM2 may hinder Aβ clearance, resulting in the exacerbation of AD.

While microglia have the capacity to coordinate the immune response and clear plaques, they may also contribute to the development of AD pathology. Evidence suggests that TAM (Axl and Mer) tyrosine kinase receptors which are situated on microglial cells may play a key role in the recognition and clearance of Aβ plaque. Typically, in normal healthy
microglia levels of Axl are lower than Mer, however when faced with an inflammatory stimulus Axl becomes more upregulated (Fourgeaud et al. 2016). Another study has suggested a mechanism wherein microglia begin to contribute to amyloid plaque deposition during the progression of the disease (Huang et al. 2021). This group describe how microglia act on diffuse and immature formations of Aβ plaque and clear these away via phagocytosis mediated by the TAM receptors. Once inside the microglia the amyloid gets processed by lysosomes and packaged into denser plaque formations. These are then released as an extracellular waste product, which may contribute to the development of mature and denser plaque deposits which further contribute to the progression of AD pathology. This further provides evidence to suggest that the role that microglia play in AD is double-edged, showing the potential to be both neuroprotective and detrimental during the development of AD.

It is clear that microglia play a crucial role in the mediation of inflammation in AD. Therefore, they can act as a useful target when considering therapeutic intervention. However, it is important to note that while microglia are critical for mediating the innate immune response in the brain, other cells such as astrocytes also produce cytokines and contribute to the response (Gao et al. 2013). Our understanding of the crosstalk between these cells and the respective cytokines they produce is limited. Developing our knowledge here could be very useful to further understand how the cell mediators of the immune system work in conjunction to respond to neurodegenerative diseases.

1.3 The gut

The gastrointestinal (GI) tract is essential for digestion and the retrieval of nutrients from the diet. A summary of the main features of the GI tract are shown in Figure 1.6. In mammals, the GI tract comprises of the mouth, the oesophagus, the stomach, the small intestines, the large intestines and rectum. The small intestines are composed of three different regions; duodenum, jejunum and the ileum and are important for continuing digestion and absorption of dietary nutrients (Drake et al. 2020). The large intestine, also known as the colon, plays an important role in water absorption (Drake et al. 2020).

The intestinal walls are made up of 4 layers of mucosal tissue which allow for key gut functions such as peristalsis to move the bolus of food through the GI tract and the absorption of water and nutrients (Roa and Wang 2011). The gut epithelial lining is covered in villi cells which increase the surface area of the gut for absorption of water and nutrients. The villi are lined with enterocytes which are secretary goblet enteroendocrine cells with microvilli on their
surface to further increase the surface area of the gut and maximise nutrient absorption (Cheng and Leblond 1974, Roth et al. 1990). Enterocytes mediate the digestion and absorption of nutrients and water via the production of enzymes on their surface and allow for transport of the digested molecules through from the lumen through to the lymphatic and circulatory system (Ziv and Bendayan 2000). Enterocytes also play a role in immunity via regulation of tight junctions to prevent pathogens passing through into the somatic circulatory system (Pitman and Blumberg 2000). One other prominent feature of both the small and large intestines is the existence of populations of gut bacteria collectively known as the gut microbiome.
1.3.1 The gut microbiome

The gut microbiome is a term used to define the genomes of the populations of microbes in the gut made up largely of bacteria, but also including protozoa, archaea and viruses, which exist in symbiosis with their host (Hooper and Gordon 2001). Using new sequencing techniques such as Next-Generation Sequencing (NGS) and projects such as The Integrative Human Microbiome Project, current knowledge of the complexity of our gut microbiome is expanding rapidly (Proctor et al. 2019). The GI tract harbours over 100 trillion
resident bacteria, which significantly outnumbers the quantity of human cells in the body (Rodríguez et al. 2015, Sender et al. 2016). This dynamic population is unique to each individual and fluctuates both as we age and as we are exposed to different environments.

The abundance and diversity of the gut microbiome gives rise to a variety of functions that the human intestinal system alone would not be able to carry out suggesting a likely cause of the evolution of this symbiotic relationship. The necessity of this relationship has also been highlighted by studies in GF rodents which show that a lack of gut bacteria results in decreased immune defence against pathogens, increased levels of anxiety and abnormal brain development (Ichinohe et al. 2011, Crumeyrolle-Arias et al. 2014, Heijtz et al. 2011).

Our microbiome increases our ability to breakdown a range of food, particularly those high in fibre allowing us to harness further nutrients from our diets and to increase the nutritional load (Flint et al. 2012). Gut bacteria also help to develop and co-ordinate metabolism and immunity (Romano-Keeler et al. 2014).

The human gut microbiome develops and varies throughout our lifetime. While the foetus is in the womb it is thought that they develop in a sterile environment since there is no clear bacterial colony transmission. Studies investigating whether or not the placenta and amniotic fluid contain their own microbiomes have produced contradicting results (Aagaard et al. 2014, Wampach et al. 2017, Lim et al. 2018). Nevertheless, it is widely accepted that upon birth the substantial development of a lifelong symbiotic relationship with micro-organisms begins. The accumulation of micro-organisms from birth and beyond appears to have a huge impact on how the microbiome of an individual develops (Yang et al. 2016). As the baby passes through the birth canal it is exposed for the first time to its mother’s vaginal and faecal microflora which largely consists of *Lactobacillus* (Dominguez-Bello et al. 2010).

This is a significant event and studies have shown that exposure to these bacteria can be very beneficial to long-term health and support the normal development of the immune system (Salminen et al. 2004). The importance of this becomes even more clear when comparing the health of babies born vaginally compared with those born via caesarean section. Interestingly, when babies are born via caesarean section they are primarily exposed to different populations of bacteria from the skin microbiome (Akagawa et al. 2019). These babies typically collect bacteria such as *Staphylococcus* and lack key commensal bacteria (Dominguez-Bello et al. 2010). Therefore, it is unsurprising that differences have been found in the health of these babies as they develop into infancy. The importance of the development of a healthy post-natal microbiome is such that it has been suggested that transfer of faecal
samples from the mother to babies born via caesarean section could be a way of introducing these beneficial bacteria (Korpela et al. 2020). This would allow babies to be exposed to new bacteria in the hope it will help to develop a stronger immune system.

The introduction to bacteria at an early age is extremely important and the population diversity continually increases through childhood, dependent on factors such as diet and environment (Kumbhare et al. 2019). Once in adulthood the gut tends to stabilise and peak at its microbial diversity. Around 90% of the adult microbiota population consists of two highly conserved key phyla; *Firmicutes* and *Bacteroidetes* (Lozupone et al. 2012, Forster et al. 2019). The rest of the population belong to the phyla *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Qin et al. 2010). This population of bacteria are distributed in varying concentrations along the digestive tract with some bacteria being more abundant in certain gut regions. For example, biopsies have shown that *Bacteroidetes* are more prevalent in the large intestine, while *Firmicutes* are more prevalent in the small intestine (Frank et al. 2007).

### 1.3.2 The microbiome-gut-brain axis

The microbiome, the gut and the brain are able to communicate through a bi-directional system mediated by nervous, chemical and immunological messages known as the microbiome-gut-brain axis (MGBA) (Rhee et al. 2009). Evidence suggests that understanding how the MGBA works exactly could help to elucidate the mechanisms behind a range of diseases, which may be in part, gut derived.

The vagus nerve which is part of the parasympathetic nervous system plays a key role in linking the brain and the gut (Carabotti et al. 2015). This has been evidenced through the link between stress and anxiety and gut related problems such as IBS (Jerndal et al. 2010). The enteric nervous system refers to the enervation of the GI tract which allows the gut to perform autonomous signalling in response to stimuli (Goyal and Hirano 1996). However, while able to act independently, the enteric nervous system can also communicate with the vagus nerve to send signals back to the CNS, allowing for the co-ordination of responses like hunger via signals such as Ghrelin from the gut to the brain (Julio-Pieper et al. 2013). For communication between these key enervated regions, neurotransmitters play a crucial role.

Neurotransmitters also play a role in MGBA communication and have been reported to be produced by gut bacteria. Evidence suggests that the *Lactobacillus brevis* strain of bacteria is able to produce glutamic acid decarboxylase (GAD) which allows for the conversion
of glutamate into gamma aminobutyric acid (GABA) (Li et al. 2010). GABA is a neurotransmitter that plays an important role in the control of nervous signalling by downregulating neuronal excitation (Olsen and Sieghart 2008). Interestingly the neurotransmitter serotonin is also thought to largely be produced in the gut via the support of gut microbes and can interact with the vagus nerve as a means of co-ordinating gut functions such as peristalsis (Yano et al. 2015, Browning 2015). One study found that serotonin levels in GF mice were depleted in comparison with wild type (WT) mice, suggesting the importance of gut microbiota to produce this key neurotransmitter (Wikoff et al. 2009). Serotonin is largely secreted by a type of enteroendocrine cell known as enterochromaffin cells (ECs) upon stimulus such a movement in the intestines and chemical signals (Bertrand 2004, Smith et al. 2006). However, the full extent of EC regulation of serotonin is still largely unknown.

Afferent ECs also play an important role by providing an interface for gut-brain communication. ECs are situated in between the villi in the gut epithelial layer and can therefore act as a mediator of the gut lining through direct interaction between with luminal gut bacteria and nerves situated in the lamina propria (Bellono et al. 2017). Interestingly, it has been found that ECs also have TLRs (Bogunovic et al. 2007). It is thought that ECs therefore play a critical part of microbiota-gut-brain communication through their ability to recognise bacterial pathogen associated molecular patterns (PAMPs) in a similar way to microglial cells. Particularly, ECs have been found to be sensitive to bacterial flagella which allow for bacterial movement, and which acts as a ligand for TLR5, a TLR exclusively found in the gut (Uematsu et al. 2006). The activation of TLR5 via flagellin leads to downstream activation of the innate immune NK-κB pathway, contributing to inflammation and cytokine release in the gut (Hayashi et al. 2001).

Evidence suggests that short chain fatty acids (SCFAs) play a role in inducing serotonin production via ECs in the gut (Reigstad et al. 2015). SCFAs have been found to play a vital role in communication in the MGBA. They are one of the key metabolic by-products produced by the gut microbiota via fermentation of indigestible dietary carbohydrates such as cellulose, typically found in plant-based food and also known as fibre (Grabitske and Slavin 2008). This highlights the importance of dietary fibre, with studies suggesting that a high fibre diet is linked to greater SCFA production which is beneficial for maintaining health (Desai et al. 2016). There are three main SCFAs produced through this microbiota mediated fermentation process: acetate, butyrate and propionate (Miller and Wolin 1996, Topping and Clifton 2001, Pryde et al. 2002). SCFAs are important additional energy sources in mammals, contributing to ~10% of total energy needs per day (Bergman 1990).
Overall, the MGBA is central for mediating the functioning of a plethora of biological functions. It is likely that there is a lot of crossover between many of these already intricate pathways. Therefore, elucidating aberrant mechanisms that may give rise to diseases is complex and at present our knowledge in this area is limited.

1.3.3 Immunity and the gut

The human gut is a hub of innate immunological activity and also contains large amounts of gut associated lymphoid tissue (GALT). GALT includes lots of adaptive immune structures such as Peyer’s patches (PPs) and isolated lymphoid follicles (ILFs) (Mowat and Agace 2014). The PPs are rich with immune cells such as T and B lymphocytes, macrophages, and dendritic cells which help to regulate inflammation and coordinate immune responses in the gut as a reaction to pathogens or pro-inflammatory stimuli (Spencer et al. 1986).

The enterocytes which make up part of the gut epithelia play a role in gut immunity. One of the main actions of the enterocyte layer is to act as a filter to prevent pathogens and other detrimental molecules from crossing over into systemic circulation and help to maintain homeostasis (Turner 2009). The gut barrier is similar to the BBB in that tight junctions between cells control passage and transport of molecules across it. Tight junction proteins such as Occludin and Claudin-5 are key for supporting selective membrane permeability to allow water and solutes from the gut lumen environment across the gut epithelium and into systemic circulation (Furuse et al. 1993, Krause et al. 2008). Loss of integrity of the gut barrier via reduced action of tight junctions between enterocytes has been reported to contribute to a phenomenon colloquially coined as “leaky gut” and has been shown in GF mice (Braniste et al. 2014) (Figure 1.7).
An increase in intestinal permeability contributes to elevated levels of inflammation through activation of immune cells. For example, inflammatory stimuli such as lipopolysaccharide (LPS) from gram negative bacteria have been shown to have the ability to cross through a comprised gut barrier via bacterial LPS extracellular vesicles (Ellis and Kuehn 2010). This can result in an upregulation of inflammatory cytokines in systemic circulation due to the immune response, including the activation of TLRs on macrophages and (and brain microglial cells if the BBB is also compromised) via PAMPs and danger associated molecular patterns (DAMPs) (Tulkens et al. 2020). However, the full extent of how leaky gut contributes to inflammation and the mechanisms behind this are yet to be fully understood.
Previous evidence also suggests that gut microbiota have an impact on maintaining gut integrity. It has been reported that one of the SCFAs produced by gut microbiota, butyrate, may have a beneficial impact gut epithelium integrity by enhancing the production of tight junctions via activation of protein kinases as seen in cultured gut epithelia cells (Peng et al. 2009). Another study found that one strain of gut bacteria Akkermansia muciniphila directly interacts with cultured enterocytes via adhesion which resulted in improved gut barrier integrity (Reunanen et al. 2015). This suggests that gut bacteria play an important role in the maintenance of gut barrier integrity as part of the regulation of gut immunity.

In addition to the physical barrier of the gut epithelium, in response to the detection of detrimental pathogens the gut can also defend itself via key mechanisms such as mucus production and release of Immunoglobulin A (IgA). The production of mucins from goblet cells in the intestinal villi create a barrier between the intestinal lining and pathogens preventing these microbes from reaching the gut barrier (Bergstrom et al. 2010). This mucus layer has been shown to be protective against pathogens such as rotaviruses (Chen et al. 1993). Goblet cells in the intestinal epithelia produce mucin glycoproteins known as mucin 2 (MUC2) which make part of the composition of the mucus layer (Johansson et al. 2008). The importance of this mucus protein as part of the intestinal protection mechanism has been highlighted via knock out studies where MUC2 knock out mice go on to develop colitis (Van der Sluis et al. 2006). Other studies have also found a link between gut microbiota and MUC2, suggesting that probiotic supplementation results in upregulation of MUC2, possibly providing increased intestinal protection (Caballero-Franco et al. 2007).

IgA is released by the intestinal mucus membrane in response to gut microbes and coats the GI epithelial lining which helps to prevents the crossing of pathogens across the epithelial barrier which could lead to an inflammatory response (Johansen et al. 1999). More recent evidence has also implicated the presence of intestinal IgA+ plasma B cells present in the CNS during disease. The study carried out by Rojas et al. (2019) showed that in the experimental autoimmune encephalomyelitis (EAE) model circulating intestinal IgA+ plasma B cells were found to be present in the CNS and helped to improve inflamed brain regions, possibly through IL-10 release. While this is interesting, the mechanisms behind this finding are not clear and further work would need to be done to determine if this is a protective mechanism coordinated by the gut and brain as a response to inflammation in the brain.

Gut bacteria are also able to regulate immune cells in the gut which can communicate with the brain to initiate the inflammatory response which in part helps to explain the
mechanisms involved in the MGBA (An et al. 2014). It is thought that after their production by the gut bacteria metabolism, SCFAs are actively transported across the gut lumen via monocarboxylate transporters to reach colonocytes to be metabolised (Iwanaga et al. 2006, Gonçalves et al. 2012). SCFAs that are not metabolised are able to enter the blood circulation (Cummings et al. 1987). SCFAs that cross BBB then have the ability to interact with G-protein coupled receptors (GPCRs) known as free fatty acid receptor (FFAR) 2 and 3 which are expressed by numerous immune cells to control cell signalling events (Uhlen et al. 2015). SFCAs can also act by inhibiting histone deacetylases (HDACs) by promotion of acetylation around histones resulting in changes of gene expression (Waldecker et al. 2008). Butyrate and propionate have been implicated in inflammation and have been shown to have the ability to downregulate the pro-inflammatory NF-κB pathway (Segain et al. 2000, Al-Lahham et al. 2010).

Gut microbiota metabolite SCFAs have also been linked to the functions of CNS microglia. Evidence suggests that SCFAs produced by gut bacteria play an important role in the maturation of microglial populations. Using RNA-seq one study found that microglial populations in GF mice showed a downregulation in genes required for microglial cellular activation and cell signalling in comparison with WT mice (Erny et al. 2015). This suggests that a lack of diversity in the gut microbiome, resulting in diminished levels of SCFA may affect microglial function and therefore levels of inflammation in those with gut dysbiosis. A further study has confirmed this, reporting that SCFAs can downregulate proinflammatory cytokines such as IL-1β and TNF-α in cell cultures of THP-1 monocytes, a cell type similar to microglia (Wenzel et al. 2020). While this link has been established, the full mechanistic relationship behind gut microbiota and microglia function is yet to be understood. This highlights the importance of gut microbiota and SCFAs in the control of inflammation and immunity in the gut. This evidence also suggests that restoring the gut microbiome could result in improved microglial function and control of inflammation. This may be very beneficial in diseases such as AD where disease associated and microglial mediated inflammation is prevalent.

### 1.3.4 Links between the gut microbiome, aging and AD

Aging is a major risk factor in the development of AD and has been shown to correlate with loss of diversity in the gut microbiome. Individuals who reach extreme old age (classed as 105 years old or above) have gut microbiomes enriched with key beneficial bacteria such
as Akkermansia and Bifidobacterium which are thought to help support health and longevity and reduce age associated problems like inflammaging (Biagi et al. 2016). This supports the idea that replenishment of the gut bacterial population with key beneficial microbes could be an appropriate method of intervention to help prevent the onset of age-related disease.

There is also growing evidence which suggests a close link between the gut microbiome and neurodegenerative diseases such as AD. Treatment of APP/PS1 mice with antibiotics altered gut microbiome composition which correlated with a decrease in Aβ plaque deposition and an increase in soluble amyloid 40/42 monomer levels (Minter et al. 2016). In another study it was shown that aged APP/PS1 GF mice display a reduction in Aβ plaque levels and activated microglia in comparison with age matched conventionally raised APP/PS1 mice (Harach et al. 2017). Both studies support the idea that the gut microbiome may play a role in the development of AD pathology and associated inflammation.

Evidence also points towards a link between the inhibition of HDACs, particularly HDAC2, and improved memory (Morris et al. 2013, Guan et al. 2009). SCFAs produced by gut bacteria play a role in the inhibition of HDAC function and downstream gene expression and it is thought that SCFAs in this context could help to improve AD symptoms such as memory impairment through HDAC2 inhibition (Waldecker et al. 2008). Another study found that the composition of gut microbiota may even be already impaired in patients with MCI which precedes AD (Li et al. 2019). The group who conducted the study hypothesise that gut dysbiosis may occur during MCI due to little difference of gut microbiota composition upon comparison of patients with MCI and AD, suggesting that the gut microbiota composition could even be used as an early biomarker for risk of AD development (Li et al. 2019).

Collectively, this evidence strongly suggests a link between the composition of the gut microbiota and the development of AD pathology and symptoms. This has led to the idea that it may be possible intervene on AD onset and progression through manipulation of the gut microbiome composition, potentially through the use of probiotic supplementation.

1.4 Probiotics

Probiotics were originally defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2002). They are commonly administered orally via capsules or tablets as dietary supplements but are also present in many food products (Meybodi et al. 2017). For example, they are often found naturally
occurring in fermented dairy products such as yogurt or kefir. The most common bacteria used as probiotics includes the strains *Bifidobacterium* and lactic acid producing bacteria *Lactobacillus* (Holzapfel et al. 2001).

It is thought that probiotics replenish the intestinal microbiome with bacteria that release by-products which are toxic to detrimental strains of bacteria that live in the gut (O’Shea et al. 2012). One group of by-products includes bacteriocins which have been shown to diminish the effects of detrimental bacterial strains such as *Listeria monocytogenes*. These effects have been shown to be due to the bacteriocin Abp118, produced by a specific strain of *Lactobacillus salivarious* (Corr et al. 2007).

While probiotics appear to have many beneficial properties, they do also have their limitations. Firstly, probiotics are only effective if they reach the gut alive and can colonise the gut as described in the original WHO definition. This can prove difficult, especially if probiotics are being taken in conjunction with drugs such as antibiotics which may have toxic effects on the probiotic bacteria (Obanla et al. 2016). However, it has also been speculated that in fact probiotics may be a useful therapeutic tool in improving the efficacy of some drugs and could be considered for use in combination with a range of current therapeutics for different diseases (Al-Salami et al. 2008, Matuskova et al. 2014).

### 1.4.1 Potential health benefits of probiotics

In recent years, the possible beneficial health effects of probiotics have been documented and appear to have the potential to be used as a therapy for some diseases and health problems including Covid-19 (Chen et al. 2015, Raygan et al. 2018, Yong et al. 2020, Baud et al. 2020). It is thought that they do this by restoring the diversity of the gut microbiome where the population has been depleted resulting in gut dysbiosis (Kumar et al. 2020). Gut dysbiosis can lead to problems which have a knock-on effect on health such as leaking of the intestinal epithelial barrier resulting in the infiltration of pathogens and an increase in oxidative stress which has been linked to several health problems (Maes et al. 2010, Stadlbauer et al. 2020). The effects of disease or the use of certain medications such as antibiotics can upset the homeostasis of the gut, resulting in dysbiosis. Use of antibiotics appear to diminish biologically beneficial bacteria in the gut while being administered to eradicate pathogenic bacteria (Bartosch et al. 2004). This can upset the homeostasis of the gut microbiome and induce a state of gut dysbiosis particularly if the antibiotics are taken in the early years of life (Neuman et al. 2018).
The most common gut disorder is irritable bowel syndrome (IBS) with an estimated 15% of the global population suffering from its effects (Lovell and Ford 2012). The symptoms typically include changes in stool consistency and frequency along with uncomfortable bloating and flatulence. Studies have shown that there is a significant difference in gut microflora in patients with IBS compared with healthy controls (Kassinen et al. 2007). A recent systematic review of clinical trials assessing the potential of probiotics as a treatment for IBS concluded that there was a trend for multi-strain probiotic treatments improving IBS symptoms and could be a promising treatment option (Dale et al. 2019).

There has been an increase in studies linking neurodegeneration with gut dysbiosis, particularly, Parkinson’s disease (PD) and AD (Hasegawa et al. 2015, Vogt et al. 2017). In recent years the literature has greatly expanded with studies supporting the benefits of probiotics in AD. One study found that probiotic supplementation combined with exercise in the APP/PS1 AD mouse model improved AD development (Abraham et al. 2019). Another study found that supplementation of rats injected with Aβ (1-42) with *Bifidobacterium* and *Lactobacillus* probiotic strains improved behaviour and showed improved levels of oxidative stress in the hippocampal region (Azm et al. 2018). Another group has reported that the supplementation of probiotic in a GF AD mouse model improved the gut integrity of the mice and improved inflammation in the gut. (Kaur et al. 2020). However, the authors did note that probiotic supplementation of the mice had little effect on AD pathology and levels of astrogliosis in this study and that further work is needed. Additionally, probiotic supplementation in rats fed a high fat diet showed lower levels of microglial activation and subsequently improved brain function, supporting that probiotics may have a positive impact on microglial activation status (Chunchai et al. 2018).

A consortium of probiotics known as Lab4 have been used in numerous studies to assess the impact of probiotics in health and disease. Previous work has shown that Lab4 probiotics have beneficial effects on cholesterol levels and arthrosclerosis (Michael et al. 2017, O’Morain et al. 2021). In addition, it has been shown that Lab4 probiotics may have neuroprotective effects, suggesting that they could be advantageous if used as a treatment for AD and other neurodegenerative conditions (Michael et al. 2019). The study utilised the human SH-SY5Y neuronal cell line to assess the effects of Lab4 probiotic application in the presence of challenges such as neurotoxic rotenone. Lab4 probiotic application resulted in improved cell viability and a decrease in the production of reactive oxygen species (ROS), suggestive of neuroprotective properties.
1.5 Research question, hypothesis and aims

Our knowledge of the gut microbiome and its link to disease has expanded in recent years, however there are still many things we do not yet fully understand and require further investigation. Also, we still lack a lot of knowledge around the use of probiotics and their potential benefits in a range of diseases. Evidence from previous studies strongly suggest that the brain and gut are closely linked and that the gut is also responsible for co-ordinating immune responses (An et al. 2014). An area that requires further study is the use of probiotics in neurodegenerative diseases, particularly in AD to explore whether it is possible for probiotics to impact the onset and progression of AD and if they can be a viable early intervention. What remains unclear is how probiotics have this beneficial impact on AD pathology and symptoms. It is likely that gut microbiota metabolites such as SCFAs have an impact on microglial health and function as part of the control of inflammation and regulation of the immune response in a disrupted MGBA.

Based on findings from previous studies which have indicated that probiotics may have neuroprotective properties, ameliorate cognitive decline and improve neuroinflammation in vitro and in vivo, we hypothesise that the Lab4 family of probiotics may help support normal microglial cell health and may ameliorate inflammatory activation of these cells.

The aims of this thesis are:

1) To use in vitro models of microglia to test whether probiotic metabolites generated in vitro can affect gene expression of key inflammatory markers and microglial cell health.

2) To assess the impact of probiotics in vivo in healthy WT mice.

3) To use in vitro models of microglia to test the ability of probiotic-derived metabolites generated in vivo to modulate amyloid-induced inflammatory effects.

To achieve these aims, initially the use of in vitro methods of experimentation were used to assess the effects of probiotics on mouse BV-2 microglial cells. BV-2 cell viability and gene expression were measured using crystal violet (CV) assay and RT-qPCR on a range of key inflammatory target genes. To validate the in vitro findings, a limited in vivo experimentation was carried out. This included assessing the behaviour of WT mice fed a diet supplemented with Lab4P probiotic and mice fed a normal chow diet over a period of two-weeks through the use of novel object recognition testing and assessment of changes in
microglial population via flow cytometry. The \textit{in vivo} work also allowed the generation of mouse serum from control and Lab4P supplemented mice. This serum was then used in downstream \textit{in vitro} assays using BV-2 cells to determine the effects of the serum on the cells without a challenge, before finally assessing the effects of serum on BV-2s in the presence of synthetic Aβ (1-42) to determine the effects of probiotic metabolites on microglial cells in the context of AD. This was measured through the use of CV assay for cell viability and RT-qPCR to determine changes in gene expression of the same inflammatory target genes.
Chapter 2

Materials and methods
2.1 Cell culture and experimental seeding

The BV-2 microglial cell line is an immortalised semi-adherent mouse microglial cell lineage that was selected for use in this project due to its relevance, as seen in the literature (Blaisi et al. 1990, Henn et al. 2009, Stansley et al. 2012). Cells were routinely cultured in T75 flasks (Sarstedt, UK) using Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, UK), supplemented with 10% (v/v) sterile filtered Foetal Bovine Serum (FBS) (Gibco, UK) and 100 U/mL Penicillin/Streptomycin (P/S). Cells were maintained in a humidified incubator at 37°C and 5% CO₂. Cells were passaged when confluency was between 80-100%. Since BV-2 cells are semi-adherent, cell culture flasks were lightly tapped to release cells from the surface to facilitate passaging. To passage, cell suspensions were subjected to centrifugation at 150 x g for 5 minutes and washed with 1 x phosphate buffered saline (PBS). A further centrifugation step was carried out (150 x g for 5 minutes) before removal of 1 x PBS and resuspension of the cell pellet in the culture medium as described above. For routine passaging, cells were diluted between 1:2 and 1:10 depending on the level of confluency of the parent flask.

For experiments, BV-2 cells needed to be transferred to assay plates at specific cell densities. Briefly, a coverslip was attached to the haemocytometer before 10μL of the stock of collected cell suspension was pipetted under the coverslip with the help of capillary force. 10X magnification was used on a Nikon Eclipse TS100 microscope (Nikon) to visualise cells, which were counted in the haemocytometer quadrants. Cells were then diluted in the same cell culture medium before placing in the relevant assay plate for experiments. Cell density was scaled up or down depending on the required plate to maintain a density of 150,000 cells per cm².

2.2 Crystal violet assay

BV-2 cell viability was assessed using the CV method. BV-2 cells were seeded into 96-well plates. After 24 hours, medium was removed and changed to medium containing the conditions for the experiments (as described in the appropriate figure legends and accompanying results). Following the treatment period, cells were washed twice in room temperature 1 x PBS. CV solution (0.1% (w/v) CV powder in 2% (v/v) ethanol) was applied at a volume of 100μL per well and incubated at room temperature for 5 minutes. Cells were then washed twice with 1 x PBS after CV solution removal. Plates were left to dry overnight and once dry 10% Acetic acid (v/v in 1 x PBS) was applied to cells and incubated at room temperature for 1 hour. Supernatant (20μL) was transferred into duplicate wells of a new 96
well plate and diluted with 180μL of 10 % (v/v) Acetic acid. Absorbance was read on a Tecan Microplate Reader (Life Sciences) at 595nm.

2.3 Lab4 consortia of probiotics

Probiotics used were provided by Cultech Ltd. (Port Talbot, Wales) as freeze-dried preparations. Lab4b probiotics included the strains: *Lactobacillus salivarius* CUL61 (NCIMB 30211), *Lactobacillus paracasei* CUL08 (NCIMB 30154), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis*, subspecies *lactis* CUL34 (NCIMB 30172). Lab4P strains comprised of: *Lactobacillus acidophilus* CUL21 (NCIMB 30156) and CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis*, subspecies *lactis* CUL34 (NCIMB 30172) and *Lactobacillus plantarum* CUL66 (NCIMB 30280).

2.4 Conditioned media preparation

Conditioned media preparation was carried out as described in Michael et al. (2019). Briefly, Lab4b and Lab4P conditioned media were prepared by inoculating 10mL of DeMan Rogosa Sharpe (MRS) broth with 10mg of the freeze-dried Lab4b or Lab4P powder. This was left to incubate anaerobically at 37˚C overnight. The bacteria were then pelleted via centrifugation (1000 x g for 20 minutes). The supernatant was then removed and replaced with 10mL 1 x PBS before being centrifuged again at 1000 x g for 20 minutes. DMEM containing no additives was added to dilute the probiotic concentration to 1 x 10⁹ colony forming units per mL (CFU/mL) for each sample. Cultures were then centrifuged at 1000 x g for 20 minutes to pellet the bacteria again. Probiotic by-products were then extracted by passing the supernatant through a 0.22μM sterile filter, then adjusting the pH to 7.4 using 1M NaOH and 1M HCl and adding 50 U/mL P/S. The resulting sterile filtrate of Lab4b or Lab4P by-products is hereafter denoted as “conditioned medium” (CM).

2.5 Synthetic amyloid *in vitro* assays

Synthetic lysophilised amyloid peptides were obtained from Abcam UK (Amyloid Beta 1-42, human (ab120301)). An amyloid stock was prepared by solubilising in 100% (v/v) Dimethyl Sulfoxide (DMSO) at a concentration of 1mM as suggested by the manufacturer.
Two concentrations of DMSO amyloid solution were tested and prepared in DMEM at 0.5μM and at 5μM (Chiozzi et al. 2019). A control containing DMEM and 0.5% (v/v) DMSO was also used. The literature suggested that synthetic amyloid could aggregate to a pathological form *in vitro* when left over time, therefore amyloid was prepared and used by two methods (Caldeira et al. 2017):

(i) Applying neat (non-aggregated) DMSO solubilised amyloid to the cells at 0.5μM and 5μM.

(ii) Allowing the DMSO solubilised amyloid to aggregate in DMEM at a concentration of 0.5μM and 5μM for 24 hours at 37°C and 5% CO₂ before application to cells.

### 2.6 RT-qPCR

BV-2 cells were seeded into 24-well plates or 48-well plates as required before the application of conditions of experiments carried out (as described in the appropriate figure legends and accompanying results). After incubation with test conditions for a given period of time BV-2 cells were gently washed in 1 x PBS once followed by the addition of 0.5mL RiboZol™ RNA Extraction Reagent (VWR Life Sciences). RNA extraction was carried out according to the manufacturer’s directions for adherent cell RNA extraction. After removing media, cells were washed once in 1 x PBS and 500µL RiboZol™ was added. Following this, samples were transferred into 1.5mL RNase free Eppendorf tubes and 100µL of pure chloroform was added. Samples were then mixed via inversion for 15 seconds to allow for phase separation before centrifugation at 12,000 g for 5 minutes. The top aqueous phase was transferred into new duplicate Eppendorf tubes and 250µL of 100% isopropanol was added. Tubes were mixed via inversion for 15 seconds and incubated at room temperature for 10 minutes. Samples were then centrifuged at 12,000 x g for 10 minutes to pellet RNA. Isopropanol was removed and pellets were washed via repeated washing steps in 70% (v/v) ethanol with centrifugation at 7,500 x g for 5 minutes. After three washes, ethanol was removed and RNA pellets were resuspended in 20μL of molecular grade H₂O and stored at -80°C until ready to use.

Quantification of RNA was carried out using a QuBit according to manufacturer’s instructions (Thermofisher). Following quantification of the RNA, a reverse transcriptase (RT) reaction mix was made using Applied Biosystem Reverse Transcriptase kit (Applied Biosystems, Thermofisher). For RT reactions, molecular grade H₂O was used to adjust the
concentration of RNA in the RT mix to create a standardised quantity of 1,000ng, 500ng or 200ng RNA for the RT reaction depending on the available concentrations of RNA. Adjusted RNA was added to the reaction mastermix containing RT buffer, random primers, dNTP mix, RNAse inhibitor and Reverse Transcriptase. Thermal cycler conditions for RT reaction were 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. The resulting cDNA generated from the RT reaction was then diluted to a standardised stock of 10ng/μL so that all samples and experiments could be accurately compared using the same concentration of total cDNA.

For qPCR analysis, 10ng of all cDNA samples were assayed in 20μL reactions by mixing with 2 x SYBR Green (Bio-Rad) with 0.25μM forward and 0.25μM reverse primers specific to gene targets. Primer sequences for the assayed genes of interest (GOI) are detailed in Table 2.1. qPCR thermal cycler conditions are detailed in Table 2.2. The cycling parameters were pre-validated from previous work carried out by the Cultech research team.

RT-qPCR data was analysed using the ΔΔCt method. Statistical analysis for all RT-qPCR data was carried out on ΔCt values. Data are presented graphically after normalisation as mean fold change relative to the control following ΔΔCt calculation for clarity and visual effect. All graphs and statistical analysis for RT-qPCR carried out on ΔCt values can be found in the appendix.

Table 2.1. Primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Species</th>
<th>Target Gene</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (5’-3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>IL-1β</td>
<td>GAGGACATGAGCACCCTTCTTT</td>
<td>GCCTGTAGTGCAAGTTGTCTAA</td>
<td>Eurofins, UK</td>
</tr>
<tr>
<td>Mouse</td>
<td>IL-6</td>
<td>GAGGATACCACCTCCAACAGACC</td>
<td>AAGTGCAATCGTCTGTCATACA</td>
<td>Eurofins, UK</td>
</tr>
<tr>
<td>Mouse</td>
<td>IL-10</td>
<td>TAAGGTTACCTGGTGCTCCA</td>
<td>GAGAATCGATGACAGGCCCC</td>
<td>Eurofins, UK</td>
</tr>
<tr>
<td>Mouse</td>
<td>β-Actin</td>
<td>ACCACCCGACACACGTCGCAAT</td>
<td>CACACCCCTGGTGCCAGAGGCCCAGATG</td>
<td>Eurofins, UK</td>
</tr>
</tbody>
</table>
Table 2.2. qPCR thermal cycle conditions used to assess gene expression.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 secs</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>15 secs</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 secs</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>5 secs</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>5 secs</td>
</tr>
<tr>
<td>Post Reaction</td>
<td>10</td>
<td>∞</td>
</tr>
</tbody>
</table>

2.7 Animal cohort

Under the authority of Home Office license P8159A562, 20 WT C57BL/6 male mice were purchased from Charles River and delivered to Biological Services (BIOSERV) at Cardiff School of Medicine. Mice (N=20) were housed in groups of 1 – 3 per cage in scatainers in a room at 21°C. Rooms were on a 12-hour light/dark cycle (7am-7pm). After one week to acclimatise in the BIOSERV facility, the mice were randomly assigned into groups of 10. The first group (N=10) had access to standard rodent chow (Teklad) with vehicle control powdered diluent Maltodextrose 20 (MD20) and water ad libitum. The other group (N=10) had access to a rodent chow diet (Teklad) supplemented with freeze-dried Lab4P probiotic mix and water ad libitum. All dilutions of probiotics were prepared in MD20 to align both the control and the probiotic feed. Lab4P probiotic was applied to the chow containing $1.4 \times 10^9$ CFU/g. Both control and Lab4P diets were mixed well before applying to cage hoppers. Mice were weighed at the start point (day 0), mid-point (day 7) and end point (day 14) of the two-week period. Average food and water intake were also calculated by weighing food pellets and water over a 24-hour period.

2.8 Mouse serum generation

Mice were culled via a Schedule 1 method (CO₂) and blood exsanguinated via cardiac puncture. Blood was allowed to clot naturally before being separated by centrifugation at 3000 x g for 5 minutes to generate serum which was removed and stored in 1.5mL Eppendorf tubes at -80°C until ready to use. Prior to use in experimentation serum was heat inactivated at 56°C for 30 minutes to destroy any complement activity that might confound data.
2.9 Mouse behavioural testing

Mice (N=20) were subjected to behavioral assessment at the start point (0 weeks) before being randomly assigned into groups fed either a control (N=10) or a Lab4P supplemented (N=10) diet. Mice were then subjected to behavioral assessment again at the end of a two-week treatment period. Initial acclimatisation of the behavioural arena was carried out for all mice on the day before testing was carried out. All behavioural testing was carried out without any noise to promote natural mouse behaviours. Both cohorts of mice were filmed using a GoPro Hero 5 camera for 10 minutes per test with 30-minute intervals. At these intervals, mice were placed back in their original housing. Behavioural arena layouts are shown in Figure 2.1. The behavioral arena (39 x 39 cm) was made of solid white plastic on all sides with one transparent panel at the front for observations. It was also lined with sawdust bedding material spread across the base to promote natural behaviours and to reduce stress in the mice (as previously identified from not using sawdust in previous work). GoPro Hero 5 cameras were plugged into mains and suspended by a solid plastic arm 39cm above the centre of the arenas to capture all movements by the mice. Mice were placed individually into the arena for testing. All objects used were similar in size and placed symmetrically on pre-marked places in the cage as not to promote left or right preferences. Sets of “familiar” and novel objects used for start point and end point behavioral assessment were changed to ensure no prior exposure to the object which might bias object recognition. Testing was carried out in three phases:

**Phase 1: Open field testing (arena habituation)**

The first phase of testing was the habituation phase. Mice were allowed to move freely in an open field (arena with no objects) and filmed for a period of ten minutes.

**Phase 2: “Familiar phase” (object habituation)**

Mice were placed into the same arena with two identical objects on the left- and right-hand side of the arena. Mice were allowed to move and interact with the objects freely in the test arena and filmed once again for 10 minutes. During this phase the objects become “familiar” to the mice.
**Phase 3: Novel object recognition (NOR)**

In the final phase of testing, mice were placed in the arena with one now “familiar” object from Phase 2 on the right-hand side of the arena. The “familiar” left hand side object was removed and replaced with one novel object to test NOR. Mice were allowed to move freely and interact with the objects while being filmed over a period of ten minutes.

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**Figure 2.1 Layout of arenas used for 3 phases of behavioural testing.** (A) Arena layout used for phase 1 of behavioural testing with no objects present and inner and outer areas defined as “perimeter” and “centre”. (B) Arena layout used for phase 2 of behaviour testing (“familiar” object habituation) where two unseen objects (blue) were placed on two pre-defined marks on the left- and right-hand side of the arena. (C) Arena layout used for phase 3 of behaviour testing (novel object recognition) showing the replacement of the left “familiar” object with a novel object (red) on the left and the same “familiar” object on the right.

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**2.10 Use of EthoVision XT™ for behavioural analysis**

Analysis of animal behavior was carried out using EthoVision XT™ Software (Noldus). Files were imported from the GoPro Hero 5 devices into the EthoVision XT™ software. Templates were set up for open field, familiar object and novel object. For open field analysis, a mask was applied to the top-down videos to separate the arena into outer and inner areas (inner being 20% inward from the perimeter). For all object recognition analysis, masks were drawn around objects with an additional 2cm radius to track interest in this area and in the objects themselves. Arena settings were micro-adjusted appropriately for each individual video file where small discrepancies in setup may have altered the layouts of items slightly.
Mouse detection was carried out automatically and non-subjectively using the in-built algorithms of the software.

Using these parameters, this allowed for the generation of values for total distance the mouse moved, cumulative time the mouse spent moving and frequency of mouse movement. Definition of the “perimeter” and “centre” regions of the arena allowed for the generation of values for time spent in each defined region or the frequency that mice entered each defined region. For object recognition tests, automatic mouse detection set up comprised of a front directional overlay, allowing the software to identify the mouse’s nose and detect when it entered the previously defined object perimeters. These parameters allowed for the generation of values for frequency of mouse interaction with object and the amount of time each mouse spent at the objects. Discrimination ratios (DR) were calculated to identify novel object preference by assessing time spent interacting with the novel object minus time spent interacting with the “familiar” object, divided by the total time spent at both object during Phase 3 of testing. After completion of behavioral testing all videos were processed through the software to generate a data spreadsheet which was exported as a Microsoft Excel .csv file for further analysis.

2.11 Isolation of cells from mouse brains

Brains were harvested from mice fed a control (N=5) or Lab4P supplemented (N=5) diet over a two-week period. One hemisphere was taken for use in flow cytometry analysis and placed in sterile 1 x PBS. PBS was removed using a pipette and brain hemisphere was homogenised by mashing into small pieces via scissors and spatula. Tissue was then digested in 1mL of Collagenase buffer (reagents outlined in Table 2.3) which was kept at 4°C until ready to use. Tissue mixed with Collagenase buffer was incubated at 37°C in a rocking water bath for 45 minutes. Following digestion each mixture was passed through a 70μm cell strainer before passing through in 20mL of cold PBS to rinse the strainer and to suspend the mixture. Tissue mixture was then centrifuged at 250 x g for 5 minutes. Supernatant was removed before resuspending the pellet in 20mL of room temperature 37.5% Percoll solution (diluted with 1 x PBS). This was then centrifuged again at 250 x g for 5 minutes to induce separation of hepatocyte and lymphocytes. Supernatant was removed and pellet resuspended in 20mL of sterile 1 x PBS and centrifuged at 250 x g for 5 minutes. Finally, the pellet was resuspended in 250μL of sterile PBS.
Table 2.3. Composition of collagenase buffer for digestion of homogenised brain tissue.

<table>
<thead>
<tr>
<th></th>
<th>Volume for 10mL</th>
<th>Final Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>9.195mL</td>
<td>N/A</td>
<td>Gibco, UK</td>
</tr>
<tr>
<td>FBS</td>
<td>500μL</td>
<td>5% (v/v)</td>
<td>Gibco, UK</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl)</td>
<td>100μL</td>
<td>5mM</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>Collagenase D</td>
<td>200μL</td>
<td>1mg/mL</td>
<td>Roche, UK</td>
</tr>
<tr>
<td>Deoxyribonuclease I (DNAse I)</td>
<td>5μL</td>
<td>50μg/mL</td>
<td>Sigma, UK</td>
</tr>
</tbody>
</table>

2.12 Preparation of cells for flow cytometry

Following cell isolation, 100μL of cell solution was added to a round bottom 96-well microplate. Leftover cell solution was pooled into one control sample well and one probiotic sample well to act as controls. The plate was centrifuged at 450 x g for 3 minutes. The supernatant was then carefully removed and 25μL of Live/Dead™ fixable Zombie Aqua (Thermofisher, UK) diluted 1:50 in 1 x PBS was added to all wells. Cells were then incubated with the Live/Dead stain for 5 minutes in the dark at room temperature. Following incubation 100μL of FACS Buffer (FB) was added to each well to wash. The plate was then centrifuged at 450 x g for 3 minutes. Supernatant was removed again and 25μL of Fc block (anti-mouse CD16/32) was added to all wells and left to incubate in the dark for ten minutes at room temperature. Cells were washed with FB as before. Then 25μL of anti-mouse cell surface antibodies diluted in FB were added to respective wells. Antibodies were not added to the control and Lab4P pooled sample wells. The antibody panel used for this work is detailed in Table 2.4. Once antibodies had been added to the wells, they were left to incubate in the dark for ten minutes at room temperature. Cells were then washed in FB as before. After washing 25μL of 4% (w/v) Paraformaldehyde (PFA) (diluted with FB) was added to each well for fixation and the plate was left to incubate in the dark for ten minutes at room temperature. Cells were then washed and 100μL of FB added to each well.

Following fixation, the plate was centrifuged at 450 x g for 3 minutes and the supernatant removed. Intracellular staining permeabilisation wash buffer (Biolegend, UK) was used at 1 x (diluted 1:10 using sterile H2O) was added to the cells at 25μL and left to incubate in the dark for ten minutes at room temperature. Then 25μL of anti-mouse intracellular antibodies diluted in 1 x cell permeabilisation buffer (Table 2.4) were added to the wells and left to incubate in the dark for ten minutes at room temperature before washing. Finally, cells were resuspended in 100μL of FB before being processed via FACS. Data was acquired using
a Thermo Attune NxT polychromatic flow cytometer (ThermoFisher, UK). Files were exported as .fcs file for further analysis in FlowJo™ (Treestar).

### Table 2.4. Anti-mouse antibody panel used for staining of cells for flow cytometry. Antibody clone, associated fluorochromes, dilution of each antibody used and source are also detailed.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reactivity</th>
<th>Clone</th>
<th>Location</th>
<th>Fluorochrome</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Anti-mouse</td>
<td>30-F11</td>
<td>Cell Surface</td>
<td>Bv605</td>
<td>1:100</td>
<td>Biolegend, UK</td>
</tr>
<tr>
<td>CD68</td>
<td>Anti-mouse</td>
<td>FA-11</td>
<td>Cell Surface</td>
<td>PerCP</td>
<td>1:50</td>
<td>Biolegend, UK</td>
</tr>
<tr>
<td>CD11b</td>
<td>Anti-mouse</td>
<td>M1/70</td>
<td>Cell Surface</td>
<td>APC-Cy7</td>
<td>1:100</td>
<td>Biolegend, UK</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Anti-mouse</td>
<td>MP6-XT22</td>
<td>Intra-cellular</td>
<td>APC</td>
<td>1:100</td>
<td>Biolegend, UK</td>
</tr>
<tr>
<td>IL-6</td>
<td>Anti-mouse</td>
<td>MP5-20F3</td>
<td>Intra-cellular</td>
<td>PE</td>
<td>1:100</td>
<td>Biolegend, UK</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-mouse</td>
<td>JES5-16E3</td>
<td>Intra-cellular</td>
<td>FITC</td>
<td>1:100</td>
<td>Biolegend, UK</td>
</tr>
</tbody>
</table>

#### 2.13 Use of FlowJo™ software for flow cytometry analysis

FlowJo™ software (v10.8.1) (Treestar) was used for analysis of flow cytometry data. After upload of .fcs files to the FlowJo workspace it was possible to begin gating for cells of interest based on microglial cell surface markers. Microglial cell markers where chosen based on previous literature for microglial gating. Initially a lymphocyte population was determined to distinguish lymphocytes amongst other cellular debris. Next the cell surface antigen CD45, a widely used marker for determining microglial cells was used in further gating with the Live/Dead to determine a population of CD45+ cells (Hickman et al. 2013, Rangaraju et al. 2018). Finally, CD11b and CD68 were chosen due to being well known microglial specific cell surface markers (Chakrabaty et al. 2010, Holness and Simmons 1993). Following this gating strategy, a population of CD11b+ and CD68+ cells was established for use in further analysis (gating strategy is summarised in Figure 2.2).

Event counts generated by FlowJo™ were used to determine mean numbers of microglial populations in the brain of mice fed a control or Lab4P supplemented diet. Additionally, expression of cytokines TNF-α, IL-6 and IL-10 in the CD68+ and CD11b+ microglial populations were also assessed. Data are presented as contour plots (2%) or as histograms (normalised to mode). After individual analysis, data files were pooled into
“Control” and “Lab4P” groups using the concatenation tool embedded in FlowJo™, for further analysis. The same gating strategy was used as described above to determine event counts and cytokine expression using the pooled data files.

2.14 Statistical analysis

All statistical analyses were carried out using GraphPad™ (Prism). Statistical significance was set at $P \leq 0.05$. All data are presented as mean ± SEM. When comparing two un-matched groups normality testing was carried out using Shapiro-Wilk testing. If data were normally distributed an un-paired t-test was used. If not normally distributed a Mann-Whitney test to compare ranks was used. To compare means of datasets with more than two groups,
data was assessed for normality using Shapiro-Wilk testing. If data were normally distributed a normal one-way ANOVA was carried out to compare differences in means between groups. This was followed by post-hoc analysis using Dunnett’s multiple comparison test. If data were not normally distributed a Kruskal-Wallis ANOVA test was used to assess differences in means between groups. This was followed by post-hoc analysis using Dunn’s multiple comparisons test. Two-Way ANOVA was required in cases where it was required to test the effects of two variables at the same time. Where Two-Way ANOVA was carried out, post-hoc analysis followed using Sidak’s multiple comparison testing.
Chapter 3

*In vitro* assessment of the effects of probiotic conditioned media on BV-2 microglial cells
3.1 Introduction

The use of \textit{in vitro} cell culture has proved to be an invaluable tool in scientific research. Cellular monoculture allows for assays to be performed to assess genetic, metabolic and phenotypic changes that occur in response to a wide range of challenges.

Microglia play a key role in mediating the inflammatory response in AD. Gliosis has been shown to be an early event during the pathogenesis of AD, perpetuating widespread inflammation as the disease progresses (Craft et al. 2006, Kamphuis et al. 2012, Martin et al. 2017). Evidence suggests that suppression of microglial activity could help to improve the outlook of AD in patients (Spangenberg et al. 2016). Therefore, investigating the inflammatory processes driven by microglia \textit{in vitro} could prove helpful in elucidating the mechanisms behind their involvement in AD and subsequent disease associated inflammation. An advantage of \textit{in vitro} cell lines by comparison with \textit{in vivo} models, is that they provide a means of manipulating specific variables in a tightly controlled manner where the level of complexity is markedly less than in an \textit{in vivo} model, providing a quicker turnover in terms of data collection (Stansley et al. 2012).

A range of microglial cell lines exist that could be useful for this purpose, including the mouse N9, rat HAPI and human CHME-5 (Righi et al. 1989, Cheepsunthorn et al. 2001, Janabi et al. 1995). However, one of the most widely cited in the literature is the mouse BV-2 cell line (Timmerman et al. 2018). The cell line was originally derived from primary neonatal cells taken from the cerebral cortex of C57BL/J6 mice and transformed by the J2 retrovirus which carries the \textit{v-raf} and \textit{v-myc} oncogenes (Blasi et al. 1990). The BV-2 cell line has comparable functionality and phenotypic properties to \textit{in vivo} microglia and therefore can act as a useful \textit{in vitro} model in the context of AD (Henn et al. 2009, Stansley et al. 2012).

Previous evidence has suggested that dysbiosis of gut microbiota is linked to the development of AD (Cattaneo et al. 2016, Zhang et al. 2017). Since probiotics can replenish the gut microbiome and improve dysbiosis it is possible that they may confer a health benefit to AD patients via improving disease associated inflammation and changes in metabolism (Azm et al. 2018, Bonfili et al. 2020). Microglia are able to produce particular cytokines which contribute to the inflammatory response and have been found to be important in the context of AD. Three key cytokines produced by microglia as part of the inflammatory response in AD chosen for their relevance to this work include:
**IL-1β**

IL-1β plays a central role in the induction of the inflammatory response and is largely produced by macrophages (Taylor et al. 2005). Activation of microglial TLRs via PAMPs or DAMPs from inflammatory stimuli initiate IL-1β transcription and translation (Akira et al. 2006). Production of IL-1β protein is a tightly controlled process and requires two signals. Initially, the first signal is induced by inflammatory stimuli interacting with DAMPs which activate TLRs which then dimerise and initiate signaling via MyD88, leading to the transcription and translation of pro-IL-1β via the NF-κB pathway (Takeda and Akira 2005). This is a priming step which prepares the microglia to produce the mature IL-1β cytokine protein. Secondly, another signal is required, such as ATP, which can activate the P2X7R receptor. This allows the NLRP3 inflammasome to activate caspase 1, which in turn cleaves pro-IL-1β to convert it into its active form (Ferrari et al. 2006, Facci et al. 2014). Finally, active IL-1β release requires the presence of phospholipase C for secretion out of the cell (Andrei et al. 2004).

IL-1β is typically known to be pro-inflammatory and can mediate inflammatory pathways. After IL-1β production, it binds with the type I IL-1 receptor (IL-1R1) on the cell surface of immune cells such as B-cells, T-cells and neutrophils to initiate inflammatory signaling cascades involving the mitogen activated protein kinase (MAPK) pathway and the activation of transcription factors such as NF-κB (Dunne and O’Neill 2003). NF-κB is a transcription factor which is required for the control of a variety of cytokine genes, therefore IL-1β mediated production of NF-κB results in the production of more cytokines, including IL-6 and induces oxidative stress (Liu et al. 2017). This complex signaling cascade therefore results in the propagation of the inflammatory response. IL-1β itself has the ability to perpetuate the inflammatory response in the presence of itself via a positive feedback loop, therefore sustaining IL-1β gene expression levels (Dinarello et al. 1987, Schindler et al. 1990).

IL-1β is particularly involved in the mediation of neuroinflammation and has been found to be upregulated in MCI and AD patients (Forlenza et al. 2010). One study suggests that IL-1β mediated sustained activation of microglia in the hippocampal region of rats induced via LPS injection, resulted in reduced nervous transmission, a decrease in BDNF and a decline in memory (Tanaka et al. 2006). This is also supported by newer evidence which suggests that chronic IL-1β production in the hippocampus results in decreased BDNF function which may be a contributing factor to cognitive decline alongside neuronal death in AD (Carlos et al. 2017).
Evidence suggests that the downregulation of IL-1β may be beneficial in AD. Zhang et al. (2021) showed that IL-1β inhibition in an AD mouse model resulted in reduction of amyloid deposition and increased neuroprotection. Additionally, previous work has also shown that probiotic supplementation of rats injected with Aβ caused a reduction in IL-1β levels, among other cytokines (Mehrabadi and Sadr 2020). Therefore, as it is an important microglial mediator of inflammation in the context of AD, IL-1β was a key gene target in this work.

**IL-6**

IL-6 is a cytokine which has pro-inflammatory properties and is released by innate immune cells such as microglia. This also occurs in response to inflammatory stimuli such as PAMPs and DAMPs recognition by TLRs (Takeda and Akira 2005). IL-6 is largely under the control of the transcription factor NF-κB with evidence suggesting the presence of highly conserved NF-κB binding sites around the IL-6 transcriptional start site (Miyazawa et al. 1998). Canonical NF-κB signaling triggered by stimuli at TLRs is very important for mediating the transcription and translation of IL-6 and is therefore a key player in facilitating inflammation (Wang et al. 2014).

After transcription and translation, IL-6 is secreted and binds to IL-6 receptors on the cell surfaces of immune cells initiating classical IL-6 signaling (Heinrich et al. 2003). However, soluble IL-6 receptors (sIL-6R) also exist in the extracellular environment and can bind to free IL-6 to mediate trans-signaling by docking onto cell membranes (Peters et al. 1998). In each case after binding, the addition of the signal transducing subunit gp130 is required to induce the IL-6 signaling pathways; JAK/STAT and MAPK (Ohtani et al. 2000, Guschin et al. 1995, Costa-Pereira et al. 2014). The release of IL-6 from microglia also contributes to the support of the adaptive immune system including a type of lymphocyte known as CD4+ T-cells where IL-6 prevents apoptosis to ensure T-cell survival (Ayroldi et al. 1998).

IL-6 has been shown to play a role in controlling T-cell phenotypes. Normally, the BBB restricts the entry of T-cells into the brain, however in AD where the BBB is compromised these cells are able to pass through as part of the inflammatory process (Ferretti et al. 2016). A distinctive subset of lymphocytes that uniquely produce IL-17 are known as T-helper 17 (T17) cells and play a major role in inflammation (Ouyang et al. 2008). These are produced via differentiation of CD4+ T-cells facilitated by IL-6 and other cytokines such as TGF-β (Bettelli et al. 2006, Veldhoen et al. 2006). Additionally, JNK pathway associated phosphatase (JKAP) has also been implicated in mediating CD4+ differentiation and dysregulation of this protein.
linked to cognitive decline in AD (Zeng et al. 2021). T_{H}17 cells are upregulated in AD patients and are thought to perpetuate chronic inflammation which is associated with the disease (Saresella et al. 2010, Oberstein et al. 2018). T_{H}17 cells do this by generating pro-inflammatory cytokines such as IL-17, IL-21, IL-23 and IFN-γ which contribute to the recruitment of activated immune cells and further propagate inflammatory signaling (Korn et al. 2007, Chung et al. 2006).

Interestingly, TGF-β which has been implicated in T_{H}17 differentiation also plays a role upregulating another CD4^+ subpopulation known as T regulatory cells (T-regls), adding to the complexity of this arm of the immune response (Fantini et al. 2004). This is conflicting because T-regls under the control of transcription factor Foxp3 help to regulate and dampen the inflammatory response via release of IL-10 (Chen et al. 2003, Hill et al. 2007). Evidence shows that a reduction in T-regls in APP/PS1 AD mouse models contributes to AD cognitive decline and prevented microglial recruitment to amyloid plaques suggesting they are vital for clearance of AD pathology (Dansoko et al. 2016). Therefore, if probiotics do have the ability to reduce IL-6 gene expression this could also help to diminish chronic disease associated inflammation by influencing the balance of CD4^+ differentiation in response to disease by either suppressing the differentiation of CD4^+ into T_{H}17 cells and promoting the differentiation of CD4^+ T-regls via TGF-β signaling. This makes IL-6 a target gene of interest in the context of AD and microglial mediated inflammation.

**IL-10**

IL-10 is an anti-inflammatory cytokine produced by a range of immune cells, including CNS microglia (Ledeboer et al. 2002). Evidence suggests that IL-10 has the ability to inhibit the pro-inflammatory NF-κB pathway which results in an anti-inflammatory effect (Driessler et al. 2004). Since chronic inflammation is associated with AD due to the development of pathology and the increase in activated microglial, IL-10 has been shown to play a potentially important role in reducing microglial activation (Cianciulli et al. 2015). However, contrary to this there is evidence to suggest that AD mouse models crossed with IL-10 knock out mice showed better cognition, thought to be due to better Aβ plaque clearance via microglia (Guillot-Sestier et al. 2015). Therefore, despite these links between IL-10 and AD, our understanding of the full effects of the role of IL-10 in AD does remain unclear at present.

Evidence suggests that probiotic supplementation may be able to help upregulate IL-10 production providing anti-inflammatory effects (Karthikeyan et al. 2012, Jensen et al. 2017).
Particularly, studies have shown that probiotic metabolites such as SCFAs may be able to promote populations of T_{H1} cells resulting in higher levels of IL-10 production (Sun et al. 2018). Therefore, assessing the effect that Lab4b and Lab4P may have on IL-10 gene expression in microglial cells is of interest in this present study.

Lab4 probiotic CM has previously shown to have neuroprotective properties in the context of AD (Michael et al. 2019). Additionally, previous work carried out at Cultech has shown that Lab4 supplementation in 3xTg mice improves gene expression of inflammatory cytokines, possibly by reducing levels of microglial activation (communicated by Dr Tom Webberley). Microglia are involved in crosstalk with neurons and are closely associated with the progression of AD via mediation of disease associated inflammation. Therefore, an investigation into the effects that Lab4b and Lab4P have on microglial cell health and cytokine gene expression may help to elucidate the mechanisms by which they contribute to neurodegeneration.

Therefore, the aims of the work presented in this chapter are as follows:

1) Determination of suitable experimental parameters to assess the impact of Lab4b and Lab4P probiotic CM on BV-2 cells.
2) To determine the response of BV-2 cells following stimulation with Lab4b and Lab4P probiotic CM in the absence of any other challenge.

These aims were achieved by establishing appropriate seeding numbers and measurement of BV-2 cell viability over different incubation periods and by assessing BV-2 cell viability and levels of gene expression of key microglial cytokine target genes after stimulation with Lab4b and Lab4P CM.
3.2 Results

3.2.1 Determining optimum conditions for *in vitro* experimentation with BV-2 cells

The BV-2 cell line was chosen as an appropriate cell culture model for the investigation of microglial health. CV assay (as described in Chapter 2.2) was used to determine the most appropriate number of BV-2 cells to seed wells for future experiments. To do this BV-2 cells were seeded at different densities and plated per well (area of 0.32cm²) in 96-well plates to assess cell viability over two timepoints (Figure 3.1). Results shown have been normalised relative to the control (0 cells per well) to account for background noise.
Figure 3.1. Assessment of BV-2 cell viability over time at different seed densities.
BV-2 cells were seeded into 96 well plates at increasing cell density and grown with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours (A) or 48 hours (B) to determine an optimum cell density for future BV-2 cell assays. Cells were processed using Crystal Violet assay to assess viability. (C) Datasets were compared to assess changes between timepoints. Cell viability was measured using Crystal Violet assay. Results shown have been normalised relative to the control (0 cells per well) to account for background noise. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included (A and B) One way ANOVA, followed by post hoc Tukey’s multiple comparisons test and (C) two-way ANOVA, followed by post hoc Sidak’s multiple comparisons test. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.
24 Hours

After a growth period of 24 hours in standard cell culture medium containing 10% (v/v) FBS and 100 U/mL P/S, assessment by CV showed a significantly greater absorbance at the 50,000 cell density compared to at 10,000 cells \( (P=0.003) \), 15,000 cells \( (P=0.006) \) and 25,000 cells \( (P=0.026) \) (Figure 3.1, A). There was no significant difference found between other cell numbers, although there was a trend for an increase in a linear manner.

48 Hours

After a growth period of 48 hours, significant differences in the absorbance of CV were found between 25,000 cells with 10,000 cells \( (P=0.003) \) and 15,000 cells \( (P=0.05) \). Significant differences were also found between 50,000 cells and 10,000 cells \( (P=0.0001) \) and 15,000 cells \( (P=0.001) \) (Figure 3.1, B).

Integration of datasets to compare both timepoints and cell densities was analysed via two-way ANOVA and showed both an increased absorbance with time (24 vs. 48 hours) from cell densities of 15,000 per well or greater (All \( P<0.05 \), Sidak’s multiple comparison test) and trends for increases in absorbance with cell density for both timepoints (Figure 3.1, C). From these data it was determined that 50,000 cells per well grown for 24 hours before the start of experimental conditions was a suitable cell density for use in downstream BV-2 cell experiments. Although these assays showed that BV-2 cells were viable up to a 48-hour growth period it was decided to select a 24-hour initial growth period to prevent cells from becoming over confluent or dying. Additionally, choosing a 24-hour growth period reduced the risk of reaching the upper limits of spectrophotometer absorbance when cells were subjected to experimental timepoints such as 4, 8 and 16-hour post initial 24-hour growth period.

3.2.2 Assessment of BV-2 cell viability in response to Lab4b and Lab4P probiotic conditioned media

After determining appropriate cells densities for BV-2 cell experimentation in vitro, the next step was to establish a suitable concentration of probiotic CM for use in downstream BV-2 assays. BV-2 cells were plated at 50,000 cells per well and incubated with increasing
concentrations of Lab4b and Lab4P probiotic CM and cell viability measured using the CV assay (Figure 3.2).

**Figure 3.2** BV-2 cell viability in response to Lab4b and Lab4P probiotic conditioned media. 50,000 BV-2 cells per well were grown in 96 well plates with DMEM (10% (v/v) FBS and 100 U/mL P/S) for a 24-hour growth period. Cells were then incubated under control conditions (DMEM, 0% CM, 100 U/mL P/S), a positive control of DMEM (10% (v/v) FBS, 100 U/mL P/S) (not shown) or increasing concentrations of Lab4b (A, B) or Lab4P (C, D) probiotic conditioned media for either 4 or 16 hours. Crystal Violet assay was used to assess cell viability. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). All data was tested for normality using Shapiro-Wilk testing. Statistical tests included (A, B and C) Ordinary One-Way ANOVA and followed by post hoc Dunnett’s Multiple Comparison Testing and (D) Kruskal-Wallis test followed by post hoc Dunn’s Multiple Comparison Testing. * = P≤0.05,
This was carried out to determine how increasing concentrations of Lab4b and Lab4P CM affected BV-2 cell viability in order to choose an appropriate CM concentration for use in further assays.

No significant differences in BV-2 cell viability were found between any of the groups at the 4-hour timepoint stimulated with Lab4b or Lab4P when compared to the control (Figure 3.2, A, C) (One-Way ANOVA and post-hoc multiple comparisons testing).

However, when BV-2 cells were incubated with Lab4b for 16 hours, a significant increase in mean absorbance was recorded following stimulation with 50% (v/v) CM in comparison with the control (Figure 3.2, B) \( (P=0.04, \text{ post-hoc multiple comparison testing}) \). No other significant differences were found. Additionally, when BV-2 cells were incubated with Lab4P for 16 hours significant differences between groups were found (Figure 3.2, D) \( (P=0.024, \text{ Kruskal-Wallis test}) \). Further analysis revealed significant differences between the control and 25% (v/v) Lab4P CM \( (P=0.018) \) and between the control and 50% (v/v) Lab4P CM \( (P=0.23) \) (post-hoc multiple comparison testing).

Based on these data it was established that an increase in Lab4b and Lab4P CM concentration over 4-hour or 16-hour period had no detrimental effects on BV-2 cell viability as measured by CV assay. It was therefore decided that the 50% (v/v) probiotic CM could be used as a suitable experimental condition for both Lab4b and Lab4P CM in downstream BV-2 and probiotic CM assays without having any negative effects on the cell line.

### 3.2.3 Assessment of changes in BV-2 gene expression in response to Lab4b and Lab4P conditioned media

After establishing 50% (v/v) as an appropriate CM concentration for use in downstream BV-2 cell experiments, this dose of CM was then applied to BV-2 cells over a period of either 4 hours (Figure 3.3) or 16 hours (Figure 3.4) following a 24-hour growth period and the cell response measured via RT-qPCR analysis. This was done to determine if treatment with probiotic CM resulted in alterations in target gene expression. Gene targets selected for these analyses included IL-1β, IL-6 and IL-10 with β-Actin being used as a housekeeper gene.

These present data are shown graphically as gene expression relative to the control (DMEM, 0% CM, 100 U/mL P/S) which has been assigned an arbitrary value of 1 for clarity and visual effect. Statistical analysis of differences in gene expression have been carried out on ΔCt values and not the relative mean fold change data. The raw ΔCt data that were used
for statistical analysis to generate the P-values can be found in the appendix (Figure A1 and Figure A2).
Figure 3.3. Assessment of BV-2 expression of target genes in response to 4-hour treatment with 50% (v/v) probiotic conditioned media (CM). BV-2 cells were grown in 24 well plates at 300,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before incubation with Lab4b (A,B,C) or Lab4P (D,E,F) probiotic CM for 4 hours. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as mean fold change relative to the control calculated via the ΔΔCt method. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical analysis shown were carried out on ΔCt values using unpaired t-test. * = P≤0.05, *** = P≤0.001.
Following a 4-hour incubation of BV-2 cells with 50% (v/v) Lab4b CM, RT-qPCR analysis showed that there was a significant increase in IL-1β gene expression (Figure 3.3 A) ($P=0.026$, unpaired t-test, mean fold change of 988) and IL-6 gene expression (Figure 3.3 B) ($P=0.046$, unpaired t-test, mean fold change of 42). However, there was no significant increase in IL-10 gene expression (Figure 3.3 C) (mean fold change of 2.7).

Following a 4-hour incubation of BV-2 cells with 50% (v/v) Lab4P CM, analysis of gene expression showed there was a significant increase in IL-1β expression (Figure 3.3 D) ($P=0.004$, unpaired t-test, mean fold change of 79). There was also a significant increase in IL-6 gene expression (Figure 3.3 E) ($P=0.028$, unpaired t-test, mean fold change of 8.6). No significant increase in IL-10 gene expression was seen in comparison with the control (Figure 3.3 F) (mean fold change of 2.3).
Figure 3.4. Assessment of BV-2 expression of target genes in response to 16 hour treatment with 50% (v/v) probiotic conditioned media (CM). BV-2 cells were grown in 24 well plates at 300,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before incubation with Lab4b (A,B,C) or Lab4P (D,E,F) probiotic conditioned media for 16 hours. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as mean fold change relative to the control as calculated via the ΔΔCt method. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical analysis shown were carried out on ΔCt values using unpaired t-test. * = P≤0.05, ** = P≤0.01, **** = P≤0.0001.
Lab4b 16hr

Following a 16-hour incubation of BV-2 cells with 50% (v/v) Lab4b CM, gene expression analysis showed that there was a significant increase in IL-1β gene expression (Figure 3.4 A) ($P=0.0056$, unpaired t-test, mean fold change of 1168) and IL-6 gene expression (Figure 3.4 B) ($P=0.039$, unpaired t-test, mean fold change of 4016). There was also a significant increase in IL-10 gene expression (Figure 3.4 C) ($P=0.025$, unpaired t-test, mean fold change of 15).

Lab4P 16hr

Following a 16-hour incubation of BV-2 cells with 50% (v/v) Lab4P CM, RT-qPCR analysis showed that there was a significant increase in IL-1β gene expression (Figure 3.4 D) ($P=<0.0001$, unpaired t-test, mean fold change of 315) and IL-6 gene expression (Figure 3.4 E) ($P=0.062$, unpaired t-test, mean fold change of 35). However, there was no significant increase in IL-10 gene expression (Figure 3.4 F) ($P=0.35$, unpaired t-test, mean fold change of 6.8).
3.2.4 Effects of low concentrations of Lab4b and Lab4P probiotic conditioned media on BV-2 cells

Following the initial CM assays, it was decided to assess the response of BV-2 cells to lower concentrations of probiotic CM to determine if the concentration of 50% (v/v) Lab4b and Lab4P CM was giving rise to a large levels of pro-inflammatory gene expression. In this experiment, concentrations ranging from 0.5% (v/v) to 5% (v/v) of Lab4b and Lab4P were applied to BV-2 cells over a period or either 4, 8 or 16 hours. BV-2 cell response to stimulation was assessed using CV assay for cell viability and RT-qPCR for gene expression (both as described in Chapter 2.2 and 2.6).

Crystal violet for assessment of BV-2 cell viability

Lab4b 4, 8 and 16hr

CV assays showed no significant differences in BV-2 cell viability after stimulation with low concentrations of Lab4b after a 4 hour and 8-hour incubation (Figure 3.5 A and B). However, CV did show that there was a significant difference in BV-2 cell viability at the 16-hour timepoint (Figure 3.5 C) (Ordinary One-Way ANOVA, $P=0.0054$). This prompted further analysis which showed a significant increase in BV-2 cell viability when cells were stimulated with 5% (v/v) Lab4b probiotic CM for 16 hours in comparison with the control ($P=0.031$, Dunnett's Multiple Comparison Test). No other significant multiple comparisons were found.

Lab4P 4, 8 and 16hr

CV assays revealed no significant differences in BV-2 cell viability when cells were stimulated with Lab4P at all three low range concentrations over 4-, 8- and 16-hour incubation periods (Figure 3.5 D, E and F) (Ordinary One-Way ANOVA).
Figure 3.5. Assessment of BV-2 cell viability in response to 4-, 8- and 16-hour treatment with low concentrations of Lab4b and Lab4P probiotic conditioned media (CM). 50,000 BV-2 cells per well were grown in 96 well plates with DMEM (10% (v/v) FBS and 100 U/mL P/S) over a 24-hour growth period. Cells were then incubated with a control (DMEM, 0% CM, 100 U/mL P/S), a positive control (DMEM (10% (v/v) FBS, 100 U/mL P/S) (not shown) or increasing concentrations of Lab4b (A, B, C) or Lab4P (D, E, F) probiotic conditioned media for 4, 8 or 16 hours. Crystal violet assay was used to assess cell viability. Values are shown as a percentage increase of absorbance relative to the control. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Following normality testing via Shapiro-Wilk testing, statistical tests included Ordinary One-Way ANOVA followed by Post hoc Dunnett’s Multiple Comparisons Testing (B, C, D, E and F) or Kruskal-Wallis test followed by Post hoc Dunn’s Multiple Comparisons Testing (A). ** = P≤0.01.
**RT-qPCR analysis for gene expression**

As before these data are shown as gene expression relative to the control (DMEM, 0% CM, 100 U/mL P/S) which has been assigned an arbitrary value of 1 for clarity and visual effect (Figure 3.6). Statistical analysis of differences in gene expression have been carried out on ΔCt values and not the relative gene expression data. The ΔCt data that were used for statistical analysis to generate the P-values for these can be found in the appendix (Figure A3).
Figure 3.6. Assessment of BV-2 expression of target genes in response to 4-, 8- and 16-hour treatment with low concentrations of Lab4b and Lab4P probiotic conditioned media (CM). BV-2 cells were grown in 24 well plates at 300,000 cells per well with DMEM and 100 U/mL P/S) for 24 hours before incubation with a low range of Lab4b (A, B, C) or Lab4P (D, E, F) probiotic conditioned media concentrations. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results shown as mean fold change relative to the control (0% CM). N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests were carried out on ΔCt values and included Ordinary Two-Way ANOVA followed by Dunnett’s Multiple Comparison Post Hoc Test for all graphs. * = P≤0.05, ** = P≤0.01, *** = P≤0.001, **** = P≤0.0001.
Lab4b

Analysis showed that the CM concentrations used had a significant effect on gene expression levels of IL-1β (Figure 3.6 A) \((P<0.0001, \text{Two-Way Anova})\), IL-6 (Figure 3.6 B) \((P<0.0001, \text{Two-Way ANOVA})\) and IL-10 (Figure 3.6 C) \((P=0.014, \text{Two-Way ANOVA})\). Stimulation of BV-2 cells with a low concentration of 0.5% (v/v) CM Lab4b over 4-hours caused a significant increase in IL-1β gene expression by comparison with 0% (v/v) CM control (Figure 3.6 A) \((P=0.002, \text{post-hoc Dunnett’s multiple comparisons, mean fold change of 68})\). Stimulation of BV-2 cells with 5% (v/v) Lab4b CM over 4-hours also caused a significant increase in IL-1β gene expression (Figure 3.6 A) \((P<0.0001, \text{post-hoc Dunnett’s multiple comparisons test, mean fold change of 771})\). 0.5% (v/v) and 5% (v/v) Lab4b CM also caused a significant increase in IL-1β gene expression after 8 hours in comparison with the control (Figure 3.6 A) \((P=0.0064, \text{post-hoc Dunnett’s multiple comparison test, mean fold change of 30})\) \((P<0.0001, \text{post-hoc Dunnett’s multiple comparison test, mean fold change of 244})\). In addition, there was a significant change in IL-1β gene expression after BV-2 stimulation with 5% (v/v) Lab4b CM over a 16-hour period (Figure 3.6 A) \((P=0.0006, \text{post-hoc Dunnett’s multiple comparison test, mean fold change of 92})\).

There was a significant increase in IL-6 gene expression when BV-2 cells were treated with 0.5% (v/v) Lab4b CM and 5% (v/v) over a 16-hour incubation period (Figure 3.6 B) \((P=0.01, \text{post-hoc Dunnett’s multiple comparison test, mean fold change of 7.5})\) \((P<0.0001, \text{post-hoc Dunnett’s multiple comparison test, mean fold change of 99})\). No other significant differences in gene expression were seen for IL-1β and IL-6 at any other Lab4b concentrations and timepoints in comparison with the 0% (v/v) CM control.

Lab4b at a concentration of 0.5% (v/v) stimulated an increase in IL-10 gene expression in BV-2 cells after a 16-hour incubation period (Figure 3.6 C) \((P=0.049, \text{Dunnett’s multiple comparisons test, mean fold change of 3.2})\). No other significant differences in IL-10 gene expression were found between all Lab4b low concentrations by comparison with the control across all three incubation timepoints (Two-Way ANOVA and Dunnett’s multiple comparisons post hoc testing).

Lab4P

No significant differences were seen in levels of BV-2 IL-1β, IL-6 or IL-10 gene expression upon comparison of cell treated with Lab4P CM to the control (Figure 3.6 D, E and
(Two-Way AVOVA and Dunnett’s multiple comparisons post hoc testing). While gene expression levels of IL-1β and IL-6 appear to be lower than when cells were treated with Lab4P compared to Lab4b, there does appear to be a great deal of variation within these relative mean fold change data. Notably, there is a lot of variation across values for IL-1β gene expression at 4 hours across the low range CM concentrations, particularly 0.05% (v/v) and 0.5% (v/v) (Figure 3.6 D). Variation was also seen across values for IL-6 gene expression at 4 hours at each low CM concentration (Figure 3.6 E).

3.3 Discussion

3.3.1 Summary of data

On the basis of the initial results, the parameters selected for in vitro testing with BV-2 cells were 50,000 cells per well (96 well plate) and the use of Lab4b and Lab4P at a concentration of 50% (v/v). It was determined that a 50% (v/v) concentration of CM stimulated the best level of cell viability after a 16-hour incubation via CV assay. This was due to the 50% (v/v) CM concentration in both Lab4b and Lab4P showing between 60-80% greater cell survival relative to the control (Figure 3.2 B and D). This therefore deemed it to be a suitable concentration for use in downstream cell assays since it had no negative effects on cell viability. However, it is important to note that while the increase in mean absorbance at 16 hours suggests an increase in the number of cells present, this could not be confirmed. In future CV assays, it would be beneficial to count cells before and after treatment to determine whether increase in the mean absorbance correlates to increased BV-2 cell proliferation. From this work alone, only an assumption on cell viability in response to Lab4b and Lab4P probiotic CM can be made.

RT-qPCR revealed high levels of BV-2 gene expression of the pro-inflammatory cytokine IL-1β at both 4-hour and 16-hour incubation with Lab4b, showing almost a 1000-fold increase compared to the control (Figure 3.3 A and 3.4 A). Additionally, Lab4b induced a significant increase in IL-6 gene expression after a 4-hour and 16-hour incubation (Figure 3.3 B and 3.4 B). This suggests that Lab4b has the ability to induce a strong pro-inflammatory response in the BV-2 cells. Assessment of the effect of Lab4P on BV-2 cell gene expression showed lower levels of IL-1β and IL-6 gene expression compared with Lab4b. However, at both 4-hour and 16-hour timepoints there was an upregulation of IL-1β and IL-6 gene expression by comparison with the control suggesting that Lab4P may also induce a pro-inflammatory response at a concentration of 50% (v/v) (Figure 3.3 D and E, 3.4 D and E).
There was a significant increase in IL-10 gene expression seen when BV-2 cells were stimulated with Lab4P at 50% (v/v) for 4 hours (Figure 3.3 F) and with Lab4b at 50% (v/v) for 16 hours (Figure 3.4 C). This suggests that Lab4b and Lab4P may have the ability to induce IL-10 gene expression in BV-2 cells. Taken together these results prompted further investigation of the effects of both Lab4b and Lab4P CM on BV-2 gene expression in order to determine if the initial chosen concentration of 50% (v/v) CM was too high and inducing a strong inflammatory response.

To do this a range of lower concentrations were applied to BV-2 cells over three separate time points. Initial CV assay revealed at low concentrations of Lab4b and Lab4P probiotic CM had no effect on BV-2 cell viability, apart from when BV-2 cells were stimulated with 5% (v/v) Lab4b CM after a 16-hour incubation period, where cells showed a significant increase in cell viability in comparison with the control (Figure 3.5 C). As mentioned above, cell counting was not carried out to confirm if this increased absorbance correlated to an increase in cell proliferation. However, despite the absence of data on cell numbers the data from the CV assays do suggest that low concentrations of both Lab4b and Lab4P CM did not have a negative impact on cell viability.

BV-2 gene expression was also assessed and after applying a range of low CM concentrations to the BV-2 cells it was found that Lab4b still appeared to stimulate high levels of IL-1β gene expression in comparison with Lab4P. Comparison of IL-1β gene expression levels after application of Lab4b CM at 5% (v/v) incubated for 4 hours showed a mean fold change of 771 relative to the control (Figure 3.6 A). This differed markedly to that seen when Lab4P CM was applied at 5% (v/v) for 4 hours, with a mean fold change in IL-1β gene expression of 9 recorded (3.6 D). Therefore, 5% (v/v) concentration of Lab4b CM appeared to induce ~85-fold greater gene expression compared to Lab4P under the same conditions. This suggests that the difference in composition of the probiotic blends and respective probiotic by-products produced by the bacteria during anaerobic respiration may contain sources of unknown inflammatory stimuli. Both Lab4b and Lab4P probiotic CM are produced using the same method. However, the results in this chapter suggest that Lab4b CM induced a stronger inflammatory response than Lab4P at the same concentrations and timepoints. This suggests that the degree of inflammation may be caused by the unique probiotic by-products produced by each probiotic blend and that Lab4b may contain a greater source of by-products that may result in an inflammatory response.
As noted previously, there was a considerable amount of variation within the Lab4P data for IL-1β and IL-6 gene expression at the 4-hour timepoint during this assay and the reason for this is unclear (Figure 3.6 D and E). It is likely a true effect and may be representative of the timescale of activation of these genes in response to stimuli as IL-1β and IL-6 are activated around 4-6 hours post inflammatory stimulation. This could be one explanation as IL-6 gene expression levels appear to stabilise at the later 8- and 16-hour timepoint when activation of IL-6 gene expression typically begins to decline. Additionally, statistical analysis showed that despite using a concentration of Lab4b CM as low as 0.5% (v/v) and 5% (v/v), there were still significantly high levels of IL-1β gene expression at 4 hours and of IL-6 gene expression at 16 hours compared to the control, again suggestive of the presence of stimuli capable of inducing inflammatory gene expression at concentrations as low as 0.5% (v/v) Lab4b CM (Figure 3.5 A and B). Two-way ANOVA confirmed statistically that the difference in gene expression were dependent on the concentration of each probiotic applied.

In the case of anti-inflammatory cytokine IL-10, no significant increases in gene expression were seen during the low CM concentration assays. Further exploration of the effects of probiotic CM on IL-10 gene expression is required.

### 3.3.2 Induction of high levels of pro-inflammatory cytokines in response to Lab4b and Lab4P probiotic conditioned media

Results in this chapter suggest that probiotic CM, particularly Lab4b, was capable of significantly inducing IL-1β gene expression. It is unclear exactly why this is, however, there are some possible explanations to consider. The process of generating probiotic CM (fully described in Chapter 2.4) includes multiple centrifugal steps to remove probiotic bacteria from the growth medium. It also includes a filtration step where the media is passed through a 0.22μm filter before storing for use. These steps should ensure the production of a cell free supernatant leaving behind only probiotic bacterial by-products. However, after applying both Lab4b and Lab4P CM to BV-2 cells in vitro high levels of pro-inflammatory IL-1β were found suggesting the possible presence of inflammatory stimuli in the CM.

This may be caused by the presence of residual bacterial fragments not removed by filtration at 0.22μm (Kmonickova et al. 2012). Thus, it is possible that fragments of gram-positive bacterial cell walls leftover from the probiotic bacteria containing lipoteichoic acid (LTA) are still present after filtration and may be a contributing factor. LTA is primarily found
as a component of peptidoglycan layer of gram-positive bacterial cell walls and shares similar lipid-linked structural and chemical properties with LPS (Coley et al. 1972). LTA has been shown to activate key inflammatory receptors such as TLR2 found on microglial cells and plays a role in priming microglia for the release of pro-inflammatory cytokines such as IL-1β and IL-6 (Schröder et al. 2003, Tang et al. 2010). Since both Lab4b and Lab4P probiotic bacterial blends contain species such as Lactobacillus and Bifidobacterium which are gram-positive bacteria, this may be one explanation for the cause of the induction of the inflammatory response seen in BV-2 cells upon application of CM. There is also evidence to suggest that fragments of bacterial quorum sensing peptides may activate microglia in vitro which may also stimulate inflammatory cytokine production (Janssens et al. 2021). Given the sensitivity of microglia to inflammatory stimuli due to the abundance of TLRs found on the cell surface, it is perhaps unsurprising that if any inflammatory stimuli are present in the CM that microglia would respond via production of pro-inflammatory cytokines. This is not to say that probiotic CM would not be suitable for use in other in vitro cell lines that are not immune cells and do not possess a high level of TLRs or other inflammatory response receptors on the cell surface.

On the contrary, while IL-1β is renowned for its pro-inflammatory properties, it is possible that the upregulation of IL-1β expression in BV-2 cells stimulated by Lab4b may be a beneficial microglial priming response. In the context of AD, the upregulation of IL-1β has previously been shown via an in vitro study with neurons to alleviate the deposition of Aβ plaque through induction of Tumour Necrosis Factor α Converting Enzyme (TACE) (Tachida et al. 2008). TACE plays a role in APP processing and this upregulation shifts the balance of APP cleavage from β to α, producing soluble APP-α fragments instead of insoluble Aβ (Buxbaum et al. 1998). Therefore, the upregulation of IL-1β via probiotic stimulation may allow microglia to be primed and ready to carry out a rescue attempt which may prevent further Aβ deposition under in vivo conditions. Another study supports this, suggesting that a sustained increase in IL-1β induced by LPS injection may help to clear Aβ plaque via increased activation of microglia (Shaftel et al. 2007).

In summary, at this point the cause of the high levels of BV-2 IL-1β gene expression in response to probiotic CM is unclear. Due to time constraints during this project the CM was not tested for presence of endotoxins such as LTA, but this should clearly be assessed in future work and may prove to be useful in trying to understand the results of this chapter. A full analysis of Lab4b and Lab4P probiotic CM components could help to elucidate any by-
products present in the CM that may be contributing to the induction of an inflammatory response in the BV-2 cells would be a useful addition to this work.

3.3.3 Use of crystal violet assay for BV-2 cell viability

CV is a well-established and widely used technique for cell viability. CV is a triarylmethane dye that binds to molecules containing ribose such as DNA and is widely used as a colorimetric cell viability assay (Feoktistova et al. 2016). The premise of the assay is that any cells that are alive remain adhered to the surface and become stained, whereas cells that are dead lose adherence and shed off following washing steps, leaving only viable, stained cells behind. The CV assay was used during initial experiments with BV-2 cells to establish a practical BV-2 cell density and viability for downstream experiments over different timepoints and CM concentrations. This allowed for the determination of any cytotoxicity following application of probiotic CM.

Therefore, from this assay it is possible to determine volume of cells that are alive based on optical density. However, a key limitation of this assay is that it does not reveal any other information. From the CV assay alone, it is not possible to determine other factors of cell health such as changes in proliferation rate or cell metabolism which would be useful additional data to fully assess the impact of probiotic CM on BV-2 cells. A useful addition to the CV work completed in this thesis would be to count cells before and after treatment to determine cellular proliferation following CM stimulation.

There are alternatives to the CV assay which could be used instead to provide more information on microglial cell health and not solely cytotoxicity. For example, the popular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is an easy-to-use alternative colorimetric cell viability assay which can also be used to assess changes in cell metabolism (Mosmann 1983). However, the MTT assay is also light sensitive and may have negative effects when applied to eukaryotic cell types which could result in inaccurate cell viability readings (Lü et al. 2012).

Another commonly used colorimetric in vitro assay for cell viability is lactate dehydrogenase (LDH) (Decker and Lohmann-Matthes 1988, Stoddart 2011). Similarly, to the MTT assay this method is quick and relatively easy to use. One issue with the use of the LDH assay for this work is that probiotic bacteria such as Lactobacillus casei contain levels of LDH has the potential to interfere with the assay (Kim et al. 1991). Therefore, although a useful
method for assessing cell viability, the CV method was more suitable for work requiring the application of probiotic CM to cells.

3.3.4 Use and limitations of the BV-2 cell line

The BV-2 cell line is frequently cited in the literature and widely used as an in vitro model for microglia (Timmerman et al. 2018). They are ideal for use in in vitro work due to their high proliferation rate and being low maintenance in terms of upkeep. Therefore, they were chosen as an appropriate cell model for this work.

However, there are some limitations of the BV-2 cell line. One challenge is that BV-2 cells are semi-adherent and exist attached to the plastic surface of cell culture flasks and plates but also can exist in suspension. During this work it was found that this property can make their use in in vitro experimentation problematic. This was particularly evident when applying washing steps during assays which may wash away any loose cells or cells in suspension. To minimise the effect of losing cells during experimentation during the application of washing steps, great care was taken to slowly remove and replace PBS or media. Additionally, plates can be spun down before washing steps to reduce the chance of this being an issue and affecting results.

Also, cell lines can continuously proliferate often due to retroviral transformation, however this may lead to genetic and phenotypic drift over multiple generations which can cause problems during in vitro assays (Geraghty et al. 2014). Since the murine BV-2 cell line is an immortalised line to prevent drifting away from the original parent cell phenotype BV-2 cells were passaged no more than 15 times and replaced by cells frozen down at low passage numbers (between p1-4). In addition, BV-2 cells were monitored routinely for phenotypic changes using a microscope during passages.

Additionally, evidence from the literature suggests that the BV-2 cells also show genetic and phenotypic deficiencies in comparison with primary microglial cells. One study by Das et al. (2016) showed via full transcriptomic sequencing using RNA-seq that BV-2 cells show differences in transcriptomic signatures following LPS mediated activation compared to primary microglia (Figure 3.7).
This study showed that primary cells expressed hundreds of unique genes required for cytokine production, transcription factors and epigenetic regulation suggesting greater genetic and biological complexity compared to the BV-2 cell line where these genes are not expressed (Das et al. 2016). Interestingly, other RNA-seq data has also shown that BV-2 cells express high levels of genes required for progression of the cell cycle, but low levels of genes required for cell adhesion in comparison with primary microglial cells (He et al. 2018). This finding appears to underpin the phenotypic profile of these BV-2 cells including high proliferation rates and a semi-adherent nature. This contrasts with primary cells which are more adherent but grow at slower rates. The same study also found that in terms of cytokine production in
response to LPS, primary microglial cells showed greater levels of IL-1β gene expression whereas BV-2 cells significantly greater levels of gene expression of IL-6 (He et al. 2018).

Another issue that should be considered is that since the BV-2 cell line is neonatal in origin, it is likely that the cells will have lower levels of “adult” gene expression, lacking the expression and phenotypic profile gained with age and maturation (Timmerman et al. 2018). Thus, when studying these cells in the context of AD which is a disease primarily associated with aging, a lack of genetics gained during the aging process could prevent a full understanding of the genetics and molecular pathways that microglia are involved in AD.

Therefore, while the BV-2 cell line has been very useful for this preliminary in vitro experimentation, it does have limitations. For future work it may be advantageous to consider alternatives to the BV-2 microglial cell line with greater genetic complexity and relevance in study of neurodegeneration, such as primary cultured cells, induced pluripotent stem cells (iPSCs) and 3D models such as organoids.

**Primary cell culture**

Primary cells typically derive from neonatal mice and are taken directly from a specific tissue of interest such as the brain cortex before being grown in culture (Giulian and Baker 1986). Additionally, primary cells can be taken from the brains of adult mice (Lee and Tansey, 2013, Woolf et al. 2021). The use of adult mouse microglia may be more beneficial when investigating the role of microglia in AD since it is primarily a disease of aging. Additionally, primary cells could be taken from AD mouse models to generate in vitro cell populations with greater genetic diversity. This could also be useful for understanding the nuances of AD progression on an individual basis. As discussed previously, primary microglial cells have shown greater genetic complexity than the BV-2 cell line which could be more beneficial as a tool for understanding the mechanisms and molecular pathways that microglia are involved in during AD onset and progression (Das et al. 2016).

However, primary cells do also have their own limitations. They can be difficult to culture and maintain in comparison with standard cell lines. Evidence suggests that after removal from their niche in the brain they begin to alter expression of some genes in response, possibly contributing to phenotypic changes and making work with them challenging (Mizee et al. 2017). In addition, due to the lack of retroviral transformation primary cells will not continue to proliferate markedly unlike their cell line counterparts, often undergoing numerous
passages before reaching a senesce state (Hayflick and Moorhead 1961). This means that to replace primary cells that have reached senesce, cells would have to be taken regularly from neonatal mice. Also, there will be a limited number of cells available in the neonate brain which can be collected. This can make the process time consuming and expensive since it relies on a ready supply of mice at the right age to provide tissue. Due to these and other factors, the use of primary cells during this present study was not a viable option but should perhaps be considered for use in future work.

As a caveat, an important consideration when undertaking any animal work is how the research aligns with the 3Rs. In the case of using primary cells, it may be difficult to justify the maintenance of animals purely for the purpose of extracting cells when the use of transformed cell lines can eliminate the need for animals altogether. However, it could also be argued that since primary cells more accurately represent how cells behave in vivo they may be able to help refine the design of in vivo experiments better than cell lines.

**Induced pluripotent stem cells - 2D and 3D models**

In recent years iPSCs have become a popular model used for a range of in vitro experiments as an alternative to standard cell lines (Sirenko et al. 2014, Abud et al. 2017, Gao and Liu 2017). While mouse derived cell lines and animal models have proved to be invaluable in AD research, evidence suggests that it is difficult to interpret this work in the context of humans due to key differences in genetics between mice and humans (Burns et al. 2015, Friedmann et al. 2018).

Previous work has shown it is possible to generate human microglial iPSCs from fibroblasts for use in in vitro experimentation (Douvaras et al. 2017). Cells can then be supplemented with growth factors such as VEGF, SCF and CSF1 to encourage progenitor formation and maturation into microglial cells with specific microglial cell surface markers such as ionised calcium binding adapter molecule (Iba-1) (Douvaras et al. 2017). Such studies have also included taking donor cells from AD patients to characterise AD phenotypes and response to treatments on an individual basis (Kondo et al. 2013).

However, while the heterogenicity of iPSCs is advantageous it can also cause the introduction of chromosomal abnormalities which may lead to confounded results (Taapken et al. 2011, Mitsui et al. 2003, Chambers et al. 2003). Additionally, this a is newer area of work and to date the protocols published vary considerably in method and yield of cells (Douvaras
et al. 2017, Muffat et al. 2016). However, the use of microglial iPSCs does appear to hold great promise for future work investigating the complex role of microglial in AD and may be an option to consider in further study.

As work has progressed with iPSCs, so has the use of these cells in the production of 3D cell culture models such as organoids. Organoids are a relatively new type of 3D cell culture model used in vitro which closely mimic the in vivo environment of organs such as the brain. One study published showed the ability to generate organoids via directed cell growth that mimic specific brain regions of interest such as the hippocampus (Di Lullo and Kriegstein 2017). This could prove to be a useful tool in AD research and some 3D organoid models have already been generated. For example, Choi et al. (2014) developed an in vitro AD organoid model containing neuronal and glial cells by upregulating genes implicated in AD pathogenesis APP and PSEN1. These 3D models offer even more complexity than iPSCs alone via cell type heterogeneity within imitated organ microenvironments that could help to elucidate the crosstalk between cells such as neurons, microglia and astrocytes in neurodegenerative diseases.

### 3.3.5 Conclusion

The use of probiotic CM during these experiments has raised some important questions. Particularly, why CM appears to have such a stimulatory effect on pro-inflammatory cytokine gene expression in BV-2 microglial cells. Further, and in this respect, why Lab4b CM appears to outweigh the effects seen with Lab4P CM. Both Lab4b and Lab4P CM showed the ability to induce some IL-10 expression under 16-hour conditions, however warrants further investigation. It is likely that the different compositions of probiotic strains in Lab4b and Lab4P give rise to different probiotic by-products which may have differing effects on immunomodulation when applied in vivo. Ultimately, the bacterial components and metabolic by-products of each probiotic blend would need be investigated further, to elucidate why this is the case, possibly through NMR analysis.

Since in vivo microglial cells would not be exposed to probiotics and their by-products as directly as was the case in the in vitro cell assays of this chapter, it was decided that an alternative, more physiologically relevant supplement was required for testing in order to validate these results.
Chapter 4

Assessment of the effects of Lab4P probiotic diet on B57BL/6J (wild-type) mouse phenotype
4.1 Introduction

Previous data from both \textit{in vitro} and \textit{in vivo} studies suggest that probiotics may impact upon brain cell health and inflammation (Michael et al. 2019, Mehrabadi and Sadr 2020). This was also found to be the case when mice fed a diet supplemented with probiotics displayed altered behaviour and immunomodulatory potential (unpublished findings communicated by Dr Tom Webberley).

To determine whether responses observed in this current work are comparable and/or translational \textit{in vivo}, WT (C57BL/6J) mice were fed a diet supplemented with probiotic over a two-week period. Funding and time limitations restricted the use of both Lab4b and Lab4P, so only Lab4P was used as it demonstrated strong efficacy in the previous \textit{in vivo} results (communicated by Dr Tom Webberley). As described in Chapter 2.7, one group of mice (N=10) were fed normal chow supplemented with Lab4P probiotic over a period of two weeks while a control group (N=10) were fed a chow diet in parallel. Following the two-week diet, phenotypic and behavioral assessment was carried out as described in this chapter. Additionally, as part of this \textit{in vivo} work, serum was generated from the mice post-mortem. This was then used as a medium supplement for \textit{in vitro} work with BV-2 cells to validate the results seen in Chapter 3 using CM. Experimentation using this serum will be described in Chapter 5.

The aims of the work outlined here in Chapter 4 were:

1) To determine if the Lab4P probiotic modulates dietary intake and bodyweight.
2) To determine if the Lab4P probiotic impacts behavioural changes \textit{in vivo} in WT mice.
3) To determine if the Lab4P probiotic has an impact on the number of brain microglial cells and expression of target cytokines \textit{in vivo}.

To achieve these aims, food and water intake were monitored and endpoint body weights of the cohort were assessed. Manual morphometric analysis and digital assessment of mouse behaviour was carried out using specialist software EthoVision XT\textsuperscript{TM}. Additionally, flow cytometric analysis was carried out on homogenised brain tissue obtained from the control or Lab4P supplemented mice.
4.2 Results

4.2.1 Assessment of food and water intake of C57BL/6J fed control or Lab4P supplemented chow for two-weeks

During the two-week supplementation regimen, food and water intake were measured over a 24-hour period to determine if dietary intake was affected by probiotic supplementation (Figure 4.1). Differences in food and water weights were calculated per cage and divided by the number of mice in the cage to give an estimated intake per mouse for both the control group and the Lab4P supplemented group (Figure 4.1). No significant differences were found in food or water intake.

![Graphs showing food and water intake](image)

**Figure 4.1. Assessment of food and water intake in C57BL/6J wild-type mice fed a control or Lab4P supplemented chow diet for two-weeks.** Difference in weight of food (A) and water (B) at the start and end of a 24-hour period were analysed from the cages of mice fed a control or Lab4P supplemented diet over a two-week period. Data are expressed as the mean +/- standard error of the mean (SEM) and represent estimated intake per mouse based on weights measured per cage. Shapiro-Wilk followed by (A) Mann-Whitney test and (B) unpaired t-test.
4.2.2 Morphometric analysis of C57BL/6J wild-type mice fed a Lab4P supplemented chow diet for two weeks

Mice were weighed before, during and after the treatment period to determine whether diet had affected weight (Figure 4.2). There were no significant differences in weight between the two groups at any time point including both raw weight comparisons (Figure 4.2 A) or after calculation of percentage relative weight changes at the endpoint normalised to the start point (Figure 4.2 B).

Post-mortem organ weight analysis was also conducted to determine whether control or Lab4P treatment affected organ weight over the two-week period (Figure 4.3). No significant differences were found between raw brain, liver and spleen weights between the control and Lab4P supplemented groups were observed (Figure 4.3 A, B & C).
Figure 4.2. Body weight assessment of C57BL/6J wild-type mice supplemented with Lab4P in a chow diet for two weeks. Mice fed either a control chow or Lab4P supplemented chow diet were weighed over a two-week period (N=10 per group) (A). Weight differences were calculated as percentage end point weight gain for both groups (B). Control group (N=10), Lab4P group (N=10). Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included (A) Two-Way ANOVA and (B) following Shapiro-Wilk testing, unpaired t-test was used.

Figure 4.3. Control chow and Lab4P chow fed mouse post-mortem organ weights after a two-week period. Mice were fed either a control chow or Lab4P supplemented chow diet were weighed over a two-week period (N=10 per group). Post-mortem the brain (A), liver (B) and spleen (C) were weighed to assess impact of the probiotic supplemented diet. Data are expressed as the mean +/- standard error of the mean (SEM). Shapiro-Wilk and unpaired t-test for all graphs.
4.2.3 Behavioural assessment of C57BL/6J wild-type mice supplemented with Lab4P in chow diet for two-weeks

Probiotic supplementation has been shown to modulate memory, behaviour and stress responses in mice in both published studies and work carried out by Cultech Ltd. (communicated by Dr Tom Webberley). Therefore, it was decided to assess the effects of a Lab4P supplemented diet on groups of WT mice via novel object recognition (NOR) to identify any changes in memory and cognition. For all behavioural data, outliers defined as values that were 2 standard deviations away from the mean in each group of mice were removed.

Phase 1: Open field testing (arena habituation)

Mice were placed in an open field with no objects and were allowed to move freely over a period of 10 minutes. Open field testing was conducted at 0 weeks on a group of 20 untreated mice and at 2 weeks after random assignment of mice into two groups of 10, either fed a control or Lab4P supplemented diet. Mouse movement (Figure 4.4) and mouse preference for the perimeter of the field (Figure 4.5) were assessed initially.
Figure 4.4. Open field movement analysis of C57BL/6J wild-type mice supplemented with Lab4P added to chow diet over two-weeks. Mice were fed a control or Lab4P supplemented chow diet over a course of two-weeks (N=10 per group). Total distance travelled (A) and differences between the start and end point in total distance travelled (B) in the open field test were recorded (using GoPro cameras) and analysed (EthoVision XT™). Cumulative movement time (C) and frequency of movements (D) were also detected by EthoVision XT™ were also assessed. The difference in mouse movement before and after treatment was calculated (B). Cumulative time spent moving (C) and mobile frequency (D) were also assessed. Data are expressed as the mean +/- standard error of the mean (SEM). (A, C and D) One-Way ANOVA and Dunnett's multiple comparisons testing. (B) Unpaired t-test.
Figure 4.5. Open field perimeter analysis of C57BL/6J wild-type mice supplemented with Lab4P added to chow diet for two weeks. Mice were fed a control or Lab4P supplemented diet over a course of two-weeks (N=10 per group). Only “arena perimeter” data are presented as “arena centre” are the inverse of the same data and graphically redundant. Time spent in the field perimeter (A) was calculated. The frequency of the mice in the perimeter was recorded (B) and overall difference in percentage time spent in the field perimeter was calculated (C). Data are expressed as the mean +/- standard error of the mean (SEM). (A, B and C) One-Way ANOVA and Dunnett's multiple comparisons testing.
Assessment of WT mouse movement in an open field arena before and after the two-week probiotic supplementation period showed no significant differences in movement patterns between mice fed a control or Lab4P supplemented diet compared to before treatment (Figure 4.4 A, C and D) (One-Way ANOVA, Dunnett’s multiple comparisons testing).

The mean difference in distance moved by the mice at the start point compared to the end point was calculated (Figure 4.4 B). The mean of the control group showed a decrease in distance moved with a mean of -230 cm, while the mean of the Lab4P group showed an increase in distance moved with a mean difference of 181 cm. However, an unpaired t-test revealed that this difference in distance moved between the two treatment groups was not significant.

The preference for the arena perimeter was also assessed (Figure 4.5). No significant differences in total time spent in the arena perimeter and total frequency the mice entered the defined perimeter regions were found between the treatment groups and the untreated group (Figure 4.5 A and B). Percentage of time that each mouse spent in the perimeter of the arena was calculated before and after the two-week treatment period. Calculation of overall percentage time spent in the arena perimeter to normalise these data revealed that after the two-week treatment period there was a non-significant difference of 0.5% time spent in perimeter between the mice fed a control and Lab4P supplemented diet (Figure 4.5 C) (unpaired- t-test).

**Phase 2: Familiar object testing (object habituation)**

In phase 2, mice were habituated with two identical objects in order to confirm no arena preferences and to allow mice to become familiar with two identical objects. Mice were placed in the arena with two identical objects and were allowed to move freely over a period of 10 minutes before and after a two-week intervention period. Following analysis of videos with Ethovision XT™ software, mouse movement (Figure 4.6) and mouse interaction with the identical objects during the familiar habituation phase (Figure 4.7) were assessed.
Figure 4.6. Movement analysis of C57BL/6J wild-type mice supplemented with Lab4P added to a chow diet for two weeks in the familiar object habituation test. Mice were fed a control or Lab4P supplemented diet over a course of two-weeks (N=10 per group). Before and after supplementation, patterns of mouse movement in the arena were recorded including total distance moved by the mice (A). The difference in mouse movement at the endpoint compared to the start point was calculated (B). Cumulative time spent moving (C) and mobile frequency (D) were also assessed. Data are expressed as the mean +/- standard error of the mean (SEM). (A, C and D) One-Way ANOVA and Dunnett’s multiple comparisons testing. (B) Unpaired t-test.
Figure 4.7. Assessment of object exploration of C57BL/6J wild-type mice supplemented with Lab4P added to chow diet for two weeks in familiar object habituation test. Mice were fed a control or Lab4P supplemented diet over a course of two-weeks (N=10 per group). Before and after supplementation the frequency that mice explored the left (A) and right (B) objects were assessed, as was total time the mice spent at the left object (C) and right object (D). Data are expressed as the mean +/- standard error of the mean (SEM). One-Way ANOVA and Dunnett's multiple comparisons testing for all graphs.
Assessment of WT mouse movement during the familiar phase of behavioural testing before and after the two-week probiotic treatment period showed no significant differences in movement between control and Lab4P fed mice compared to untreated mice (Figure 4.6 A, C and D) (One-Way ANOVA, Dunnett’s multiple comparison test). After the two-week period difference in distance moved by the two groups of treated mice at the start and end point was calculated and showed no significant difference between the groups (unpaired t-test).

Object interaction was also assessed as part of the familiar field analysis to determine whether the mice had any preference for the left or right objects in the testing arena. (Figure 4.7 A, B, C and D). No significant differences were found upon comparison of control and Lab4P supplemented groups with the initial untreated group (One-Way ANOVA, Dunnett’s multiple comparisons test). Furthermore, overall time spent at the left and right objects by the untreated start group and treated control and Lab4P supplemented end point groups were graphed to determine any difference in time spent at each object (Figure 4.7 E). No significant differences in time spent at each object by the groups were found (One-Way ANOVA, Dunnett’s multiple comparisons test).

Discrimination ratios were calculated to determine left and right object preferences during phase 2 of testing. The mean discrimination ratio for left object preference in the untreated start point group was 0.42 (Figure 4.8 A). After the two-week treatment period the mean discrimination ratio for left object of the control group was 0.44, while in the Lab4P group it was also 0.44 (Figure 4.8 A). The right-hand object mean discrimination ratios for each group are the inverse of those stated for the left-hand object (Figure 4.8 B). Discrimination ratios for left and right object preference showed no significant differences upon comparison of untreated start point group with the control and Lab4P supplemented end point groups (One-Way ANOVA, Dunnett’s multiple comparisons test).
Phase 3: Novel object recognition testing

Finally, following mouse exposure to both the open arena (Phase 1) and the now “familiar” objects (Phase 2), mice were subjected to NOR testing where one of the identical objects was swapped with a novel object. Mouse movement in the arena with these objects (Figure 4.9) and NOR (figure 4.10) were assessed using EthoVision XT™.
Figure 4.9. Movement analysis of C57BL/6J wild-type mice supplemented with Lab4P added to chow diet for two weeks during novel object recognition testing. Wild-type mice were fed a control or Lab4P supplemented diet over a course of two-weeks (N=10 per group). Before and after treatment Novel Object Recognition (NOR) behavioural assessment was carried out. Mouse movement patterns in the novel object arena were recorded including total distance moved by the mice (A). The difference in mouse movement at the endpoint and start point of treatment was calculated (B). Cumulative time moving (C) and frequency of movement (D) were also assessed. Data are expressed as the mean +/- standard error of the mean (SEM). (A, C and D) One-Way ANOVA and Dunnett's multiple comparisons testing. (B) Unpaired t-test.
Figure 4.10. Analysis of object exploration of C57BL/6J wild-type mice supplemented with Lab4P added to chow diet for two weeks during novel object recognition testing. Wild-type mice were fed a control or Lab4P supplemented diet over a course of two-weeks (N=10 per group). Novel object interaction frequency (A) and familiar object interaction frequency (B) were assessed along with total time interacting with the novel object (C) and total time spent interacting with familiar object (D). Data are expressed as the mean +/- standard error of the mean (SEM). One-Way ANOVA followed by Dunnett’s multiple comparison test were used as statistical tests for all graphs. * = P≤0.05.
Assessment of WT mouse movement patterns in the NOR arena before and after the two-week probiotic treatment period was carried out (Figure 4.9 A, C and D). No significant differences in movement patterns were found between the untreated start group of mice and the end point control and Lab4P supplemented groups. After the two-week period, difference in distance moved by the two groups of treated mice at the start and end point showed no significant difference (unpaired t-test).

Mouse interaction with the novel object (Figure 4.10 A and C) and “familiar” object (Figure 4.10 B and D) were also assessed. Analysis of novel object interaction frequency showed a significant decrease in interaction in the endpoint Lab4P supplemented group compared to the untreated start group ($P=0.047$, Dunnett’s multiple comparison test) (Figure 4.10 A). No other significant differences between groups were found.

Discrimination ratios based on time spent at novel object were calculated part of the NOR analysis (Figure 4.11). The mean discrimination ratio based on time spent at novel object in the start point untreated group of mice was 0.57. The mean discrimination ratio for the end point group fed a control diet was 0.54, while the Lab4P supplemented mice were found to have a mean NOR discrimination ratio of 0.53. No significant differences were found in discrimination ratios between the start point untreated group and end point treated groups (One-Way ANOVA).
Figure 4.11. Discrimination ratios for object preference in the novel object recognition test. Wild-type mice were fed a control or Lab4P supplemented diet over a course of two-weeks (N=10 per group). Before and after treatment NOR was carried out. As part of the familiar phase testing discrimination ratios were calculated for left “novel” object preference at the start and end of the two-week period mice fed a control diet and mice fed a Lab4P supplemented diet. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included One-Way ANOVA followed by Dunnett's multiple comparisons testing.
4.2.4 Flow cytometric analysis of homogenised WT mouse brains

Confirmation of a live microglial population was carried out via gating for microglial specific cell surface markers (as described in Chapter 2.13). Following this, microglial event counts from the homogenised brains taken from groups of control and Lab4P supplemented mice were determined using FlowJo™ software (Figure 4.12).

![Figure 4.12](image)

**Figure 4.12. Flow cytometry analysis of populations of CD68$^+$ and CD11b$^+$ microglial cells from homogenised brains of control and Lab4P supplemented wild-type mice.**

Two groups of WT mice (N=5) were fed either a control or Lab4P probiotic treated diet for two weeks. Following treatment one brain hemisphere was removed, homogenised and stained for flow cytometry to assess impact of Lab4P on microglial cell number. (A) Microglial populations from Control (red) and Lab4P (green) treated brains were detected using microglial cell specific surface markers CD11b and CD68. Cells that were CD11b$^+$ and CD68$^+$ were determined to be microglial populations. (B) Graphs represent the mean percentage of CD11b$^+$/CD68$^+$ microglial events in each group and (C) mean microglial event count. N=5 per treatment group. Data are expressed as the mean +/- standard error of the mean (SEM). (B) and (C) Unpaired t-test.
Following the gating strategy for CD11b+ and CD68+ microglial populations, all samples were analysed in the same manner (Figure 4.12.A). The percentage of CD11b+/CD68+ microglial events in each mouse was determined by FlowJo™, as was the microglial event count. From these data it was possible to compare the mean percentages of CD11b+ and CD68+ cells in each group and the microglia event counts (Figure 4.12.B and C). In both cases, no significant differences were found in mean percentage of microglia and mean event counts (Shapiro-Wilk and unpaired t-test).

As part of the assessment of microglial populations, after gating for CD11b+ and CD68+ microglial cells, these cells were analysed for intracellular cytokine expression. Initially TNF-α expression was measured across all five mice in each group (4.13 A). Comparison of median fluorescence intensity (MFI) showed no significant differences between the control and Lab4P supplemented group (4.13 B) (Shapiro-Wilk and unpaired t-test).

The same analysis was carried out for IL-6 (Figure 4.14 A) and IL-10 (Figure 4.15 A). In both cases comparison of the MFI of each cytokine from control and Lab4P supplemented mouse brains showed no significant difference (Figure 4.14 B and 4.15 B).
Figure 4.13. Assessment of TNF-α expression in microglial populations from mice fed a control and Lab4P supplemented diet. Groups of WT mice (N=5) were fed either a control or Lab4P probiotic supplemented diet for two weeks. Following treatment one brain hemisphere was removed, homogenised and stained for flow cytometry in order to determine differences in inflammatory cytokines in control and Lab4P groups. (A) Frequency of TNF-α expression in groups of control or Lab4P supplemented mice. (B) Median fluorescence intensity of TNF-α. Data are expressed as the mean +/- standard error of the mean (SEM). (B) Shapiro-Wilk followed by unpaired t-test.
Figure 4.14. Assessment of IL-6 expression in microglial populations from mice fed a control and Lab4P supplemented diet. Groups of WT mice (N=5) were fed either a control or Lab4P probiotic supplemented diet for two weeks. Following treatment one brain hemisphere was removed, homogenised and stained for flow cytometry in order to determine differences in inflammatory cytokines in control and Lab4P groups. (A) Frequency of IL-6 expression in groups of control or Lab4P supplemented mice. (B) Median fluorescence intensity of IL-6. Data are expressed as the mean +/- standard error of the mean (SEM). (B) Shapiro-Wilk followed by unpaired t-test.
Figure 4.15. Assessment of IL-10 expression in microglial populations from mice fed a control and Lab4P supplemented diet. Groups of WT mice (N=5) were fed either a control or Lab4P probiotic supplemented diet for two weeks. Following treatment one brain hemisphere was removed, homogenised and stained for flow cytometry in order to determine differences in inflammatory cytokines in control and Lab4P groups. (A) Frequency of IL-10 expression in groups of control or Lab4P supplemented mice. (B) Median fluorescence intensity of IL-10. Data are expressed as the mean +/- standard error of the mean (SEM). (B) Shapiro-Wilk followed by unpaired t-test.
Due to the observation of low microglial event counts in individual samples, it was decided to concatenate the data into groups using the concatenation tool embedded in FlowJo™. This allowed for the pooling of five biological repeats into one group, subsequently increasing the number of microglial events and allowing for more accurate gating using the same gating strategy as described in Chapter 2.13.

Analysis of the CD11b⁺ and CD68⁺ microglial population from the pooled groups following gating (Figure 4.16 A) allowed for the determination of the percentage of CD11b⁺ and CD68⁺ microglia and a microglial event count (Figure 4.16 B and C). This was followed by determining populations of microglial cells expressing intracellular TNF-α, IL-6 and IL-10 (Figure 4.17 A, B and C). As before, MFI of each cytokine was determined using FlowJo™ for both the control and Lab4P supplemented groups (Figure 4.17 D, E and F).
Figure 4.16. Flow cytometry analysis of concatenated populations of CD68$^+$ and CD11b$^+$ microglial cells from homogenised brains of control and Lab4P supplemented wild-type mice. Two groups of WT mice (N=5) were fed either a control or Lab4P probiotic treated diet for two weeks. Following treatment one brain hemisphere was removed, homogenised and stained for flow cytometry to assess impact of Lab4P on microglial cell number. (A) Microglial populations were detected using microglial cell specific surface markers CD11b and CD68. Cells that were CD11b$^+$ and CD68$^+$ were determined to be microglial populations. (B) Graphs represent percentage of CD11b$^+$/CD68$^+$ microglial events in each group and (C) microglial event count.
Figure 4.17. Use of concatenated flow cytometry data to assess microglial cell populations positive for inflammatory biomarkers. Groups of WT mice (N=5) were fed either a control or Lab4P supplemented chow diet for 2 weeks. Following treatment one brain hemisphere was removed, homogenised and stained for flow cytometry in order to determine differences in TNF-α, IL-6 and IL-10 frequency in control and Lab4P groups. Data was concatenated to pool all 5 mice from each group into one data file. Frequency of CD11b+ and CD68+ microglial cells positive for (A) TNF-α, (B) IL-6 and (C) IL-10 was analysed. Fluorescence intensity for (D) TNF-α, (E) IL-6 and (F) IL-10 was determined using FlowJo™.
4.3 Discussion

4.3.1 Summary of data

This preliminary *in vivo* study aimed to assess the effects of a two-week high dose Lab4P supplemented diet compared with the effects of a control chow diet on 3-month-old healthy, WT mice.

During the treatment period mouse food and water intake was estimated by measuring the weight of food and water from each cage at the start and end of a 24-hour period (Figure 4.1 A and B). Analysis of these findings suggested that there were no significant differences in the food and water intake of both the control and Lab4P group. This was an encouraging finding which suggests that the addition of the probiotic to the chow was palatable, did not deter the mice from consumption and had no impact on appetite or thirst, providing confidence that the Lab4P group did receive the Lab4P supplemented diet.

The bodyweights of each mouse were measured at the start point, mid-point and end point of the two-week treatment period (Figure 4.2 A). It appeared that in both groups of mice weight gain was seen over the two-week period, however this was not significantly different. This weight gain is likely due to the mice being young and therefore not yet reaching a steady adult bodyweight. Despite the changes in bodyweight not being significantly different between the groups over the two-week period, there was a slightly lower but non-significant overall percentage weight gain in the Lab4P treated mice (Figure 4.2 B), in line with previous research carried out at Cultech (Michael et al. 2020, Michael et al. 2021). Additionally, upon culling the weights of the brain, liver and spleen from mice in each treatment group were weighed. No significant differences were found suggesting that the two-week treatment had little effect on the organ phenotype of mice in each treatment group.

To further assess the impact on the Lab4P supplemented diet, behavioural assessment was carried out on groups of WT at the start (untreated, N=20) of the two week period. Mice were then randomly assigned into groups either fed a control or Lab4P supplemented diet for two-weeks. Following treatment, behavioural assessment was carried out again on the two groups of treated mice. Before and after the treatment period, mice were subjected to three phases of behavioural assessment. All testing of mouse movement patterns across all three phases of behavioural testing revealed that there were no significant differences found in movement between the start point untreated and the endpoint control or Lab4P supplemented groups (Figure 4.4, 4.6 and 4.9). This suggests that the Lab4P supplemented diet did not have any impact on mouse locomotor activity.
In the initial phase of testing (Phase 1 – arena habituation) the time the mice spent in the arena perimeter and the frequency they entered the defined perimeter region was assessed and revealed no significant differences between the untreated and treated groups (Figure 4.5). This suggests that there was no evidence of a preference for the inner or outer regions of the arena following supplementation.

Analysis of the second phase of testing (Phase 2 – object habituation) for interaction with the identical left and right objects revealed that there were no significant differences for each object between the start-point untreated and endpoint treated groups (Figure 4.7 A, B, C and D). To confirm that the mice did not spend a greater amount of time at either object, overall time spent at each object by the groups were assessed (Figure 4.7 E). This showed that there was no significant difference in left or right object interaction within the control and Lab4P supplemented groups. However, calculation of discrimination ratios based on time spent at each object during phase 2 of testing did suggest a preference towards the object on the right-hand side in both the control and Lab4P supplemented mice. Additionally, this preference appeared to be the same at the start point and end point of the testing period. It is unclear why this is the case, especially since testing of overall time spent at each object during phase 2 did not show any significant differences.

In the last phase of testing (Phase 3 – NOR), assessment of mouse interaction with the novel and “familiar” objects was carried out (Figure 4.10). Analysis of frequency of interaction of mice with the novel object did show that there was a significant decrease in interaction with the novel object in comparison with the untreated start point group (Figure 4.10 A). This suggests that the mice fed a Lab4P supplemented diet may not have been as interested in the novel object after the two-week treatment period. No other significant differences in object interaction were found. Finally, discrimination ratios were calculated to determine mouse object preferences during phase 3 testing. The discrimination ratios for all groups suggest a slight preference for the novel object (>0.5). No significant differences in discrimination ratios for novel object recognition were found between the untreated start point and control or Lab4P supplemented end point groups. Overall, behavioural testing did not show any significant changes in mouse activity, anxiety or object interaction, suggesting that the Lab4P supplemented diet had little effect on the memory, cognition and motor activity of the mice after two-weeks of supplementation.

Finally, flow cytometry was carried out which revealed populations of microglia as defined by positive staining for CD68 and CD11b present in samples of homogenised brain.
from control and Lab4P supplemented mice. From these data, mean event counts of CD68\(^+\) and CD11b\(^+\) cells revealed a trend of greater incidence of microglial cells in the Lab4P supplemented mouse brain samples. However, this difference in mean event counts between the two groups was not significantly different. Additionally, assessment of intracellular cytokine expression levels via MFI revealed no significant differences in cytokine expression between the groups. Due to overall low levels of CD68\(^+\) and CD11b\(^+\) microglia as evidenced by the event counts determined in each group, it was decided to assess these data using the concatenation tool embedded in FlowJo\(^{TM}\). Concatenation of the data files allowed the merger of all five individual sample files from each treatment group into one file in order to increase the microglial event count and provide more accurate gating of cell populations. The result from these concatenated data suggests that there may be a greater number of CD68\(^+\) and CD11b\(^+\) microglial cells present in the brains of mice supplemented with Lab4P based on the event counts, however statistical analysis could not be carried out to confirm this. Additionally, expression levels of all three cytokines were found to be low, possibly as a result of the low event counts. This part of the experimentation has been a preliminary exploration into the use of flow cytometry for assessment of microglial populations and cytokine expression in control and Lab4P supplemented mouse brains.

4.3.2 Use of young wild-type mice

Despite the overall focus of this work on the role of Lab4P probiotics on neurodegeneration, a WT mouse strain was selected for this component of the work. It was decided to use young and healthy mice as a cleaner model in this study, rather than using a mouse model of neurodegeneration. In a diseased model, if Lab4P does implicate upon disease development, this might produce combined effects on microglial cells which would be more difficult to interpret. For these reasons, unmodified, young and healthy WT mice were selected. The mice used in this study were 3 months old. This is typically when mice begin to enter their maturity but are still relatively young, with this life stage being acknowledged to be equivalent to a 20–30-year-old human brain (Fox et al. 2007).

While these data have highlighted non-significant trends and the possible potential of Lab4P to impact upon microglial cell numbers following preliminary flow cytometry work, future experimentation delineating roles of the Lab4P consortia in neurodegeneration should focus on older mice, particularly ones harbouring genetic mutations modelling neurodegeneration. Widely used AD mouse models that could be considered for use in future studies include the
3xTg, 5XFAD and APP/PS1 models (Oddo et al. 2003, Oakley et al. 2006, Radde et al. 2006). Since AD is most prevalent in humans after the age of 65, the use of mice of an equivalent age (around 18 months or more) would be useful when considering how to take this work further (Fox et al. 2007).

This is important since the immune system becomes more activated and dysregulated with age (Franceschi et al. 2000). Since it is of interest how probiotics affect microglial cells during periods where they are chronically activated and contributing to low grade chronic inflammation, as seen in AD, the use of aged mice in future studies would be useful.

In vivo AD models are all more biologically complex than in vitro cultures and help to bridge the gap between in vitro work and clinical trials. In the context of further work based on this present study the use of aged AD mouse models offers an opportunity for additional assessment of the effects of the Lab4 probiotic consortium on microglia in vivo.

4.3.3 Length of study and dose of probiotic

The mouse cohort (N=20) was administered either a control (N=10) or Lab4P probiotic (N=10) supplemented diet over a two-week period. Dosage was based on a previous study using Lab4P carried out using a rat model (Baker et al. 2021). The results presented in this chapter suggest that the dose of probiotic given did not have any negative effects on the mice. The dose given contained more CFU/g than usually given during in vivo study with mice and treatment occurred over a shorter duration (communicated by Dr Tom Webberley). The dose was chosen to increase the likelihood of the Lab4P probiotic having an impact on the gut microbiome of the supplemented mice and to increase the likelihood of the presence of probiotic metabolites in the serum generated. However, it was not confirmed whether the two-week period and high dose of probiotic were sufficient to confer changes to the gut microbiome of the mice and this is a limitation of this work.

In mice, reports of short periods of probiotic supplementation which have positive effects on mouse health have been reported. One previous study assessing the impact of probiotics on the mouse gut microbiota revealed significant changes in composition after treating 2-month-old WT female C57BL6/J mice over a period of 5 days using oral gavage to administer three cultures of probiotic bacterial strains (Lactobacillus helveticus, Lactobacillus paracasei and Bifidobacterium bifidum) as a suspension (Taverniti et al. 2021). Another study assessed the effects of 3 strains of Lactobacillus probiotic alongside a high fat diet on mice.
over a period of 45 days and administered the probiotics through supplementation of water with the probiotic at a dose of $\sim 1 \times 10^8$-$4 \times 10^8$ CFU per day and found significant changes in animal weight and decreased levels of pro-inflammatory cytokines (Fabersani et al. 2021).

Further evidence supporting the use of probiotics in health includes one report of a two-week study carried out where aged ICR/CD1 mice were fed a probiotic fermented milk drink (Hunsche et al. 2019). Mice received $\sim 4 \times 10^7$ CFU per day of three probiotic strains including *Lactobacillus casei*. The study found that a two-week period was sufficient to induce improved exploratory behaviours and appeared to reduce levels of oxidative stress compared to untreated mice.

In this present study the treatment period was carried out over two weeks which may explain why few significant differences were seen between the control and Lab4P treated groups across all subsequent *in vivo* analyses. However, these reports suggest that significant changes in the mouse gut microbiota composition, mouse behaviour and levels of cytokine levels over a short period of probiotic supplementation is possible.

A clear limitation of this work is how the gut microbiota underpins the observed responses. While faecal pellets were extracted from these animals, there were logistical difficulties in analysing these for viable bacteria by carrying out microbial enumeration and next generation sequencing of bacterial populations. It would be essential to confirm any modulation of the gut microbiome the Lab4P supplementation may have impacted in order to validate the findings outlined in this chapter. However, the observations made in this chapter helps to act as a useful baseline for future work.

4.3.4 Behavioral analysis

While the Lab4P intervention period was only two weeks long, if probiotics were able to affect C57B/6J mouse brain phenotypes, this might be reflected in behavioural responses which automated software such as EthoVision XT™ may be able to determine.

Mice were given time at the start of this study to acclimatise to the arena to minimise anxiety during the assessment period as part of standard procedure. During phase 1 testing, preference for the arena perimeter was assessed. Typically, mice that are more timid will have a preference for the perimeter of the field (Lipkind et al. 2004). Therefore, testing for mouse preference of the perimeter can offer a means of assessing anxiety, as well as cognitive
function in mouse models of cognitive decline (Sterniczuk et al. 2010). There were no trends or differences indicating effects on normal exploratory behavior.

During the second phase of testing (object habituation) mouse interaction with two objects was assessed. No significant differences in time spent at each object between the untreated, control and Lab4P supplemented groups. However, calculation of the discrimination ratios for left and right object preference did appear to show a bias towards the object on the right-hand side (Figure 4.8 A and B). Since two identical objects are used in the familiar phase of the Behavioural testing, discrimination ratios for objects should be equal during this phase (0.5 each). However, analysis of object interaction during phase 2 of testing revealed a preference for the right object. It is unclear why this is the case. It may be due to environmental factors such as noise, however the testing was carried out in a quiet area under a class II laminar flow hood with minimal noise disturbances other than the background hum of the air pumps. Additionally, this preference for the right-hand object appeared to be present at the start point in the untreated group and endpoint of testing in both control and Lab4P supplemented groups. Since there is object interaction preference for the right object seen during this testing phase, it may be the case that the results of the NOR testing carried out where the novel object was placed on the left-hand side of the arena have been dampened due to a pre-existing object bias.

The NOR method of cognitive assessment is widely used and was adopted in this study to determine whether Lab4P supplementation could impact upon mouse behaviour (Aggleton 1985, Ennaceur and Delacour 1988, Leger et al. 2013, Grayson et al. 2015). NOR tests are used to assess the ability of mice to identify novel (previously unseen) objects amongst objects they have previously been exposed to. NOR testing can be used to identify normal ‘inquisitive’ and ‘unstressed’ behaviour in mice but is most commonly used to identify memory deficits and cognitive decline in mouse models of AD (Miedel et al. 2017, Zhang et al. 2012). Evidence has shown that the region of the brain that is being tested during NOR is the hippocampus as it plays a major role in memory and object recognition (Cohen et al. 2013). Since the mice used in this study were WT and were not expected to exhibit any cognitive decline, the plethora of behavioural tests and analyses conducted were used as an indicator of any potential deviations from normal behaviour that the Lab4P supplemented group might have compared to the control group. In the presence of disease, one study carried out using NOR as a means of assessing how probiotic bacterial strain benefit AD-related decline with promising results (Go et al. 2021). The fact that few significant changes were observed may indicate that any effects Lab4P might have in modulating the gut-brain-axis may require an
already diseased starting point. It would be interesting to assess the effects of Lab4P supplementation mouse models of neurodegeneration as mentioned above.

The NOR behavioural assessment of control and Lab4P supplemented mice during this in vivo study showed very few statistically significant differences between the untreated and treated groups. Typically, in healthy mice there should be an increase in interaction with the novel object and both the start point untreated and the endpoint control and Lab4P supplemented groups did show discrimination ratios of over 0.5, suggesting a slight preference for interaction with the novel object. However, there were no significant differences in discrimination ratios between the untreated group and the treated groups, suggesting that Lab4P supplementation had no effect on mouse memory and cognition during NOR.

However, it is interesting to note that in most cases across the phase 2 and phase 3 object recognition analyses, the mean interaction frequencies and mean time spent at the objects were slightly lower in both the control and Lab4P groups in comparison with the start point untreated group. This suggests that treated mice may have lost interest in object interaction at phase 2 and phase 3 of object testing after the two-week period. This may be due to previous exposure of object tests at the start point (0 weeks). However, different objects were used during tests with objects at the start and end points in order to prevent this. Therefore, it is unclear what may have caused this. In future testing it may be beneficial to not only change the objects used at the start and end point of testing, but also to move the positions of the objects (eg, top and bottom rather than left and right) to keep the mice interested in the test after the initial exposure at the start point.

Since the intervention period was short, it was perhaps not surprising that Lab4P supplementation did not elicit any clear responses. Longer supplementation time and testing at regular ongoing intervals might allow for better identification of effects and is a limitation of this work. It may also be useful to conduct a wider battery of tests in the future to validate this work. For example, the Morris water maze, which is a test that has been used extensively as a means of assessing learning, memory and anxiety in mice (Brandeis et al. 1989). These are additions that could be considered for future work.

**4.3.5 Use of flow cytometry**

Flow cytometry is an important tool for cell analysis using varieties of unique cell surface and intracellular cell markers for cell isolation and purification (Adan et al. 2017). After
establishing microglial populations through gating, it was possible to assess the microglial cells for intracellular inflammatory biomarkers. Cell markers for inflammatory biomarkers in this study were chosen based on the markers used for qPCR work as discussed previously in Chapter 3. TNF-α was used instead of IL-1β due to availability of reagents at the time of experimentation. TNF-α however is another pro-inflammatory marker of interest in the context of this study. Assessment of microglial cells positive for inflammatory biomarkers showed no significant differences between the control and Lab4P supplemented groups.

One of the main problems faced during the analysis of the flow cytometry data in this present study was the low event counts of microglia present in both control and Lab4P samples. Due to low microglial event counts, the concatenation tool was used on FlowJo™ to pool all control samples and all Lab4P treated samples into respective groups. This allowed for better gating and an improved assessment of differences in microglial cell numbers between groups. However, analysis of cytokine expression on individual data showed no differences in microglial event counts or cytokines expression. Again, this is likely due to low event counts of microglial cells. In the future, it may be more beneficial to perform qPCR analysis on these primary cells to assess changes specifically in cytokine expression instead of flow cytometry or optimise the homogenisation procedure to yield greater numbers of cells for analysis.

It has been reported that cell isolation protocols to extract microglia from tissues should be performed as quickly as possible as time is critical for better cell survival and yield (Cossarizza et al. 2019). It is possible that time taken to perform cell isolation during this experimentation influenced the counts of microglia which could be detected. Guidelines also suggest that during the cell isolation and staining procedures it is useful to measure changes in cell number to determine if cells are lost throughout the process (Cossarizza et al. 2019).

In this present study, cells were not counted during the brain cell isolation or cell staining processes, so it is difficult to determine the true cause of the suspected low microglial yield. Not counting cells also meant that it was not possible to determine initial cell yields from each group which meant that the event count determined by FlowJo™ had to be used to establish incidence of microglial cells per group. This is a key limitation of this flow cytometry work, and in future work it would be critical to carry out cell counting to determine accurate cell yield and validate the observations of this preliminary work.

Refinement and further practice of the cell isolation and staining protocols and techniques could help to enhance microglial cell yield in future experiments. Protocols also
exist where microglial isolation can be carried out via the use of magnetic bead columns which define microglial cells by distinguishing them based on the CD11b cell surface marker which prevent any unwanted upregulated pro-inflammatory gene expression effects caused by FACS (Bordt et al. 2020). The lack of refinement, optimisation and operator expertise could all be contributing factors in the low event counts detected and represent a limitation to this work. In the future, more optimisation, sourcing of alternative protocols or pooling more tissue for analysis could prove useful in expanding this work and providing improved outcomes.

While some trends were observed, it is not possible to make any robust claims from these preliminary results due to the limitations stated here. Additional and more refined flow cytometric analysis on the brain cells of mice fed a control or Lab4P supplemented diet in the future is required. Therefore, whether Lab4P has an impact upon microglial cell numbers and phenotype in vivo remains unclear.

4.3.6 Conclusion

This preliminary in vivo study has provided some initial insights into the effects of the Lab4P probiotic on WT mice over a two-week period. This work suggests that probiotic supplementation (i) had no significant effect on mouse food and water consumption, body weight and organ weight and (ii) does not affect normal exploratory behaviour of the mice. It is unclear from this work whether numbers of microglial cells in the brains of the mice were altered by supplementation. More refined flow cytometric analysis would be required to elucidate this.

Due to this being a short-term study with young, healthy mice, these results are not unexpected. It is unconfirmed from this work whether this two-week period of Lab4P supplementation made substantial changes in the gut bacteria populations of these mice. In future work changes in the compositions the mouse gut microbiota would need to be tested to confirm and validate these results. Additionally, a larger cohort of mice and perhaps the use of aged or diseased mice would be useful for future studies. However, this pilot study did confirm that the two-week Lab4P probiotic supplementation did not induce any negative effects on phenotypes or behavioural effects on the mice that received the supplemented chow diet and acts as a useful baseline for future work.

This in vivo study also allowed for the collection of serum from control and Lab4P supplemented mice, which was used for further in vitro study to test the effects of probiotic
metabolites on BV-2 cells as a comparative supplement to validate the findings from the *in vitro* work in Chapter 3.
Chapter 5

Assessment of BV-2 microglial cell response to mouse serum and amyloid (1-42) challenge
5.1 Introduction

For probiotics to have a beneficial health effect after consumption they must reach the intestines alive in order to add to the diversity of the gut microbiome (FAO/WHO 2002). Probiotic bacteria produce metabolites such as SCFAs which contribute to the maintenance of gut barrier integrity and immunity which are important for overall health (Silva et al. 2020). These metabolites can be absorbed through the gut epithelium and enter the bloodstream, allowing for their involvement in a wide variety of different biological processes ranging from immunity to metabolism (den Besten et al. 2013).

Under circumstances where there is gut dysbiosis or a loss of integrity of the gut barrier, inflammatory molecules or bacterial components may pass through into the bloodstream and may contribute to disease associated inflammation in AD (Campos-Bedolla et al. 2015). Evidence from previous work carried out at Cultech (personal communication from Dr Tom Webberley) suggests that probiotic supplementation may improve the health status of microglial cells and levels of cytokine gene expression that contribute to AD associated inflammation.

One of the main problems encountered previously in Chapter 3 was the detection of high levels of pro-inflammatory IL-1β which appeared to be induced via stimulation with Lab4b and Lab4P probiotic CM when applied to BV-2 cells in vitro. During this initial testing it was suspected that CM may contain unknown pro-inflammatory stimuli, making it difficult to interpret the effects of the probiotic CM on BV-2 cells. To address this issue and validate the previous in vitro results, it was decided that a more physiologically relevant supplement was required.

One possible approach is to use serum from probiotic treated mice to allow for testing of the effects of probiotic by-products circulating in the bloodstream on BV-2 cells in vitro. Previous studies have shown the use of rat serum to test the effects of diet induced serum metabolite changes via application to BV-2 cells in vitro (Fisher et al. 2017). As part of the preliminary in vivo study detailed in Chapter 4, mouse serum was obtained post-mortem after mice were fed either a control or Lab4P supplemented diet over a period of two-weeks. Therefore, the aims of the work presented in this chapter are as follows:
1) Test the effect of control and Lab4P mouse serum on BV-2 cells in vitro.
2) Stimulate BV-2 cells with synthetic amyloid β (1-42) and assess effects on gene expression of key targets.
3) Assess whether Lab4P mouse serum used as an in vitro supplement on BV-2 cells can modulate amyloid β (1-42) driven effects.

5.2 Results

5.2.1 Assessment of BV-2 gene expression in response to control and Lab4P treated mouse serum

To assess the effects of mouse serum obtained from control and Lab4P supplemented mice on BV-2 cells, a range of concentrations were applied and incubated for either 4 hours or 16 hours (Figure 5.1). This was done initially to determine if mouse serum alone had any effect on endogenous gene expression of BV-2 cells and to find an appropriate concentration of mouse serum to be used in further assays. Data shown are graphed as mean fold change relative to the control (0% (v/v) mouse serum) for visual effect and clarity. Statistical analysis has been carried out on ΔCt values as before and can be found in the appendix (Figure A4).
Figure 5.1. Effect of control and Lab4P mouse serum on BV-2 cytokine gene expression over a 4 and 16-hour treatment period. BV-2 cells were grown in 48 well plates at 150,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before removal of medium and incubation with either control or Lab4P mouse serum stated concentrations of mouse serum (MS) for 4 hours (A, B & C) or 16 hours (D, E & F). Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as mean fold change relative to the control (0% MS). N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Two-Way ANOVA and Sidak’s multiple comparison test for all graphs. * = P≤0.05
4 hour treatment

After BV-2 cells were stimulated with serum taken from mice fed a control or Lab4P supplemented diet, it was found that serum type had a significant effect on IL-1β gene expression (Figure 5.1 A) \((P=0.026, \text{ Two-Way ANOVA})\) and IL-6 gene expression (Figure 5.1 B) \((P=0.048, \text{ Two-Way ANOVA})\). Serum type did not appear to have the same effect on IL-10 gene expression (Figure 5.1 C).

A significant increase in IL-1β gene expression after incubation with 1% (v/v) Lab4P serum for 4 hours was seen (Figure A4) \((P=0.0011, \text{ Sidak's multiple comparisons test})\). However, normalisation of these data shown relative to the control as mean fold change did not reflect this difference. All other direct comparisons of serum concentrations (Control vs Lab4P) did not show any significant differences (Sidak’s multiple comparisons test). It is important to note that a fair amount of variation was seen among the normalised mean fold change data shown here.

16 hour treatment

Stimulation of BV-2 cells with serum taken from mice fed a control or Lab4P supplemented diet over a 16-hour incubation period showed that serum type had a significant effect on IL-10 gene expression (Figure 5.1 F) \((P=0.0065, \text{ Two-Way ANOVA})\). This effect was not seen when IL-1β (Figure 5.1 D) and IL-6 (Figure 5.1 E) were assessed.

A significant difference was found in IL-10 gene expression when cells were incubated with control and Lab4P serum at a concentration of 0% (v/v) for 16 hours (Figure 5.1 F) \((P=0.029, \text{ Sidak's multiple comparisons test})\). All other comparisons of serum concentrations (Control vs Lab4P) did not show any significant differences.
5.2.2 BV-2 cell response to synthetic amyloid β (1-42) treatment

Synthetic Aβ (1-42) (Abcam, UK) was prepared in DMSO as described in Chapter 2.5. This was applied to BV-2 cells in vitro at a concentration of 0.5μM or 5μM. Amyloid was prepared by two different methods as an optimisation step to establish appropriate conditions for use of amyloid in further assays:

1) Pre-incubated in cell growth media for 24 hours prior to application to allow time for aggregation of oligomers.

2) Applied directly to the BV-2 cells in a monomeric form on the day of the assay (therefore pre-incubated for 0 hours).

The different methods allowed for the assessment of amyloid efficacy in both formats to elicit any changes in gene expression. Since amyloid is known to impact upon cell health and survival, causing cell death and necrosis when aggregated, CV cell viability assays were also performed alongside gene expression assays.

**BV-2 cell viability**

Cell viability of BV-2 cells in response to synthetic Aβ (1-42) was assessed via CV assay (Figure 5.2). BV-2 cells showed a trend for increased cell viability after a 4-hour incubation (Figure 5.2 A) and a 16-hour incubation (Figure 5.2 B) for all 4 treatment conditions in comparison with the control (DMEM, 0.5% (v/v) DMSO). Therefore, it was determined that DMSO solubilised amyloid did not show any detrimental effect on BV-2 cell viability and could be used in assays for further investigation of its effects of BV-2 cells. This pilot experiment was only repeated once due to time constraints to get an impression of cell survival in response to amyloid. This meant that statistical analysis could not be carried out. Error bars on the graph represent the mean across 6 technical repeats. While more biological repeats are required, data suggest that cell viability was higher in comparison to the control, as shown by mean absorbance.
**BV-2 gene expression in response to synthetic amyloid treatment**

After establishing that the DMSO solubilised Aβ (1-42) was not cytotoxic to cells the response of the BV-2 cells to synthetic amyloid was tested via RT-qPCR using a range of key inflammatory biomarkers following a 4-hour or 16-hour incubation (Figure 5.3). These included IL-1β, IL-6 and IL-10 with β-actin used as a housekeeper gene. Due to time constraints and sample availability, only one repeat was carried out for this work and therefore statistical analysis could not be performed. However, these preliminary data appear to show that unaggregated amyloid applied directly (0hr) may elicit more gene expression of pro-inflammatory markers such as IL-1β (Figure 5.3, A) and IL-6 (Figure 5.3 B and E).
Figure 5.3. BV-2 gene expression in response to synthetic amyloid treatments. BV-2 cells were grown in 24 well plates at 300,000 cells per well in DMEM (10% (v/v) FBS, 100 U/mL P/S). Synthetic 1-42 Amyloid-β was prepared in DMSO and DMEM (10% (v/v) FBS, 100 U/mL P/S) and either incubated for 24h (to allow for aggregation - blue) or 0hrs (to be applied in a monomeric form – purple) before application to BV-2 cells for 4 (A, B, C) or 16hrs (D, E, F) following a 24-hour incubation after seeding. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. N=1 biological repeat for all graphs. Error bars represent the mean across 3 technical repeats following normalisation using ΔΔCt method. Data are shown as mean fold change relative to the control.
5.2.3 Assessment of the effects of mouse serum on BV-2 cells with the addition of synthetic amyloid β (1-42) challenge

The hypothesis underpinning this thesis was based on preliminary data arising from work using the Lab4 consortia of probiotics suggesting that AD mouse models fed a probiotic supplemented diet showed improved profiles of inflammatory cytokines (data communicated by Dr Tom Webberley). From this work it was hypothesised that the Lab4 consortia of probiotics may have an impact on microglial cell health since microglia play a central role in coordinating the inflammatory response in the brain.

To determine this in vitro the final experiment of this thesis aimed to assess the effects of mouse serum obtained from control and Lab4P supplemented mice on BV-2 microglial cells in the context of AD via the presence of synthetic Aβ (1-42). BV-2 cells were incubated with the appropriate mouse serum for either 4 or 8 hours in the presence of a range of Aβ concentrations (Figure 5.4).
Figure 5.4. Effects of control and Lab4P mouse serum on BV-2 cytokine gene expression in the presence of amyloid β (1-42) challenge over a 4- and 8-hour treatment period. BV-2 cells were grown in 48-well plates at 150,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before incubation with stated concentrations of synthetic amyloid-β (Aβ) and either control or Lab4P treated mouse serum for 4 hours (A, B, C) or 8 hours (D, E, F). Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as mean fold change relative to the control (0μM Aβ). N=3 for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Two-Way ANOVA and Sidak's multiple comparison test for all graphs.
4 hour incubation

No significant effects due to serum type or concentration were found following a 4-hour incubation of BV-2 cells with control and Lab4P mouse serum (Figure 5.4 A, B and C) (Two-Way ANOVA). Additionally, no significant differences were found upon comparisons of gene expression after 4-hour incubations with control and Lab4P mouse serum (Figure 5.4 A, B and C) (Sidak’s multiple comparisons test).

However, there did appear to be a ~3-fold reduction in IL-6 gene expression when BV-2 cells were treated with Lab4P serum and 0.05μM Aβ or 0.5μM for 4 hours in comparison with control serum (Figure 5.5 B). While statistical analysis of ΔCt values did not highlight this as a significant difference (Appendix Figure A5), it is an interesting observation that has been drawn out via normalisation of these data (presented here as mean fold change).

8 hour incubation

No significant effects due to serum type or concentration were found following an 8-hour incubation of BV-2 cells with control and Lab4P mouse serum on IL-1β (Figure 5.4 D) and IL-10 gene expression (Figure 5.4 F) (Two-Way ANOVA). However, a significant effect caused by Aβ concentration appeared upon analysis on IL-6 gene expression (Figure 5.4 E) ($P=0.0033$, Two-Way ANOVA). No significant differences by expression of all three target genes treated with control and Lab4P treated serum were found (Figure 5.4 D, E and F) (Sidak’s multiple comparison test).

After normalisation of the data (shown as mean fold change), cells appeared to show lower levels of IL-6 (Figure 5.5 E) and IL-10 (Figure 5.5 F) expression in the presence of amyloid and both control and Lab4P treated mouse serum relative to the control of 0% Aβ.

5.3 Discussion

5.3.1 Summary of data

Work described in this chapter aimed to assess the effects of Lab4P probiotic metabolites present in serum taken from mice fed a probiotic supplemented diet on BV-2 microglial cells this was deemed to be a more physiologically relevant supplement by comparison with CM. One outcome of this was to be able to judge whether similar effects BV-2 cells were observed between CM and serum. Due to the limited supply of mouse serum only
gene expression was carried out to assess the initial effects of mouse serum on BV-2 cells in the absence of a challenge.

Initial application of serum taken from control and Lab4P supplemented mice applied to BV-2 cells showed that serum type did have a significant effect on endogenous IL-1β and IL-6 gene expression levels after a 4-hour incubation (Figure 5.1 A and B) and IL-10 gene expression levels after 16 hours (Figure 5.1 F), suggesting that serum composition may be different and may be inducing different effects on gene expression based on whether or not the mouse diet was supplemented with the Lab4P probiotic. This is interesting as it suggests that the two-week diet probiotic supplemented diet may have had an impact on probiotic metabolite levels in the serum, however further analysis of serum composition would be required to confirm this.

However, it was also found that there was a significant difference in IL-10 gene expression when serum was applied at 0% (v/v) and incubated with cells for 16 hours, acting as the experimental control. The finding of this significant difference suggests clear variation which may have impacted upon the mean fold change data presented here, as the values were normalised against these control (0% (v/v) values via the ΔΔCt method. Due to a limited volume of serum available it was not possible to repeat any biological Ns to remove any anomalous data which has likely given rise to the variation seen here. It would be essential in future experimentation to carry out further repeats to tighten these data and reduce the impact this variation may be having.

At the 16-hour timepoint gene expression levels of IL-6 and IL-10 cytokines were found to be lower than at the 4-hour timepoint (Figure 5.1 B and E, C and F). Typically, after the initiation of the inflammatory response cytokine levels peak at different times as seen in THP-1 macrophages (Figure 5.5).
According to Chanput et al. (2010) cytokine gene expression of IL-1β, IL-6 and IL-10 in THP-1 macrophages peaks at ~4-6 hours following stimulation with LPS before declining and reaching a plateau after 18 hours (Figure 5.5 A). Other studies parallel these findings, agreeing that IL-6 gene expression peaks at ~4-6 hours following introduction of inflammatory stimuli (Pulido-Salgado et al. 2018). However, time taken for IL-10 production is thought to be slightly later with gene expression peaking at ~6-8 hours and cytokine production at around
24 hours after exposure to inflammatory stimuli (Rasley et al. 2006). Based on this evidence it is likely that in this case at the 16-hour timepoint cytokine gene expression had passed their peak following initial inflammation and therefore gene expression levels were lower overall than at the 4-hour timepoint. In future experimentation it will be important to keep these timepoints in mind to capture effects on cytokine gene expression and subsequent protein production at what would be their peak in response to challenges.

Next, the effect of DMSO solubilised synthetic Aβ (1-42) on BV-2 cells was assessed. Again, due to time constraints and availability of Aβ, only one biological repeat was performed for both the cell viability assay and gene expression assay, therefore statistical analysis could not be carried out. Despite this, these assays provided information which indicated that synthetic amyloid appeared to have no negative effect on BV-2 cell viability and allowed the selection of concentrations and preparation methods of amyloid for future assays. Since the synthetic amyloid was solubilised in DMSO there was concern this may affect BV-2 cell viability. However, CV assay showed this was not the case and that this preparation method was suitable for in vitro experiment as all preparation methods showed an increase in viable cells in comparison with the negative control at 4 hours and 16 hours (Figure 5.2 A and B). In future work it would be paramount to complete further biological repeats to validate these initial observations.

After determining the individual effects of mouse serum and synthetic Aβ on BV-2 cells, a final experiment was carried out to combine both factors. Control and Lab4P mouse serum and DMSO solubilised synthetic Aβ (1-42) were applied to BV-2 cells together in order to determine the effects of Lab4P probiotic metabolites on BV-2 cells in the context of AD over a 4-hour and 8-hour incubation period (Figure 5.4). It appeared that serum type and concentration had no effect on BV-2 gene expression after a 4-hour and 8-hour incubation with serum taken from mice fed a control or Lab4P supplemented diet and the presence of Aβ challenge (Figure 5.4). However, one interesting trend observed during these assays was that when Lab4P treated mouse serum was applied to BV-2 in the presence of an amyloid challenge it appeared to reduce the fold change of IL-6 gene expression by comparison with that seen in the control serum after a 4-hour incubation period, however this was not a statistically significant finding (Figure 5.4 B). In order to try to capture cytokine gene expression at the peak following exposure to synthetic amyloid an 8-hour timepoint was used instead of a 16-hour incubation period for this final assay. However, no significant differences were seen between the control and Lab4P groups or change in overall gene expression was seen on exposure to all three amyloid concentrations. This suggests that perhaps gene expression of
these cytokines is occurring earlier between 4-6 hours and therefore these are timepoints that should be focused on in the future.

5.3.2 Impact of Lab4P mouse serum on BV-2 gene expression in the presence of amyloid (1-42)

Application of serum from control and Lab4P supplemented mice to BV-2 cells did not induce any significant changes in IL-β, IL-6 or IL-10 gene expression over a 4 or 8 hour incubation period (Figure 5.4). However, an initial non-significant trend was observed when serum obtained from mice supplemented with Lab4P was applied to BV-2 cells in the presence of 0.05μM and 0.5μM of synthetic amyloid which appeared to induce ~3-fold decrease in IL-6 gene expression after a 4-hour incubation (Figure 5.4 B). Although this was not shown to be a statistically significant reduction, it is an interesting initial observed trend. As a caveat, from the ΔCt values it is apparent that there is clear variation in the control and Lab4P values at 0% (v/v) (Appendix Figure A5 B, D and F) and this may have had an impact when normalising data and expressing values relative to the control in order to present data as mean fold change. During this experimentation only three biological repeats were carried out for the serum and amyloid assay and therefore, any anomalous results could not be excluded (ie. using a method such as finding outliers based on being 2 standard deviations away from the mean). In future work it would be important to repeat experiments where large variations are present to tighten these data. This may include carrying out more than 3 technical repeats per biological N or adding more biological repeats. In the work presented in this chapter it was not possible to do this due to the supply of mouse serum and Aβ (1-42) being limited.

However, if after further investigation to validate this observation, it is found that Lab4P does have a significant impact on IL-6 gene expression this would support previous studies. For example, similar results have suggested that the probiotic bacteria *Lactobacillus paracasei* and *Bifidobacterium* subspecies *animalis* may influence IL-6 downregulation (Čitar et al. 2015). This is also particularly relevant as IL-6 has been heavily implicated in AD onset and progression. It has been found to be upregulated in the cerebrospinal fluid and serum of AD patients and found in large concentrations in post-mortem AD brains (Brosseron et al. 2014, Cojocaru et al. 2011, e Silva et al. 2021). Additionally, evidence suggests that IL-6 is found during the early stages of Aβ plaque deposition with higher incidence of IL-6 found in diffuse plaques in comparison with mature compact plaque, suggesting it is a key player during AD onset (Huell et al. 1995). A 2021 study published in Nature looking specifically at IL-6 found
that suppression of IL-6 and inhibition of the transcription factor STAT3 helped to improve memory impairment in AD mice (e Silva et al. 2021). This suggests the downregulation of IL-6 is beneficial and improves AD symptoms. This evidence collectively gives a strong indication of IL-6 involvement in AD, however the role of IL-6 in chronic inflammation and the molecular mechanisms behind exactly how it contributes to disease onset and progression are complex and with mechanistic aspects such as molecular crosstalk unclear at this time.

There is also evidence to suggest that IL-6 impacts microglial recruitment in AD. One study showed that microglia isolated from aged mice showed higher levels of IL-6 (Garner et al. 2018). It has also been acknowledged that IL-6 is involved in gliosis, a phenomenon in AD where an increase in active microglial cells occurs in response to Aβ plaques in an attempt to eliminate them (Chiang et al. 1994). Gliosis is not an inherently detrimental process, with evidence suggesting that early on in disease it can be beneficial for Aβ plaque removal and may even enhance phagocytic abilities of microglia in AD mouse models (Chakrabarty et al. 2010, Cho et al. 2014). The problem stems from sustained microglial activation over time where according to previous evidence, the glial phenotypes flip from protective to pro-inflammatory and cytotoxic (Jimenez et al. 2008). This includes the upregulation of IL-1β and TNF-α which further potentiates AD related inflammation (Morihara et al. 2005, Yamamoto et al. 2007). One interesting study also suggested that long term exposure to IL-6 may cause desensitisation of microglia, the infiltration of T cells and a consequential increase in pro-inflammatory cytokines which may account for the “switch” in microglial phenotype described by Jimenez et al. (Recasens et al. 2021). In future work it could be interesting to measure microglial cell proliferation using a proliferation marker such as Ki-67 and microglial specific markers such as Iba-1 to determine if gliosis is reduced by the Lab4P probiotic or if gliosis occurs, but IL-6 gene expression is downregulated. This may help to elucidate part of the molecular mechanisms behind this observation.

Further work would be required to strengthen and statistically validate this preliminary observation, possibly through in vivo investigation using AD mouse models such as 3xTg or APP/PS1. However, it is interesting to speculate that probiotic metabolites may offer a method of downregulating disease associated inflammation and prevention of microglial recruitment through altering regulation of pro-inflammatory cytokines such as IL-6, potentially resulting in the slowing or prevention of AD onset. This therefore would be interesting to investigate further in future work.
5.3.3 Use of mouse serum

In this chapter, serum from Lab4P supplemented mice was used to determine whether CM responses could be reproduced in vitro using this different method of probiotic metabolite delivery to BV-2 cells. Probiotic CM is a useful in vitro tool and a number of previous studies have utilised probiotic cell-free supernatants to assess the effects of probiotic by-products on the function of cells, including macrophages (Taweechotipatr et al. 2009, Seenappanahalli Nanjundaiah et al. 2016). However, in this present study high levels of pro-inflammatory gene expression were seen in response to BV-2 cell stimulation with Lab4b probiotic CM in particular (Chapter 3).

One benefit of the use of serum over conditioned media is that probiotic bacteria do not inherently produce metabolites such as SCFAs, which evidence suggests play a major role in regulating metabolic processes and inflammation via the MGBA (An et al. 2014). For gut bacteria to produce SCFAs the presence of dietary fibre is required to act as a substrate in the fermentation process which occurs in the gut (Grabitske and Slavin 2008). Therefore, it is more likely that metabolites such as SCFAs will be present in serum taken from mice supplemented with probiotics compared to probiotic CM which is cultured anaerobically in vitro and without substrates such as fibre to act upon.

The volume of serum generated from each group of mice was very limited which meant prioritising which assays could be carried out and limiting as far as possible the variables for each assay, such as the concentration of serum used. Therefore, testing of BV-2 cell viability with the mouse serum was by-passed and gene expression analysis prioritised.

Preparation of the mouse serum involved heat inactivating to destroy complement activity which might present as a confounding factor in understanding the data and filter sterilisation via 0.22μm filter to remove debris. The concentration of mouse serum used in the final serum and amyloid assay was chosen based on the initial serum assays carried out. Ideally a concentration of 10% (v/v) mouse serum in the cell growth medium would have been used as a comparator to the typical concentration of 10% (v/v) FBS in normal cell growth medium. One previous study where mice were fed a walnut diet before serum collection and application to BV-2 cells used 10% (v/v) mouse serum, matched with 10% (v/v) FBS supplemented DMEM as a control (Fisher et al. 2017). However, in this chapter due to a limited total volume of mouse serum available it had to be used sparingly. The initial assays in this chapter where three concentrations of mouse serum were applied to cells were done to determine if a concentration lower than 10% (v/v) mouse serum could be used. The assay
showed that there was little difference between levels of cytokine gene expression induced at 4 hours between 5% (v/v) and 10% (v/v) mouse serum concentration (Figure 5.1 A, B and C). Therefore, it was decided to conserve as much mouse serum as possible 5% (v/v) mouse serum would be used in the final experiment.

It would certainly be beneficial to analyse the control and Lab4P treated mouse serum in the same way as the CM, possibly using NMR spectrophotometry, to assess the metabolites present and to determine the presence of endotoxin or other inflammatory stimuli that may be inducing false positive or false negative results in future studies.

5.3.4 Use of DMSO solubilised synthetic amyloid (1-42)

The nature of AD pathology is a key factor when attempting to carry out in vitro study in the context of the disease. In this present study the final experiment aimed to assess the effects of Lab4P probiotic metabolites on BV-2 cells in the context of AD through the use of a synthetic amyloid (1-42) as a challenge. Before starting this assay, the preparation and use of synthetic amyloid in vitro had to be carefully considered. Initial literature searches suggested a multitude of methods for the solubilisation and use of Abcam synthetic Aβ 1-42 in vitro (Karthick et al. 2019, Zhang et al. 2019).

Previous studies suggested using amyloid at a concentration of around 5μM and to aggregate by incubating it over a period of 24 hours (Chiozzi et al. 2019, Caldeira et al. 2017). The effects of amyloid beta (1-42) at 0.5μM and 5μM, aggregated over 24 hours and unaggregated when applied to BV-2 cells were assessed. Due to time constraints this optimisation work was only carried out once (N=1), hence no statistical analysis could be carried out. However, from this initial assay no notable negative effects on BV-2 cells were seen. In the final serum and amyloid assay unaggregated synthetic amyloid (1-42) at a concentration of 0.5μM was used based on a lack of negative effects on BV-2 cell viability and minimal differences between effects on BV-2 cells of aggregated and unaggregated amyloid seen in the initial optimisation. This decision was also based on time constraints and availability of synthetic Aβ (1-42) and mouse serum.

One of the problems with using synthetic amyloid seen in recurring studies is that generally the assay is done over a short period of time such as 24 hours and is used at variable concentrations. Based on literature searches it was decided in this present study to initially optimise the use of synthetic amyloid before use in further assays with mouse serum. Ideally,
more refinement of the use of synthetic Aβ should be carried out to improve future experiments. In this present study it was not confirmed whether the 24-hour incubation of amyloid beta in DMEM resulted in aggregated amyloid (1-42) oligomers. Other studies have confirmed the presence of amyloid oligomers via the use of SDS-PAGE before application to cells (Wickstead et al. 2020). Other methods of amyloid oligomer visualisation could include the use of staining procedures such as Congo Red or Thioflavin S to confirm aggregation and these should be considered in any future work (Klunk et al. 1999, LeVine 1999).

Additionally, in these present assays only one form of amyloid was used (1-42), due to it being the most relevant in AD pathology. Aβ 1-42 is the dominant isoform of amyloid which is able to conform into the amyloid fibril structure more easily than other isoforms and so makes up the majority of the composition of mature amyloid plaques in AD (Roher et al. 1993, Iwatsubo et al. 1994). However, there are other isoforms that contribute to the deposition of the amyloid plaques. One such isomer is Aβ 1-40 which although is more biologically available does not conform into the fibril structure as quickly and so does not contribute as dominantly to amyloid plaque pathology in AD (Mori et al. 1992). The ratio between the 1-40 and 1-42 amyloid isoforms found the CSF is sometimes used as a biomarker in AD patient diagnosis, with an increase in the 1-42 plaques suggesting worsening of disease severity and the development of mature plaques (Wiltfang et al. 2007, Kuperstein et al. 2010, Dumurgier et al. 2015). Aβ plaques also exist in different states during AD development such as immature diffuse plaques to mature dense core plaques which may also have different roles in disease progression and the extent of inflammation they cause (Wang et al. 2002, D’Andrea and Nagele 2010). To further highlight the complexity of Aβ plaque pathology, proteomic studies have shown that AD amyloid plaques have specific protein compositions including a higher volume of complement proteins compared to amyloid plaques found in age matched patients who did not have AD (Xiong et al. 2019).

Therefore, it is difficult to replicate the complexity and diversity of different amyloid plaque structures and protein composition in vitro and the sole use of the 1-42 Aβ isomer may be too simplistic as an approach. Time is another factor to consider when using Aβ in vitro. As mentioned earlier, in the human brain amyloid takes years to aggregate and produce amyloid plaques. To replicate this in vitro one study suggested the aggregation of amyloid over a period of days (Hughes et al. 2020). However, other studies deem a 24-hour incubation period to be sufficient for amyloid aggregation under various conditions (Stine et al. 2010).

A final consideration for the use of synthetic amyloid in vitro is the concentration of amyloid used. Previous studies suggest the use of around 1-5μM amyloid concentration in
vitro (Chiozzi et al. 2019). However, other studies argue a lower concentration in the range of 1 to 10 pM is more useful (Hughes et al. 2020). This is a key consideration as a high concentration of Aβ in vitro may induce effects on cells that would not occur in vivo, giving rise to false results. However, one study noted that the use of lower amyloid concentrations had minimal pro-inflammatory effects compared to stimulation with LPS (Wickstead et al. 2020). Therefore, further optimisation to determine an appropriate concentration for in vitro experimentation is required.

Consideration of all these factors is important to generate a suitable and reliable in vitro model of AD pathology. Nevertheless, this initial in vitro work acts as a useful starting point to assess probiotic metabolites on microglial cells in the presence of oligomeric amyloid. Following further in vitro investigation, it would be interesting to see whether work in vivo using AD mouse models with more biologically complex amyloid pathology such as the 3xTg or APP/PS1 models could help to validate these in vitro findings.

5.3 5 Conclusion

Application of mouse serum to BV-2 cells under various conditions did not induce any notable significant changes in cytokine gene expression of the three target genes. While not statistically significant, an interesting preliminary trend observed in this chapter was that when serum derived from Lab4P supplemented mice was applied to BV-2 cells in the presence of synthetic Aβ (1-42) at 0.05μM and 0.5μM and incubated for 4 hours, gene expression levels of IL-6 appeared to be reduced by ~3-fold. This observation may suggest that the Lab4P probiotic blend has the potential to improve inflammation associated with AD through down regulation of IL-6, which is thought to be involved in the early stages of disease. However, further work would need to be carried out to statistically validate and strengthen this observed trend before any robust claims could be made. If Lab4P can impact IL-6 gene expression levels in BV-2 cells, it remains yet to be elucidated as to why this is the case. Further work would need to be done to understand the molecular mechanisms such as transcriptional control that may be behind the beneficial properties that probiotics appear to have on microglia in the context of AD.

Ultimately, due to the small panel of inflammatory biomarkers used during this work, it is not possible to make any conclusive comments on the extent of the effect that probiotic metabolites may have on microglial cytokine gene expression and how this impacts inflammation in the context of AD. However, this preliminary work does point in the direction
that serum taken from Lab4P treated mice may have a different effect on gene expression by comparison with control serum. These observations warrant further and more extensive study for validation.
Chapter 6

General discussion
6.1 Project summary

The research presented in this thesis focuses on the study of microglial cells and the potential to improve microglial function through modulation of the gut microbiome via the use of probiotics. Microglia have been closely implicated in AD development due to their contribution to brain inflammation seen in conjunction with developing AD pathology. There is now a well-established link between gut dysbiosis and microglial cell health and function. Therefore, the aims of the project were:

1) To use in vitro models of microglia to test whether probiotic metabolites generated in vitro can affect gene expression of key inflammatory markers and microglial cell health.
2) To assess the impact of probiotics in vivo in healthy WT mice.
3) To use in vitro models of microglia to test the ability of probiotic-derived metabolites generated in vivo to modulate amyloid-induced inflammatory effects.

To achieve these aims the BV-2 mouse microglial cell line was chosen as an in vitro culture model of microglia. This choice was based on the widespread use of this line to study microglial biology in the literature. Initially, BV-2 cells were exposed to Lab4b and Lab4P probiotic CM over various time points to measure cell viability and expression of key inflammatory cytokines IL-1β, IL-6 and IL-10. After measuring high levels of IL-1β expression following treatment with probiotic CM, a dose response experiment was completed by stimulating BV-2 cells with a range of CM concentrations to explore this effect further.

Given this data, it was decided to refine the in vitro experimental assays by trying to bring them closer to physiological conditions. To this end, WT C57BL/J mice were fed either a control or Lab4P probiotic supplemented diet over a two-week period. The primary aim of this procedure was to generate mouse serum carrying gut absorbed metabolites influenced by a probiotic supplemented microbiome which could then be used in BV-2 cell based assays. A number of other parameters were also measured including mouse body weights, food and water intake, organ weight, behaviour and microglial cell populations in the brain.

Cell assays that had initially been carried out with in vitro derived probiotic CM were then repeated, using mouse serum instead of CM and the same gene targets assessed via RT-qPCR. At this time, the BV-2 cell response to synthetic amyloid (1-42) was also tested.
Finally, an experiment was carried out to explore whether serum taken from control and Lab4P probiotic supplemented mice could impact amyloid challenged BV-2 cells.

**6.2 Summary of main research findings**

Initial research findings from Chapter 3 showed that the two different probiotic consortia CM stimulated pro-inflammatory cytokines to different extents. Lab4b appeared to induce high levels of IL-1β in comparison with Lab4P at a range of concentrations. This is likely due to the fact that the two consortia have a different strain composition giving rise to different probiotic by-products. The high levels of IL-1β gene expression in BV-2 cells prompted the need for testing with a physiologically relevant supplement alternative to validate the *in vitro* findings.

The *in vivo* work involved supplementation of WT C57BL/J6 mice with a Lab4P probiotic in their diet over two weeks and showed no significant effects on mouse body weight, organ weights, food and drink intake and exploratory behaviour. There was also no significant difference between microglial populations measured via flow cytometry, however this work was preliminary and requires further investigation and refinement to substantiate the initial findings. This *in vivo* work did allow for the generation of mouse serum for use in work carried out in Chapter 5.

Application of serum obtained from control and Lab4P supplemented mice onto BV-2 cells did not significantly impact changes in cytokine gene expression. However, an interesting observed trend from Chapter 5 was that BV-2 cells appeared to show a decrease in IL-6 gene expression (mean fold change) in response to stimulation with serum taken from Lab4P supplemented mice in the presence of synthetic Aβ (1-42). While this observation was not statistically significant, likely due to variation in the data, it may be interesting to explore further. If Lab4P is able to impact IL-6 gene expression in microglia, this may suggest that Lab4P could potentially modulate inflammation associated with AD.

**6.3 Future work**

**6.3.1 Further work *in vitro***

*Protein analysis and expansion of genes of interest panel*

Investigation of the gene expression changes will need to be confirmed at the protein level as it has been shown that gene expression may not always fully reflect protein
functionality due to a plethora of downstream and post-transcriptional mechanisms (Vogel and Marcotte 2012, Maier et al. 2009). While the results suggest that levels of IL-1β and IL-6 gene expression were stimulated to a high-level using CM it is unclear whether levels of IL-1β and IL-6 cytokine protein production parallel this finding. This would be interesting to investigate further since evidence suggests that IL-1β is initially produced by microglia in an inactive form in response to inflammatory stimuli and requires a secondary signal such as ATP to produce the active IL-1β protein (Chauvet et al. 2001, Ferrari et al. 2006). It is unclear whether in vitro the IL-1β protein has become active and this should be investigated further. Confirmation of IL-6 protein levels in experiments where serum from Lab4P-supplemented mice appeared to show the potential to reduce IL-6 gene expression would also be useful in further identifying any anti-inflammatory potential of Lab4P in microglial cells. Cell culture supernatants from both CM and serum assays still remain from this work and could therefore be assayed for protein by ELISA, western blot or Luminex Multiplex protein assays.

The panel of inflammatory cytokine genes assessed during this project was limited due to time constraints. The key cytokines that were prioritised and focused on during this work were IL-1β, IL-6 and IL-10. These genes were all selected due to their roles in the inflammatory response in AD based on extensive literature searches and as a continuation from previous work. While exploration of these three cytokines has generated some interesting results in this present work, in future work it would be necessary to expand the panel of genes of interest further to capture additional changes in BV-2 gene expression after stimulation with probiotic CM or serum. This will help to further elucidate how the Lab4 consortia of probiotics may influence changes in microglial gene expression and how this affects the microglial mediated aspects of the inflammatory response. Other gene targets that should be considered for future investigation due to their relevance in this work are detailed in Table 6.1.

In addition to gene expression assays to explore levels of ROS it would also be useful to measure ROS production more directly. There are kits available such as the Total ROS Assay kit 520nm (Thermofisher, UK) and the ROS-Glo™ H₂O₂ Assay (Promega, UK) which are suitable for application to in vitro cell cultures and should also be used alongside gene expression analysis to directly measure the production of ROS in BV-2 cells under in vitro experimental conditions.
Table 6.1 Summary of genes of interest for future work.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in AD</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour Necrosis Factor Alpha (TNF-α)</td>
<td>• Pro-inflammatory cytokine produced by microglia.</td>
<td>Welser-Alves and Milner 2013</td>
</tr>
<tr>
<td></td>
<td>• Has been shown to cause apoptosis of neuronal cells via mediation of iNos production.</td>
<td>Combs et al. 2001</td>
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<tr>
<td></td>
<td>• Perpetuates microglial activation via positive feedback loop.</td>
<td>Kuno et al. 2005</td>
</tr>
<tr>
<td></td>
<td>• Upregulated in AD patients.</td>
<td>Swardfager et al. 2010</td>
</tr>
<tr>
<td>Interleukin 4 (IL-4)</td>
<td>• Anti-inflammatory cytokine produced by microglia.</td>
<td>Lively and Schlichter 2018</td>
</tr>
<tr>
<td></td>
<td>• Has shown ability to decrease activity of pro-inflammatory microglia receptors in the presence of LPS.</td>
<td>Zhao et al. 2006</td>
</tr>
<tr>
<td></td>
<td>• Has shown potential to reduce levels of reactive oxygen species (ROS) which may help to improve neuronal survival.</td>
<td></td>
</tr>
<tr>
<td>Brain derived neurotrophic factor (BDNF)</td>
<td>• Neurotrophic factor which drives neuronal survival and plasticity in the brain.</td>
<td>Parkhurst et al. 2013</td>
</tr>
<tr>
<td></td>
<td>• BDNF expressed by microglia has shown to be neuroprotective due its role in synaptic modulation.</td>
<td>Wu et al. 2020</td>
</tr>
<tr>
<td></td>
<td>• BDNF upregulation may reduce microglial activation associated with aging.</td>
<td>Michael et al. 2019</td>
</tr>
<tr>
<td></td>
<td>• Lab4 probiotics have neuroprotective effects, possibly due to changes in BDNF expression.</td>
<td></td>
</tr>
<tr>
<td>Reactive oxygen species (ROS)</td>
<td>• ROS are produced by microglia in response to TLR recognition of DAMPs, including Aβ.</td>
<td>Schilling and Eder 2011</td>
</tr>
<tr>
<td></td>
<td>• Chronic activation of microglia results in high levels of ROS which may impact neuronal survival.</td>
<td>Spencer et al. 2016</td>
</tr>
<tr>
<td></td>
<td>• Genes such as inducible nitric oxide synthase (iNos) and Super oxide dismutase (SOD) could be useful targets.</td>
<td></td>
</tr>
<tr>
<td>Cluster of Differentiation 68 (CD68)</td>
<td>• Microglial activation marker.</td>
<td>Frackowiak et al. 1992</td>
</tr>
<tr>
<td></td>
<td>• Microglia tend to increase in activation status around Aβ plaques as part of gliosis.</td>
<td>Hendrickx et al. 2017</td>
</tr>
<tr>
<td>Transmembrane protein 119 (TMEM119)</td>
<td>• Microglial activation marker.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Could also be useful for determining microglial activation status and gliosis.</td>
<td>Satoh et al. 2016</td>
</tr>
<tr>
<td>Ki-67</td>
<td>• Proliferation marker.</td>
<td>Frautschy et al. 1998</td>
</tr>
<tr>
<td></td>
<td>• AD pathology results in gliosis, where the numbers of microglial cells increase around Aβ plaques.</td>
<td>Gerdes et al. 1983</td>
</tr>
<tr>
<td></td>
<td>• Useful for assessing increases in microglial proliferation which may correspond to gliosis.</td>
<td></td>
</tr>
</tbody>
</table>
**Transcriptomic analyses**

In order to provide a broader assessment of the effect of probiotic supplementation on microglial gene expression, it would be useful to extend the panel of biomarkers through the use of whole transcriptome profiling. Methodologies of use include well established technologies such as microarray analyses or newer techniques such as RNA sequencing. These approaches can help to pick out multiple genes and gene families of interest that may be upregulated or downregulated. The addition of post experimental bioinformatics would allow for specific signaling pathways to be delineated which are difficult to elucidate from a single gene RT-qPCR approach. These methods could be applied following in vitro use of the BV-2 cells line after stimulation with CM or mouse serum. Additionally, this method could also be applied following in vivo work, allowing for assessment of gene expression in specific brain regions such as the hippocampus in mice fed a probiotic supplemented diet.

Previous microarray work has been carried out to determine the transcriptomic profiles of microglia in AD and other neurodegenerative disease which has revealed some useful information to date including the effects of gut microbiota on microglia (Erny et al. 2015, Tabassum et al. 2019, Maleszew ska et al. 2020). Therefore, these approaches could be extremely useful in helping to decipher microglial cell biology under the influence of probiotic dietary modification.

**Microglial phenotypic changes**

Microglia are highly flexible in their ability to respond to alterations in their surroundings. At rest they exist in a relaxed, stratified shape with long spindle like processes which extend out to survey their surroundings (Nimmerjahn et al. 2005). In contrast, activated microglia exist in an amoeboid shape and retract in their processes (Davis et al. 1994). These distinctive differences in appearance are an aspect of microglial morphology that in future work could be used to assess the response to microglial cells to Lab4b and Lab4P probiotics.

Phenotypic changes could be captured through the use of immunocytochemistry (ICC) or immunohistochemistry (IHC). Antibodies for site specific markers of microglia such as Iba-1 or markers specifically upregulated in activated microglia such as CD68 and TMEM119 could be useful to assess microglial activation states and levels of gliosis (Ito et al. 1998, Hendrickx et al. 2017, Satoh et al. 2016). Software such as ImageJ (Fiji) and QuPath (GitHub) allow for standardised analysis of cells from images and have been used previously for
microglial characterisation and quantification (Young and Morrison 2018, Morriss et al. 2020). Changes in cell surface area, cell length and cell number could help to establish the extent of microglial activation upon stimulation with Lab4b or Lab4P probiotic metabolites. Generating data on the phenotypic changes would also help to validate these gene expression data.

Transwell and TEER assays

The in vitro assays at present utilised the BV-2 microglial cell line for cell viability and gene expression assays. While useful as a starting point for initial assessment of the effects of probiotic metabolites on BV-2 cells it may be useful in future experimentation to increase the complexity of the assays. Here BV-2s were monocultured and CM or serum applied directly to the cells diluted in the cell growth medium DMEM.

One way of increasing the complexity and physiological relevance of the assays could be to introduce transwells that could mimic the endothelial BBB. In vivo, the probiotic metabolites are unlikely to directly interact with microglial cells at high concentrations. The introduction of transwells could help to mimic endothelial barriers such as the gut wall and the BBB (Czupalla et al. 2014). Endothelial cell lines such as the human cerebral microvascular endothelial cell line (hCMEC/D3) develop tight junctions via Claudin-5 and Occludin and can be used as a BBB model (Weksler et al. 2005, Poller et al. 2008). Cell viability and gene expression assays could also be performed to assess the effects of the probiotic on BV-2s after filtration through the endothelial cell layer in such a cell culture system.

Previous work using BBB models have shown that probiotics may have a beneficial effect by supporting barrier integrity (Hoyles et al. 2018). Barrier integrity could also be measured assessed using transendothelial electrical resistance (TEER) assays which measure electrical resistance and determine the integrity of the cell barrier (Benson et al. 2013). Measurement of tight junction proteins such as Claudin-5 via staining or gene expression could help to determine if the probiotics are also having any effect on BBB function which could also help to elucidate in part the effects that probiotics may have. Typically, in AD the BBB becomes compromised and “leaky” which contributes to inflammation due to the passage of pro-inflammatory molecules into the brain (Carrano et al. 2011). Therefore, assessment of the ability of probiotics to improve tight junction functionality could also in part help to elucidate the mechanism behind the beneficial role they appear to play in AD.
Co-culture of microglia cells with astrocytes and neurons

Complexity of the in vitro experiments could be increased through co-culture. Microglia are in constant cross talk with astrocytes and neurons. Therefore, co-culture of cells could help to introduce further complexity of cellular signaling pathways which may provide a fuller picture of the effects that probiotics may have on inflammation and cell health.

Development of AD pathology around neurons leads to cell damage and death, leading to loss of cognitive function and brain atrophy (Masliah et al. 1994, Scheff et al. 2007). In the brain, microglia and astrocytes reside within the CNS milieu to protect neurons. Therefore, it is unsurprising that to ensure appropriate defence mechanisms the three cells communicate through complex cellular cross talk, much of which is still unclear.

To support normal neuronal functioning astrocytes contribute to the control of synaptic plasticity through the release of factors such as BDNF (Wu et al. 2004). They also play a key role in the mediation of the inflammatory response and evidence suggests that similarly to microglia, astrocytes undergo astrogliosis which has also been linked to the development of AD and cognitive impairment (Orre et al. 2014). Astrogliosis is considered to be a neuroprotective process (Goss et al. 1998, Nakase et al. 2004). However, the neuroprotective properties of astrocytes have been attributed to control via microglia (Chen et al. 2020). The pro-inflammatory cytokines produced by microglial cells in response to Aβ plaque such as IL-1β and IL-6 are important for astrocytic regulation and promotion of tissue repair (Shinozaki et al. 2017). One study has also shown that the AD risk gene TREM2 in microglia is an important gene required for communication with astrocytes and provides a means of regulating astrocytic synaptic modulation (Jay et al. 2019). Additionally, another study found that in the EAE mouse model a metabolite produced by gut bacteria had an influence on microglial production of TNF-α which in turn reduced the pro-inflammatory properties of astroglia, promoting neuroprotection (Rothhammer et al. 2018).

Therefore, when considering the possible neuroprotective and anti-inflammatory effects of probiotics in AD, it is key to keep in mind the cellular communication between microglia and astrocytes that play a role in neuronal defence. Previous work carried out shows that co-culture of these cells is possible and may provide useful insights into the cellular crosstalk in the context of neurodegenerative disease (Goshi et al. 2020). Future work should perhaps include co-culture models that allow for more extensive analysis of both cell types in this context or the use of organoids as discussed in Chapter 3.3.4. The effects of probiotics
on the relationship between microglia and astrocytes would be just as important to analyse as the effects on microglia alone.

6.3.2 Further work *in vivo*

*Expansion of two-week *in vivo* mouse study*

As discussed in Chapter 4.3.3, one of the limitations of the *in vivo* work carried out in this study was the duration of the study and the fact that a healthy non-diseased model was used. However, previous work carried out by Dr T. Webberley of Cultech Ltd. and Dr T. Hughes of Cardiff University has shown positive effects of using Lab4b and Lab4P probiotics in mouse models of neurodegeneration. Using the 3xTg mouse model of disease, probiotic formulations appear to improve are able to slow cognitive decline and improve neuroinflammation in these mice (unpublished work). This work has also examined the roles of probiotics in the modulation of metabolism (analysis of liver gene expression) and in modulation of the overall gut microbiota (by 16S sequencing of faecal samples).

Further *in vivo* analysis in this context could involve 16S sequencing of mouse faecal pellets from the work presented in this thesis to (i) align these findings with other work in diseased models already completed, and (ii) provide insight into the role of the gut microbiota and liver metabolites in the modulation of microglia functionality and phenotype. IHC staining used to identify microglial cell markers in brain tissue from these diseased animal models supplemented with the probiotic consortia in previous work could be utilised on samples obtained during in this work. Again, this could be useful to identify protein markers of microglia cell functionality and phenotype such as Iba-1, CD68 or TMEM119 as mentioned above.

During the dissection of the mice in this present study various organs including the brain, the duodenum, the ileum, Peyer’s patches and liver were taken, frozen down in liquid nitrogen and stored at -80°C. These tissue samples were intended for further use in the analysis of gene expression through RNA extraction and RT-qPCR to determine changes in gene expression of genes of interest in those tissues but has not yet been completed. It would be interesting to determine if the Lab4P probiotic supplementation over a two-week period induced any changes in gene expression in organs such as the brain and intestines.
Clinical Trials

In the longer term it would be interesting to expand and translate these *in vivo* studies further to include the use of human clinical trials. To date the clinical trials that have been carried out to assess the impact of probiotics on patients with AD have been limited, however some have shown promising results. One preliminary clinical trial where twenty mixed sex AD patients were supplemented with probiotics for four weeks found that the probiotic impacted upon the gut microbiota and resulted in higher serum levels of the gut microbiota metabolites thought to cause an increase in macrophage activity (Leblhuber et al. 2018). Another clinical trial looked at AD patients following 3 months of probiotic supplementation and found that those that received the probiotic scored better in a mini mental state exam, however there was little difference found in inflammation levels (Akbari et al. 2016).

While clinical trials would be informative to pursue in this research context, working with patient cohorts can be very challenging for a number of reasons including (i) the ability to fully consent to participation in a trial, (ii) difficulty in recruiting willing participants, (iii) ethical approval required for such work and (iv) the cost of correctly powered trials. Methods for use in potential future clinical trials should carefully be considered. For example, the use of methods that are comparable between mice and human could be very informative, for example the Cambridge Neuropsychological Test Automated Battery (CANTAB) could be used to directly compare *in vivo* work with mice to clinical trials carried out with humans (Sahakian et al. 1988, Romberg et al. 2013). Using behavioral testing methods such as the CANTAB could be a helpful way of eventually translating the findings from animal work into clinical trials with humans, allowing for translation and validation of *in vivo* findings.

However, at present, although increasing evidence does appear to suggest a beneficial role for probiotics in AD, our knowledge of the mechanisms behind this is still very limited. Perhaps it is therefore more important at this stage to prioritise improving our understanding of the mechanisms underlying the effects that probiotics appear to exert at *in vivo* level before moving work into clinical trials.

6.4 Context and long-term impact of present study

This thesis is a contribution to a larger AD study being carried out at Cardiff University in collaboration with Cultech Ltd. The work presented in this thesis aimed to assess changes in the inflammatory profile of microglial cells upon treatment with the Lab4 family of probiotics
based on previous work and will add to work already carried out to create a fuller picture of the beneficial impact that probiotics may have on the onset and progression of dementia.

The main research findings from this present study suggest that Lab4b and Lab4P can modulate levels of cytokine gene expression in BV-2 cells. Additionally, an initial trend was observed when serum taken from mice fed a Lab4P diet was applied to BV-2 cells in the presence of synthetic Aβ (1-42) as gene expression levels of IL-6 appeared to be reduced, however this was not found to be a statistically significant decrease and warrants further investigation. If statistically confirmed through further work, this may suggest that probiotics may play a beneficial role in impacting upon microglial cell function and the role microglia play in mediating inflammation in the context of AD. However, a great deal of further work needs to be carried out to validate these observations and elucidate the mechanisms behind these findings to understand why this is the case.

Our current knowledge of the potential of probiotics to impact on neurodegenerative diseases is expanding rapidly and to date many studies have shown promising results (Bonfili et al. 2017, Distrutti et al. 2014). Since the deposition of AD pathology occurs years, often decades, before a patient is diagnosed, probiotics would have to be taken early on in the disease if they were to confer protective benefits against AD onset and progression. It is important to keep in mind that probiotic supplementation is not necessarily a therapeutic strategy but an approach that could slow or at least minimise the onset and development of AD pathology, possibly through the improvement of disease associated inflammation. This strategy supports the idea that prevention is better than cure. This approach could also be of economic benefit, saving money and improving the current and anticipated strain on healthcare services that AD imposes.

In recent years the idea of using predictive and personalised medicine to treat patients with a range of ailments has emerged (Hu et al. 2013, Wang et al. 2021). Under these circumstances of discreet patient stratification, probiotics could prove to be beneficial tools for developing a multi-faceted approach to minimising the progression of AD. The gut microbiome of individuals is typically distinctive and is largely influenced by individual dietary habits and environmental factors (Kashtanova et al. 2016, Rothschild et al. 2018). There is potential for probiotic blends to be personalised for those who require supplementation of specific gut bacterial strains or if it transpires that certain strains of probiotic bacteria give rise to specific metabolites that have disease preventing potential. Advances in our understanding of risk genes could also be useful in applying probiotics to impact upon dementia onset and
progression. Alternatively, as one study showed, a prediction of AD development could potentially be made from the gut microbiota of individuals, as gut microbiota composition has also been shown to impaired in patients with MCI (Li et al. 2019). Probiotics could be administered before any substantial AD pathology has had a chance to begin developing or help to lessen increases in levels of inflammation during the development of the disease, which may result in an improved AD prognosis. This could potentially help to improve the outlook for AD patients and alleviate the need for more intensive treatments further down the line.

6.5 Conclusion

From this work it appears that the Lab4b and Lab4P probiotics have the ability to modulate BV-2 cell cytokine gene expression to some degree. Currently, the extent of the impact and involvement that these probiotic induced immunomodulatory changes may have on the onset and progression of AD are not clear. This work provides preliminary insights into the potential effects probiotics may confer on microglial cells. However, further work is required to improve our understanding of the impact of probiotics on microglial cell function and the mechanisms behind their effects.

At the current rate, AD is projected to affect over 100 million people by 2050, if there is no improvement in current treatment options (Brookmeyer et al. 2007). Evidence suggests that probiotics as an intervention strategy do hold potential to impact upon dementia onset and progression. However, there are still many unanswered questions surrounding this topic. The complexities of the relationship between gut microbiota, microglial cell function and AD pathology are yet to be fully understood. Developing our understanding of this area further could help to change the landscape of how neurodegenerative disease are managed. Particularly, in the early stages of disease before the manifestation of pathology and symptoms to help work towards disease prevention rather than cure.
Figure A1. Assessment of BV-2 expression of target genes in response to 4-hour treatment with 50% (v/v) probiotic conditioned media (CM). BV-2 cells were grown in 24 well plates at 300,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before incubation with Lab4b (A,B,C) or Lab4P (D,E,F) probiotic CM for 4 hours. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as ΔCt values. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included unpaired t-test for all data. * = P≤0.05, *** = P≤0.001
Figure A2. Assessment of BV-2 expression of target genes in response to 16-hour treatment with 50% (v/v) probiotic conditioned media (CM). BV-2 cells were grown in 24 well plates at 300,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before incubation with Lab4b (A,B,C) or Lab4P (D,E,F) probiotic CM for 16 hours. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as ΔCt values. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included unpaired t-test for all data. * = P≤0.05, ** = P≤0.01, **** = P≤0.0001.
Figure A3. Assessment of BV-2 expression of target genes in response to 4-, 8- and 16-hour treatment with low concentrations of Lab4b and Lab4P probiotic conditioned media. BV-2 cells were grown in 24 well plates at 300,000 cells per well with DMEM and 100 U/mL P/S) for 24 hours before incubation with a low range of Lab4b (A, B, C) or Lab4P (D, E, F) probiotic conditioned media concentrations. Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as ΔCt values. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests were carried out on ΔCt values and included Ordinary Two-Way ANOVA followed by Dunnett’s Multiple Comparison Post Hoc Test for all graphs. * = Ps0.05, ** = Ps0.01, *** = Ps0.001, **** = Ps0.0001.
Figure A4. Effect of control and Lab4P mouse serum on BV-2 cytokine gene expression over a 4 and 16-hour treatment period. BV-2 cells were grown in 48 well plates at 150,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before removal of medium and incubation with either control or Lab4P mouse serum stated concentrations of mouse serum (MS) for 4 hours (A, B & C) or 16 hours (D, E & F). Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as ΔCt values. N=3 biological repeats for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included Two-Way ANOVA and post-hoc Sidak’s multiple comparison testing. * = P≤0.05.
Figure A5. Effects of control and Lab4P mouse serum on BV-2 cytokine gene expression in the presence of amyloid β (1-42) challenge over a 4- and 8-hour treatment period. BV-2 cells were grown in 48 well plates at 150,000 cells per well with DMEM (10% (v/v) FBS, 100 U/mL P/S) for 24 hours before incubation with stated concentrations of synthetic amyloid-β (Aβ) and either control or Lab4P treated mouse serum for 4 hours (A, B, C) or 8 hours (D, E, F). Expression of IL-1β, IL-6 and IL-10 were assessed via RT-qPCR. β-Actin was used as a housekeeper gene. Results are shown as ΔCt values. N=3 for all graphs. Data are expressed as the mean +/- standard error of the mean (SEM). Statistical tests included Two-Way ANOVA and post-hoc Sidak’s multiple comparisons.
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