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The effect of probiotic supplementation on cognition and mood in healthy older adults, and an exploration into microbially-derived metabolites

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

The gut microbiota (GM) has emerged as a key player in human health, with growing evidence to suggest a role in cognitive function and mental wellbeing via a host of bidirectional microbiota-gut-brain (MGB) pathways. As such, dietary interventions such as probiotic supplements are gaining attention for the potential to modulate the GM and therefore influence neural function and behaviour. In particular, concurrent age-related changes in the GM and cognitive function in older adults render this a population that may benefit from probiotic supplementation. One potential MGB pathway via which probiotics may take effect is through the bacterial production and modulation of neuroactive metabolites, where previous *in vitro* work suggests that bacteria found enterically have the capacity to produce neurotransmitters. However, it is unclear at present whether bacteria have the capacity to produce neurotransmitters under physiologically relevant conditions, or how the addition of probiotic bacteria may influence neuroactive metabolite production.

As such, the current work combined *in vitro* and *in vivo* approaches to further our understanding of bacterially derived neurotransmitters as a potential mechanism and explore the effect of a probiotic supplement on cognitive function and mood in healthy ageing adults. *In vitro*, the results provide evidence for the production of several neurotransmitters in faecal microbiota under conditions relevant to the human colon, but limited evidence for an effect of additional probiotic bacteria. In healthy older adults, chronic supplementation with a multi-species probiotic was associated with reduced cognitive reactivity to sad mood and potential benefits to executive function under high cognitive demand. Acute probiotic supplementation was associated with improved reaction times during executive function. This work therefore provides novel insight into bacterial production of neurotransmitters, and evidence to support a beneficial effect on executive function and cognitive reactivity to sad mood following probiotic supplementation within a healthy older adult population.

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Table of Contents

Declaration .... **ii**

Abstract .... **iii**

Acknowledgements .... **iv**

Table of Contents .... **v**

Table of Figures .... **ix**

Table of Tables .... **x**

Chapter 1 – An introduction to the microbiota-gut-brain axis and probiotics **1**

1.1 General introduction *1*

1.2 The microbiota-gut-brain axis *1*

1.2.1 pre-clinical evidence 3

1.2.2 Clinical evidence 5

1.2.3 Microbiota-gut-brain pathways 7

1.3 An introduction to probiotics *14*

1.4 An in vitro approach to studying the effect of probiotics on the microbiota-gut-brain axis *17*

1.4.1 Batch cultures 17

1.4.2 Continuous culture models 18

1.5 Thesis objectives and specific research questions *20*

1.5.1 Objectives 20

1.5.2 Research questions 20

Chapter 2 – the effect of probiotics on cognitive function across the human lifespan: A systematic review .. **23**

2.1 Introduction *23*

2.2 Method *25*

2.3 Results *27*

2.3.1 Study characteristics 27

2.3.2 Infants and children 42

2.3.3 Young and middle-aged adults 42

2.3.4 Ageing adults 45

2.4 Discussion *47*

2.4.1 Single versus multi-strain supplements 50

2.4.2 Species/strains 51

2.4.3 Dose 52

2.4.4 Length of intervention period 52

2.4.5 Areas of cognition 53

2.4.6 Mechanisms of action 54

2.4.7 Limitations and considerations for future work 55

2.4.8 Conclusions 57

Chapter 3 – The effect of probiotic bacteria on the composition and metabolite production of faecal microbiota using in vitro batch cultures 59

3.1 Introduction 59

3.2 Method 62

3.2.1 Preparation of probiotic strains 62
3.2.2 Faecal sample preparation 62
3.2.3 Batch culture fermentation 63
3.2.4 Preparation of samples 63
3.2.5 Fluorescence in situ hybridisation with flow cytometry (flow-FISH) 64
3.2.6 Gas Chromatography 65
3.2.7 Liquid Chromatography Gas Spectroscopy 66
3.2.8 Statistical analysis 68

3.3 Results 68

3.3.1 Donors 68
3.3.2 Enumeration of bacteria with flow-FISH 68
3.3.3 Short-chain fatty acids 72
3.3.4 Neurotransmitters 74

3.4 Discussion 76

3.4.1 Conclusion 80

Chapter 4 – Exploring the effect of a multi-strain probiotic supplement on bacterial community and metabolite production in the faecal microbiota of older adults, using *in vitro* gut models 81

4.1 Introduction 81

4.2 Methods 84

4.2.1 Continuous 3 stage model 84
4.2.2 Multi-strain probiotic supplement 85
4.2.3 Preparation of samples 86
4.2.4 Fluorescence in situ Hybridisation with Flow Cytometry (flow-FISH) 86
4.2.5 16s rRNA sequencing 88
4.2.6 Gas Chromatography 89
4.2.7 LCMS/MS 90
4.2.8 Data analysis 91

4.3 Results 91

4.3.1 Donors 92
4.3.2 Fluorescence in situ Hybridisation 92
4.3.3 16s rRNA Sequencing 92
4.3.4 Short-chain fatty acids 92
4.3.5 LCMS 94

4.4 Discussion 97

Chapter 5 – the effect of a multi-strain probiotic on cognitive function and mood in healthy older adults 102

5.1 Introduction 102

5.2 Method 104

5.2.1 Participants 104
5.2.2 Design 105

5.2.3 Procedure ... 107
5.2.4 Outcome measures ... 108
5.2.5 Data analysis ... 116

5.3 Results ... 119
5.3.1 Participant demographic data ... 119
5.3.2 Missing data ... 120
5.3.3 Cognitive measures ... 121
5.3.4 Mood measures ... 132
5.3.5 Additional measures ... 136
5.3.6 Summary of results ... 140

5.4 Discussion ... 142

Chapter 6 - Final discussion ... 149

6.1 Assessing microbial production of neurotransmitters and exploring how probiotic bacteria might influence the production of neuroactive metabolites and microbial community ... 149

6.2 Exploring the effect of a multispecies probiotic supplement on cognition and mood in healthy older adults ... 153

6.3 Limitations ... 158
6.3.1 Limitations of *in vitro* models ... 158
6.3.2 Limitations of the behavioural trial ... 158

6.4 Future work ... 160
6.4.1 *in vitro* considerations ... 160
6.4.2 Considerations for future RCTs ... 161

6.5 Final conclusions ... 162

References ... 163

Appendices ... 208

Appendix 1 – Quality Criteria Checklist from the Academy of Nutrition and Dietetics ... 209
Appendix 2 – Data extraction template for primary research from the Academy of Nutrition and Dietetics ... 211
Appendix 3 – Results of the Quality Criteria Checklist from the Academy of Nutrition and Dietetics ... 212
Appendix 4 – Batch culture data (short-chain fatty acids, neurotransmitters and bacterial enumeration) presented by donor for comparison. ... 218
Appendix 5- Krona charts representing change in relative abundance between steady state 1 and steady state 2 for *Lactococcus* (A) and *Roseburia* (B) ... 231
Appendix 6 – Gut model data (short-chain fatty acids, neurotransmitters and bacterial enumeration) presented by donor for comparison. ... 233
Appendix 7 – Study information document ... 242
Appendix 8 – Study consent form ... 245
Appendix 9 – UREC ethics committee approval ... 246
Appendix 10 – Intervention randomisation schedule ... 247
Appendix 11 – Demographics questionnaire ... 248
Appendix 12 – Montreal Cognitive Assessment ... 249
Appendix 13 – Participant compliance diary ... 250
Appendix 14 – End of study questionnaire ... 251

Appendix 15 – Debrief.. 252
Appendix 16 – Balanced Latin Square for alternative task battery randomisation.................................. 253
Appendix 17 – RAVLT word lists.. 254
Appendix 18 – Linear mixed model results tables 255

Table of Figures

Figure 1.1 Schematic of gut-brain axis pathways……8
Figure 1.2 Schematic of a batch culture fermentation model……18
Figure 1.3 Schematic of three-stage continuous culture model……19
Figure 2.1 PRISMA flow diagram……27
Figure 3.1 SCFA concentrations after 0, 8 and 24 hours of fermentation……72
Figure 3.2 SCFA concentrations after 0, 8 and 24 hours of fermentation (without positive control)……74
Figure 3.3 Neurotransmitter concentrations after 0, 8 and 24 hours of fermentation……75
Figure 4.1 SCFA concentrations at SS1 and SS2……93
Figure 4.2 Neurotransmitter concentrations at SS1 and SS2……95
Figure 5.1 Schematic of RCT design……106
Figure 5.2 TST accuracy by trial type……127
Figure 5.3 TST RTs……128
Figure 5.4 TST RTs by trial and switch type……129
Figure 5.5 Aggression, hopelessness and rumination scores (LEIDS-r)……134
Figure 5.6 Relative abundance at the level if order in faecal microbiota pre- and post-intervention……138
Figure 5.7 Relative abundance of *Lactococcus* pre- and post-intervention……139

Table of Tables

Table 2.1 Key characteristics of included studies……28
Table 2.2 Cognitive domains significantly affected following probiotic intervention……39
Table 3.1 Fluorescence in situ Hybridisation probes……65
Table 3.2 LCMS/MS parameters……67
Table 3.3 Enumeration of bacterial groups assessed using FISH ……70
Table 4.1 Fluorescence in situ Hybridisation probes……87
Table 4.2 LCMS/MS parameters……91
Table 4.3 Enumeration of bacterial groups assessed using FISH ……96
Figure 5.1 Participant demographic information……120
Figure 5.2 RAVLT data……122
Figure 5.3 CBTT data……125
Figure 5.4 TST data……130
Figure 5.5 Go/No-Go data……131
Figure 5.6 Mood outcome data……135
Figure 5.7 Results summary……140
Figure 5.8 FFQ data……147

Chapter 1 – An introduction to the microbiota-gut-brain axis and probiotics

1.1 General introduction

It has long been appreciated that the gut has an influence on human health, but more recently the gut microbiota has emerged as a key player in this gut-host relationship. One aspect of this is communication between the gut and brain known as the gut-brain axis. As the field gains traction, more research is concerned with how the gut microbiome may be leveraged to improve host health through the GBA. As such, probiotic bacteria are now widely being investigated as a potential therapeutic, both to alleviate clinical disorders and optimise health in the absence of disease. However, despite the recent surge in research, relatively little is understood about the mechanisms via which probiotics interact with the intestinal microbiota to influence host health and behaviour. The aim of this work was therefore to gain a better understanding through *in vitro* models of how probiotic bacteria may influence human health and behaviour via neuroactive metabolites, and to explore the potential for a multispecies probiotic supplement to improve cognitive function and mood in ageing adults. Explicit research questions are outlined in chapter 2, section 5.

1.2 The microbiota-gut-brain axis

The gut-brain axis, a concept perhaps recognised for many centuries, refers to the complex bidirectional relationship between the brain and the gastrointestinal tract (GIT) influencing many aspects of human health and behaviour including food intake, digestion, immune function, and sleep (Margolis, Cryan & Mayor., 2021). The brain influences the gut in a top-down manner via several routes, including the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system, to ensure homeostasis of the GIT and regulate gut motility, intestinal transit time, and intestinal permeability (Mayer & Tillisch., 2011). The effect of psychological stress on the gut is well documented, enhancing proinflammatory response leading to increased gut permeability and bacterial translocation (Herselman, Bailey & Bobrovskaya., 2013; Lyte et al., 2010) and altering the metabolism of tryptophan in the gut to favour the kynurenine pathway, which produces neurotoxic

quinolinic acid (Kennedy et al., 2017). This brain-gut connection is further implicated in gastrointestinal disorders such as inflammatory bowel disease (IBD), where mucosal mast cells in the gut translate stress signals from the brain into proinflammatory responses, leading to chronic inflammation and hyperpermeability of the intestinal barrier (Farhadi et al., 2007). Symptoms if IBD are often exacerbated by stress, and it is thought that initiation and perpetuation of the disease is stress mediated (Hart & Kamm., 2002).

In tandem, the gut is able to influence the central nervous system from a bottom-up perspective. A specific role of the gut microbiota (GM) in this communication network was highlighted in cases of hepatic encephalography (HE), where poor cognitive symptoms were improved by altering the gut microbiome using probiotics (introduced in section 1.3), and in cases of antibiotic-induced psychosis, where the provision of various antibiotics led to patients presenting with episodes of psychosis (Cumming 1986; Reeves 1992; McCue 1992). Since then, research has converged over the last couple of decades to establish the gut microbiota as a key mediator of gut-brain interactions. The GM plays a critical role in determining overall host health (Jandhyala et al., 2015) and is shaped by a number of factors across the lifespan, including mode of delivery, host genetics, age, diet and stress (Long-Smith et al., 2020). Residing in the human GIT, the GM is a complex ecosystem of microorganisms including bacteria, eukarya and archaea. Due to recent advances in sequencing technologies, we are now able to determine with good accuracy which microorganisms are present in the human microbiome from a phylum level to genus and even species (Gibbs., 2020). There are a number of compositional analysis techniques that can be used to characterise the gut microbiota. The most common indices include the relative abundance of specific microbes, the diversity of the microbes, where α-diversity describes the diversity of a single sample and β-diversity described the diversity between samples, and robustness – a measure of the degree of change in an ecosystem. The difference or similarity between microbiota samples can also be visualised using principal component analysis (PCA), while functional metagenomics – analysis of the genetic material within a sample - provides an estimate of the genetic function of microbiome, giving insight not only into what is there, but also into what these microbes are doing and how the microbial community may affect the host. Although the previously well-cited prediction that microbes outnumber human cells by 10:1 has recently been revised in favour of a figure closer to 1:1, estimates still suggest there are 100 times more genes in the gut microbiome than the human genome (Gilbert et al., 2018), highlighting the functional potential of these organisms. It is increasingly clear that this top-down, bottom-up exchange between enteric microbiota and the brain, often now referred to as the

microbiota-gut-brain axis (MGB axis), represents a fragile, symbiotic relationship that contributes to both host health and disorders of the body and the brain.

1.2.1 pre-clinical evidence

Evidence for a microbiota-gut-brain axis to date stems largely from studies in germ-free (GF) mice (Luczynski et al., 2016). GF mice are raised in a sterile environment, leaving the animals devoid of intestinal microbiota. These models therefore provide a critical tool in understanding the effect of the GM on the central nervous system (CNS), allowing for the assessment of physiological and behavioural phenotypes both in the absence of intestinal microbiota and following the selective re-introduction of bacteria. In a landmark study, Sudo and colleagues (2004) found GF mice to elicit an exaggerated stress response to acute restraint stress which was normalised following the provision of *Bifidobacterium infantis*, suggesting the GM is implicated in the hypothalamic-pituitary-adrenal axis. Paradoxically, despite this hyperactivity in endocrine signalling, GF mice have consistently been reported to exhibit less anxiety-like behaviours than conventionally colonised mice (Neufeld et al., 2010; Heijtz et al., 2011; Clarke et al., 2013; Arentsen et al., 2015). In addition, alterations in cognitive functions have been observed in GF models. For example, studies report impairment in short term and working memory using measures such as the novel object recognition and T-maze tests (Gareau et al., 2011), as well as altered social cognition, where GF mice demonstrated no preference for novel over familiar mice, which would ordinarily be the case (Desbonnet et al., 2014; Luczynski et al., 2016).

Faecal microbiota transplant (FMT) refers to the medical practice of transferring the faecal microbiota from one host to the colon of another (Allegretti et al., 2019). Importantly, colonization of GF mice via FMT from mice with a normal gut microbiota reversed these observations, such that the behavioural and cognitive phenotypes of the previously germ-free mice are altered post-transplant to that seen in the conventional mice (Heijtz et al., 2011; Clarke et al., 2013). Further evidence for a microbiota-gut-brain connection is found in FMT research of disease state models where neural function or behaviour is negatively affected. For example, FMT treatment from healthy mice was found to attenuate declines in learning and memory, reduce amyloid-B deposition and phosphorylation of tau proteins, and restore gut microbiota diversity and function in a mouse model of Alzheimer’s disease (AD) (Sun et al., 2019). Similar observations have been made in models of Huntington’s and Parkinson’s disease (PD) (Kang et al., 2021; Gubert et al., 2022), suggesting that targeting the GM through FMT may provide a potential therapeutic tool for several clinical

conditions affecting the CNS. Perhaps most excitingly, Zheng and colleagues (2016) demonstrated that transferring pooled faecal microbiota from human males with major depressive disorder (MDD) into GF mice resulted in depressive-like behaviour in the humanised mice, compared with GF mice who were colonized via FMT from healthy males without a mental health diagnosis. This depressive symptomology was coupled with an altered GM profile two weeks post-FMT that mirrored that as seen in the MDD donors, suggesting the depressive phenotype to be transmissible via the gut microbiome. This transfer of depressive-like behaviour from humans to GF animals via FMT has since been replicated in rats, coupled with findings of altered tryptophan metabolism (Kelly et al., 2016), hippocampal neurotransmitters, HPA axis function and inflammatory cytokines (Liu et al., 2020). In addition to transference of behavioural phenotypes, neuroimaging techniques have observed alterations in brain structure and function following FMT from humans with attention deficit hyperactive disorder (ADHD) in GF mice. These structural changes observed in the ADHD colonized mice were in line with previously reported characteristics of the human ADHD brain, and correlated with altered GM profile following FMT (Tengeler et al., 2020).

Further to germ-free animal models, antibiotic interventions provide an additional experimental approach to study the role of the GM. Antibiotics are known to disturb the microbiota ecology leading to dysbiosis, and therefore allow for the study of brain and behaviour in models of microbial depletion. Consistent pre-clinical evidence for altered social behaviour, increased psychiatric symptoms, and impaired cognitive function is reported following antibiotic induced dysbiosis, and these effects are evident from early post-natal models through to adults (Bercik & Collins., 2014; Zhan et al., 2018; Zhao et al., 2020; Karakan et al., 2021). Changes in behaviour and cognition were often correlated with GM characteristics, and rebalancing of the GM via FMT from normally colonized microbiota or probiotics (to be discussed in section 1.3) led to an amelioration of these deficits. While there are limitations to the use of antibiotics for studying the role of the GM on neural function (Champagne-Jorgensen et al., 2019), the effects of microbial depletion mirror those seen in GF models and provide further evidence for a microbiota-gut-brain axis.

The accumulating evidence from germ-free, microbiome depleted and FMT animal models provides strong indication for a bidirectional relationship between the gut microbiome and the central nervous system which in turn impacts neural health and behaviour. However, it is important to emphasise that while these models provide an important and necessary tool for exploring the association between microbiota and the CNS, findings from pre-clinical research do not necessarily translate to humans.

1.2.2 Clinical evidence

Several cross-sectional cohort studies have investigated the association between the human GM and psychiatric and neurological disorders. Typically, these studies have reported alterations in faecal microbiota in clinical populations compared with healthy controls, and this is true across various disorders including PD (Elfil et al., 2020), AD (Varesi et al., 2022), schizophrenia (Fond et al., 2020), MDD (Malan-Muller et al., 2018; Valles-Colomer et al., 2019), ADHD (Carmen Cenit et al., 2017) and autism spectrum disorder (ASD) (Xu et al., 2019). However, findings are often somewhat conflicting between studies assessing the same condition. For example, in individuals with MDD, researchers have reported both increased (Jiang et al., 2015) and decreased bacterial diversity (Kelly et al., 2017) compared to non-MDD individuals. These discrepancies highlight that, despite advances in sequencing technologies, understanding the association between the gut microbiota and human disorders is complex, and diversity metrices may not provide the most meaningful approach since they do not indicate how the bacterial community has changed in terms of what microbes are present, only that the diversity of species is measurably different or not. Indeed, there are several covariates shown to affect microbiome ecology including medication, diet, and lifestyle factors, many of which are not measured or accounted for when exploring the association between GM and clinical disorders (Falony et al., 2016). Additionally, the markers of a 'healthy' microbiome are still under debate, making it challenging to assess where there may be deviations in clinical populations (Falony et al., 2016). It may be that alterations in the GM, either as a cause or a consequence of disease, are not evident in measures of bacterial composition, and instead species interactions and metabolic output, such as short chain fatty acids, bile acids, tryptophan derivatives, and many others may be of greater significance in finding disease markers in the human GM.

Neuroimaging techniques provide an additional tool for exploring MGB interactions beyond microbiota-disease associations. Irritable Bowel Syndrome (IBS) is a chronic condition characterised by abdominal pain and altered bowel habits and is highly comorbid with psychiatric conditions such as anxiety and depression (Fadgyas-Stanculente et al., 2014). In line with previous work, Labus and colleagues (2017) identified sub-groups of irritable bowel syndrome (IBS) patients based on differences in the relative abundance of the *Firmicutes: Bacteroidetes* ratio, where some IBS patients presented similarly to healthy controls and others demonstrated a distinct microbial signature with higher abundance of *Firmicutes*-related taxa (IBS1). Using magnetic resonance imaging (MRI), the authors found structural brain differences between the IBS1 and other subjects, particularly in the

sensory integration and salience network regions, which correlated with their altered microbial taxa. Similarly, microbiota profiles in adolescents and adults with attention deficit hyperactive disorder (ADHD), characterised by higher relative abundance of *Bifidobacterium* and lower abundance of Clostridiales were associated with reward processing during an fMRI task of reward anticipation, where an a priori hypothesised increase in bacterial gene functionality for the encoding of the enzyme cyclohexadienyl dehydratase in the ADHD group was significantly associated with altered striatal activation during the reward task (Aarts et al., 2017). This particular enzyme is involved in the synthesis of dopamine, which is heavily implicated in reward processing and the mesolimbic reward pathway, of which the ventral striatum is a part. Finally, Tillisch and colleagues (2017) demonstrated that, in a group of healthy women, distinct GM profiles could be clustered based on the abundance of *Bacteroides* or *Prevotella,* and these GM profiles were associated with differing hippocampal responses to negative images during fMRI and differences in white and grey matter density in regions of the brain implicated in emotional, attentional, and sensory processing. These findings were also complimented by responses to the Positive and Negative Affect Schedule completed after viewing images, as women with a greater abundance of *Prevotella* and less hippocampal engagement, thought to be associated with increased emotional arousal, showed greater emotional response to negative images. The results of such studies should be interpreted with caution, not only due to being small in sample size and cross-sectional in design, but often microbiota-brain correlations are not specified a priori, or hypothesis driven. However, the findings from these brain imaging studies do provide preliminary evidence for involvement of specific taxa and their predicted metabolites in contributing to these structural changes, both in clinical and healthy populations.

Perhaps the most compelling evidence for a MGB axis comes from an open-label pilot FMT studies in humans. In an open-label clinical trial, 18 individuals aged 7-16 diagnosed with autism spectrum disorder (ASD) and comorbid gastrointestinal issues received FMT treatment from standardised healthy human faecal microbiota for 8 weeks (Kang et al., 2017). Subjects were randomised to receive FMT orally or rectally, and received an initial high dose followed by daily lower dose treatments for the remainder of the study. On average, participants reported an 82% improvement in gastrointestinal symptoms after treatment, which remained at follow-up 8 weeks post-treatment. ASD related behaviours also significantly reduced following treatment, and these changes were once again maintained at follow-up. FMT led to improved microbial diversity and increased relative abundance of the beneficial genera *Bifidobacterium* and *Prevotella,* where initial underrepresentation of *Bifidobacterium* in ASD subjects was corrected to match that seen in age and gender matched neurotypicals. Strangely the authors also reported an increase in the relative

abundance of *Desulfovibrio* following FMT – a genus not typically thought of as beneficial to the host, and one that has been related to the pathology of ASD (Finegold., 2011), although reports do vary (Kang et al., 2013). Most impressively, the authors conducted a follow up in all participants and found that alterations in GM composition and the reduction in both gastrointestinal issues and ASD behaviours were maintained 2 years post-treatment (Kang et al., 2019). Although the results of this study are noteworthy, it should be highlighted that the trial was open-label and used the antibiotic vancomycin as a preparation step prior to FMT, which itself has been reported to induce a benefit to ASD symptoms in children (Sandler et al., 2000). Since all participants combined vancomycin treatment with FMT, it is not possible to ascertain the individual contribution of each treatment. However, despite the preliminary nature of this work, these results do implicate changes in microbial composition following FMT in the alteration of behavioural phenotypes in ASD and highlight the profound potential for an effect of GM composition on the brain and behaviour. Several small studies have now also assessed the potential for FMT to ameliorate comorbid symptoms of depression and anxiety in those with IBS or irritable bowel disease (IBD), as reviewed by Settanni and colleagues (2021). Generally, the results of these trials were promising, with most IBS patients treated with active FMT displaying an improvement in depressive symptomology post-treatment. However, it should be noted that only two were RCTs, and improvements in depressive symptoms post-treatment were typically not maintained at later follow-ups. Similar conclusions were drawn in a recent narrative review, where it is suggested that altering the GM via FMT provides a promising therapeutic intervention in psychiatric disorders, but a greater number of larger, well controlled clinical trials are needed to assess efficacy across conditions (Vasiliu., 2022).

1.2.3 Microbiota-gut-brain pathways

While there is a plethora of evidence to support the existence of a microbiota-gut-brain axis, the precise mechanisms via which our gut microbes might exert such marked effects on neural function and behaviour are still somewhat elusive. Understanding how the GM influences the brain is of critical importance to being able to harness the microbiota as a tool for ameliorating disease and supporting brain health (Chakrabarti et al., 2022). The key pathways identified to date include the vagus nerve, immune signalling, neuroendocrine system, and microbially derived metabolites, which include neuroactive compounds, their precursors, and short chain fatty acids (SCFAs) (figure 1.1). Most evidence stems from pre-clinical work, although the number of human trials including neurochemical measures to assist in the understanding of MGB axis pathways is beginning to increase.

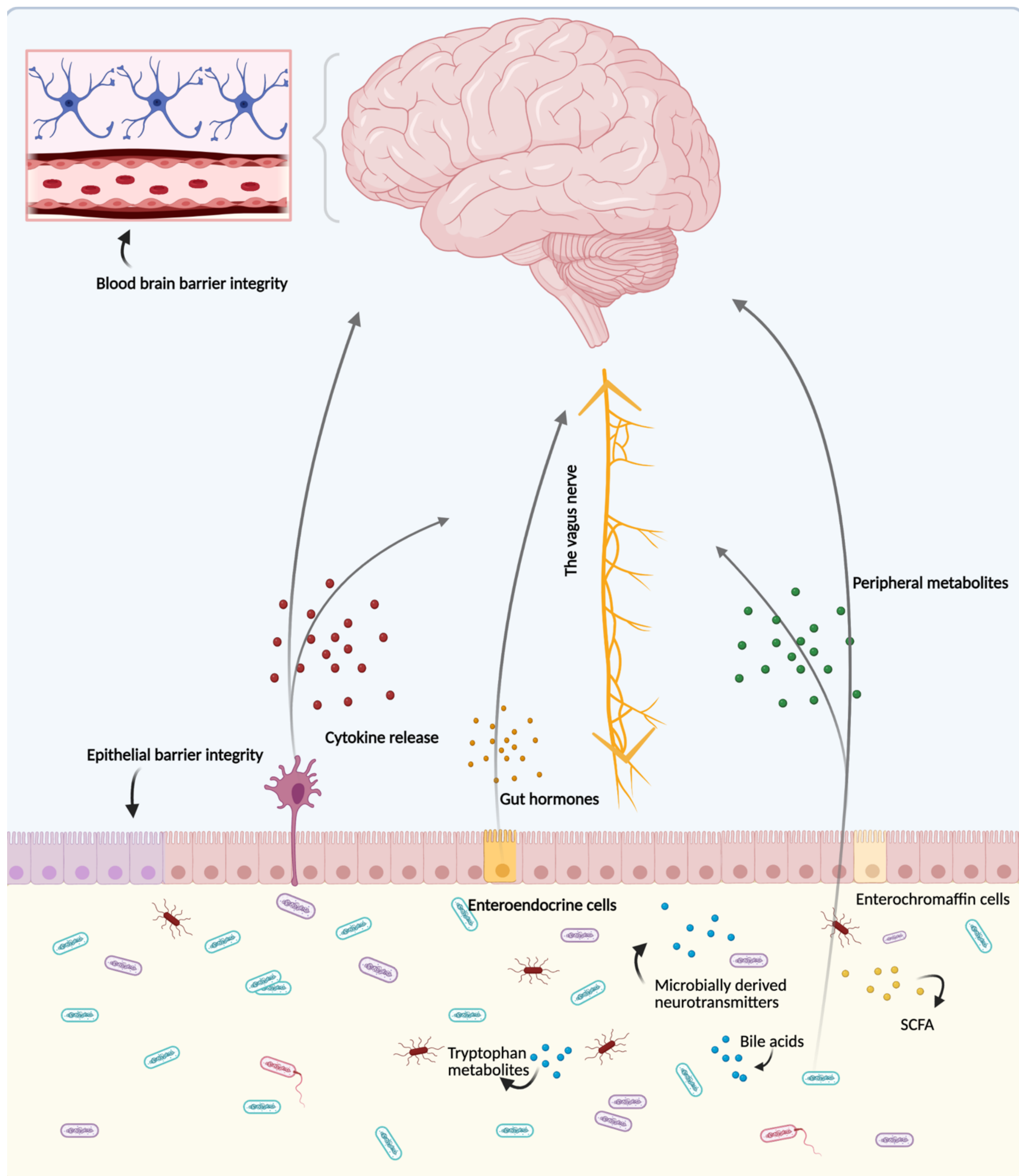


Figure 1.1 – Schematic outlining the key microbiota-gut-brain communication pathways that may be modulated by probiotic interventions to affect cognitive health, including immune and enteroendocrine signalling, vagal signalling, gut epithelial barrier and the blood brain barrier integrity, and synthesis of short-chain fatty acids (SCFAs), neurotransmitters, and other neuroactive precursors (illustration made with BioRender.com).

1.2.3.1 Microbially derived metabolites

Short Chain Fatty Acids

Short chain fatty acids are the most predominant of the microbially derived metabolites and are produced by enteric microbes as a product of indigestible dietary fibre fermentation (Tan et al., 2014). The most common SCFAs are acetate, propionate, and butyrate, which are generally found in a 60:20:20 ratio in the colon and stool, respectively (Den Besten et al., 2013). SCFAs are thought to affect gut-brain signalling via several mechanisms. Firstly, SCFAs can enhance gut barrier integrity through the improvement of epithelial tight junctions (Peng et al., 2009), which in turn reduces the risk of inflammation in the peripheral and central nervous systems and the subsequent risk for neurological disorders such as multiple sclerosis, PD and AD. SCFAs can also bind to several receptor types, including G-protein coupled free fatty acid receptors 2 (FFAR2) and 3 (FFAR3). FFAR3 is expressed on the blood brain barrier (BBB), which provides a protective boundary between the brain and the periphery. Physiologically relevant concentrations of propionate were found to elicit a protective effect on the BBB by mitigating against inflammatory stimuli and oxidative stress in *ex vivo* and *in vitro* models (Hoyles et al., 2018). In addition, SCFAs are thought to affect the synthesis of neuroactive compounds such as serotonin in a couple of ways. Firstly, SCFAs promote the transcription of tryptophan hydroxylase (TPH1) – a rate limiting step in the synthesis of serotonin – in enterochromaffin cells, which in turn influences circulating serotonin (Reigstad et al., 2015; Yano et al., 2015). Secondly, they may regulate the expression of enzymes involved in the biosynthesis of neurotransmitters such as tyrosine hydroxylase (Nankova et al., 2014), although it is unclear to what extent this may occur *in vivo*. Research has also shown that SCFAs, particularly acetate, can cross the BBB and trigger peripheral nervous system signalling (Frost et al., 2014; Logsdon et al., 2018). There is currently little research exploring whether physiologically relevant concentrations of SCFAs may cross the BBB *in vivo* and directly affect the brain, but it remains an avenue of interest. Finally, SCFAs play a role in immune modulation, as discussed in section 1.2.3.3. Combined, this research highlights the benefits of SCFAs and their importance in protecting against susceptibility to neurological and psychological diseases.

Neuroactive metabolites

Serotonin, tryptophan and the kynurenic pathway

Serotonin (5-HT) is a neurotransmitter with a wide range of roles in the human body, from GI effects such as gut motility to wider roles in the peripheral and central nervous systems such as circadian rhythm, emotion regulation and cognitive function (O'Mahony et al., 2015). Most of the body's serotonin is produced via enterochromaffin cells in the GIT (Shajib & Khan., 2015), with which the GM interact to influence the serotonergic system. GF mice display lower concentrations of 5-HT in the lumen and cecum compared to conventional mice, which is rapidly increased to normal levels upon exposure to non-GF (conventional) microbiota (Yano et al., 2015; Hata et al., 2017). On the other hand, GF mice evidence higher than normal levels of faecal and serum tryptophan – an amino acid and the sole precursor to 5-HT. This is potentially explained by reduced expression of tryptophan hydroxylase 1 (TPH1) (the enzyme for 5-HT biosynthesis within enterochromaffin cells), limiting the synthesis of serotonin and leading to an accumulation of tryptophan (Yano et al., 2015). For example, in GF mice circulating concentrations of tryptophan were higher than in conventional mice, but when colonized via FMT levels of tryptophan fell and a concurrent sex-dependent increase in hippocampal serotonin was observed (Clarke, Grenham, Scully., 2013). As discussed previously, the GM may also influence levels of 5-HT through the promotion of TPH1 transcription in enterochromaffin cells by SCFAs (Reigstad et al., 2015).

Despite being the sole precursor for serotonin, only a small proportion of dietary tryptophan is metabolised this way. The majority of tryptophan is funnelled into the kynurenine pathway (Chen et al., 2021), where it may be degraded into other neuroactive compounds including kynurenic acid, 3-hydroxykynurenine and quinolinic aid (Schwarcz et al., 2012). Both kynurenic and quinolinic acid have been implicated in mental health disorders and cognitive function. Serum concentrations of tryptophan and kynurenic acid have been negatively correlated with depression severity (Liu et al., 2018), and lower than normal concentrations of kynurenic acid are associated with disorders such as Parkinson's and AD (Szabó et al., 2011). Low dose kynurenic acid administration has also been associated with beneficial effects to cognitive function in mice (Martos et al., 2022), suggesting that at normal levels kynurenic acid can be neuroprotective. On the other hand, high levels of kynurenic acid have been implicated in the pathology of Schizophrenia (Plitman et al., 2017). Quinolinic acid, however, is a neurotoxin, and has been associated with atrophy and loss of neurons (Shear et al., 1998), cognitive deficits (Shear et al., 1998; Cathomas et al., 2021) and diseases such as depression,

AD and Huntington's disease (Lugo-Huitron et al., 2013). The GM can influence the kynurenic pathway both through modulating the availability of tryptophan, and through indirect regulation of indoleamine-2,3-dioxygenase (IDO-1) activity – an intestinal enzyme involved in the conversion of tryptophan to kynurenine. Additionally, computational analysis of bacterial genomes highlight that several bacteria express a version of this tryptophan metabolism pathway, suggesting these microbes may play a role in the utilisation of tryptophan for the kynurenine pathway (Kaur et al., 2019). Finally, tryptophan can also be metabolised through the indole pathway by the intestinal microbiota, producing a number of indole-derivatives such as indole-3-pyruvate, indole-3-aldehyde and indole 3-propionic acid which have been shown to play in role in enhancing epithelial barrier integrity and tight junction formation, and modulate both immune and inflammatory responses, typically through aryl hydrocarbon receptor activation (li et al., 2021). Given that these properties are relevant to the pathology of neurological diseases such as AD and PD, the microbial synthesis of indole derivatives provides yet another possible GBA pathway that may be exploited to attenuate disease (Pappolla et al., 2021).

GABA

GABA (γ-aminobutyric acid) is the primary inhibitory neurotransmitter in the CNS, acting to counterbalance the excitatory neural activity of glutamate. Maintaining this neural balance between GABA and glutamate is essential for human health, as demonstrated by disorders of the CNS where this balance is disrupted, such as epilepsy, ASD and anxiety (Samardzic et al., 2018). Gut microbes play a direct role in the regulation of the GABAergic system, since enteric bacteria have been found to encode for the glutamic acid decarboxylase (GAD) gene. The conversion of glutamate to GABA involves pumping the precursor L-glutamic acid into the organism via Glutamate/GABA antiporter, decarboxylation of glutamate into GABA which is catalysed by the GAD enzyme, and exportation of GABA from the intracellular environment via Glutamate/GABA antiporter (Diez-Gutiérrez et al., 2020). A number of bacteria have been identified as having the capacity to utilise this GAD system, including *Lactobacillus* spp. (Das & Goyal., 2015), *Eschericha coli* (Yu et al., 2019) and *Bacteroides* spp. (Otaru et al., 2021). In fact, this system provides a protective mechanism for the bacteria, allowing the microbe to tolerate the acidic conditions of the GIT. Microbially-produced GABA is reported to ameliorate symptoms of metabolic disease and reduce depressive-like behaviours in mice (Patterson et al., 2019). Additionally, supplementation with *Lactobacillus rhamnosus*, known GABA-producing bacteria, led to elevated levels of GABA in the brain, but only after 4 weeks of

supplementation (Janik et al., 2016). Given that microbially derived GABA is unlikely to cross the BBB *in vivo* (Knudsen et al., 1998; Boonstra et al., 2015), modulating gut-derived GABA may instead influence the activity of the enteric nervous system via the vagus nerve, which has been shown to modulate brain GABA (Ben-Manecham et al., 1995) and its receptors (Marrosu et al., 2003).

The potential for bacteria to produce neuroactive metabolites other than GABA has been explored *in vitro* for a number of bacterial strains, with the successful detection of dopamine (Ozogul et al, 2012), serotonin (Ozogul et al., 2012), histamine (Landete et al., 2007) norepinephrine (Tsavkelova et al., 2000) and acetylcholine (Girvin et al., 1954) from bacteria of various genera including *Lactobacillus*, *Bifidobacterium* and *Lactococcus* being reported. As such, the assertion that gut microbes can produce neurotransmitters has received a lot of attention. This would directly implicate the GM in a host of neuroactive pathways affecting all aspects of neural function, making gut-derived neurotransmitters an exciting avenue for exploration (Strandwitz., 2018). However, most research to date is carried out under physiologically irrelevant conditions, making it challenging to assess whether bacterial synthesis of these neuroactive compounds could be possible *in vivo*. Moreover, even in the case of microbially derived GABA, how metabolites synthesised in the gut might affect neural function is not well understood, given that neurotransmitters have not yet been proven to cross the BBB. Given the potential impact that gut derived neurotransmitters could have on brain and behaviour, exploring the potential for gut bacteria to produce neuroactive metabolites is a key focus in this thesis (chapters 3 and 4).

The GM is implicated in the production of a number of other metabolites, including bile acids and further derivatives of the tryptophan pathway, which may influence neural function and behaviour. However, since these compounds were not investigated in the current work, a review of potential interactions with the GBA falls outside the scope of this thesis and are therefore not discussed (Connell et al., 2022).

1.2.3.2 The vagus nerve

The vagus nerve is one of 12 cranial nerves in the human body, providing an efficient and bidirectional pathway between the brain and the gut via a composition of afferent (~80%) and efferent (~20%) nerve fibres (Agostoni et al., 1957). As such, it serves to transmit information from internal organ systems to the brain, and vice versa. Mapping techniques now demonstrate that the vagus nerve provides afferents to the intestinal wall from the proximal through to the distal and

transverse colon, therefore interacting with different areas of the GIT and subsequent microbiota (Wang & Powley., 2007). Although these afferent fibres do not appear to interact directly with gut microbes, it is thought that they receive signals from the GM via the binding of bacterially derived metabolites, either directly to the afferents or to nearby cells in the gut epithelium such as enteroendocrine and enterochromaffin cells (Browning., 2015; Sgritta et al., 2019; Margolis et al., 2021; Cook et al., 2021). The role of the vagus nerve in facilitating MGB interactions is highlighted in studies of vagotomised mice, where the vagal connection between gut and brain is severed. Bravo and colleagues (2011) reported altered behaviour and GABA expression in the brain in mice supplemented with a probiotic strain, but found these effects were not demonstrated in vagotomised mice, suggesting a role of the vagus nerve. On the other hand, others found increased hippocampal brain-derived neurotropic factor (BDNF) expression and exploratory behaviour to be independent of whether mice received a vagotomy or not (Bercik et al, 2011), which suggests the vagus nerve may only be a partial mediator in these gut-brain interactions.

1.2.3.3 Immune function

Gut microbes produce a number of metabolites which directly impact both innate and adaptive immune responses (Dorrestein, Mazmanian & Knight., 2014). The synthesis of SCFAs, particularly propionate, appears to have a direct impact on the availability and function of regulatory T cells, which are an essential part of the adaptive immune system. In addition, SCFAs have been shown to promote an anti-inflammatory environment and reduce the production of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 through the activation of G-protein coupled receptors and inhibition of histone deacetylases (Rooks and Garrett., 2016; Li et al., 2018).

The GM has also been implicated in the maturation and activation of microglia – specialised immune cells in the CNS which provide the first line of defence for bacterial or viral pathogens. Deficits in the microbiota, such as that seen in antibiotic-induced dysbiosis and GF models, have been linked with impaired microglia formation and reduced immune responses to bacterial toxins, which has been observed to be mitigated upon recolonization (Erny et al., 2015).

The interplay between the gut microbiota and integrity of the epithelial barrier is also an important factor in host immunity. A primary example can be seen in the case of metabolic endotoxemia – a form of low-grade inflammation linked with increased levels of circulating lipopolysaccharide (LPS) – a molecule found in the cell wall of Gram-negative bacteria. Translocation of LPS due to poor

intestinal barrier function has been implicated in a number of diseases, including the progression of liver disease, diabetes and HIV (Pinzone et al., 2012; Ghosh et al., 2020). Research now suggests that translocation of LPS across the epithelial barrier may also be implicated in the pathology of neurodegenerative disease such as AD. For example, AD patients present with much higher plasma concentrations of LPS (Zhao et al., 2018; Andre, Laugerette & Feart., 2019), and animal models suggest that LPS may act on toll-like receptor 4 to produce Nuclear factor kappa B, which in turn increases the abundance of inflammatory cytokines and leads to the characteristic amyloid plaques and myelin injury found in the AD brain (Zhan et al., 2018; Zhao & Lukiw., 2018). This highlights the importance of gut barrier integrity in the GBA, which is partly mediated, as discussed above, by the GM.

1.2.3.4 Neuroendocrine system

The hypothalamic-pituitary-adrenal (HPA) axis is the main endocrine stress system in humans, and is implicated in mediating cognitive function, mood and emotional regulation (Keller et al., 2017; Raymond et al., 2018). The link between the GM and HPA axis appears profoundly bidirectional, as exemplified in cases of IBD where altered microbial community is implicated in disease pathology, but physical and psychological stress are also known to exacerbate IBD GI symptoms (Labanski et al., 2020). GF mice, and indeed those with clinical conditions linked with gut dysbiosis, often present a hyperactive stress response to acute stressors (Murray et al., 2004; Clarke et al., 2014), which can be attenuated by recolonization or modulation of the GM via probiotics (Ait-Belgnaoui et al., 2014). Possible mechanisms via which microbes may interact with the HPA axis and thus the brain include stress-induced cytokines (Dinan et al., 2006), bacterial LPS (Vakharia and Hinson., 2005), and SCFAs (van de Wouw et al., 2018).

1.3 An introduction to probiotics

Probiotics are currently defined by the World Health Organisation as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (Guarner et al., 2012). These beneficial bacteria must reach the colon, where they may then interact with enteric microbiota in a beneficial manner. Previously, colonisation of probiotic bacteria was deemed essential for a beneficial effect, but it is now understood that the effect of probiotics is typically

more transient and instead the functional activity, such as interaction with commensal microbes, stimulation of the epithelium and production of metabolites, is likely of more importance (Sanders et al., 2018; Kristensen et al., 2016). Probiotic bacteria can be consumed as a dietary supplement administered in various forms, including powder, liquid, and capsule supplements. Additionally, fermented foods such as kefir, kimchi, and sauerkraut, are now recognised as a source of beneficial live microorganisms, either due to the select bacteria used to initiate the fermentation process, or due to microbes that are naturally present and enriched during the fermentation process (Marco et al., 2017). Several bacterial strains have now been classified as probiotic after evidencing a beneficial effect to the host in clinical trials, the majority of which belonging to the *Lactobacillus* and *Bifidobacterium* genera (O'Toole, Marchesi & Hill., 2017).

The mechanisms through which probiotics may exert effects on the CNS are not well understood, with much of the current evidence originating from studies in animal models. Bacterial species, including those deemed as probiotic, may produce or be involved in the production pathways for a number of neurotransmitters including GABA, dopamine, serotonin and norepinephrine (Barrett et al., 2012; Holzer and Farzi, 2014), as well as modulating the availability of precursors such as tryptophan (Yano et al., 2015). Probiotics may also increase the availability of neuroactive compounds indirectly by stimulating metabolites that promote biosynthesis (Yano et al., 2015). A study in adult male mice demonstrated that chronic supplementation with *L. rhamnosus* was associated with altered expression of GABA receptors in the brain and consistent reductions in stress-related behaviour and corticosterone output (Bravo et al., 2011). Additionally, magnetic resonance spectroscopy (MRS) research in mice found that supplementation with *L. rhamnosus* led to a significant increase in functional metabolites in the brain, including glutamate, N-acetyl aspartate and GABA. These studies indicate that probiotic induced changes to the gut likely led to functional changes in the brain, providing some mechanistic insight into behavioural changes, but exactly how changes in gut derived metabolites mediates altered neurochemistry remains unclear.

In addition to altered neurotransmitter production, it is thought that probiotics may influence the production of other bacteria-derived metabolites, particularly short-chain fatty acids (SCFAs), which are thought to be heavily implicated in gut-brain axis communication (Dalile et al., 2019; Silva et al., 2020). *In vitro* models have demonstrated an increase in SCFAs (particularly acetate, butyrate and propionate) as a result of probiotic bacteria (Sivieri et al., 2013; Nagpal et al., 2018). In a RCT supplementation with a multispecies probiotic, in mothers for the last 6 weeks of pregnancy and in their children for the first 3 months after birth, was associated with lower incidence of the child

developing eczema and higher abundance of faecal acetate, propionate and butyrate compared to a placebo (Kim et al., 2015). Additionally, in a non-controlled trial, 4-week supplementation with *Lactobacillus plantarum* was found to significantly increase faecal concentrations of acetate and propionate, but not butyrate, in young, middle-aged, and older adults, where concentrations remained higher than at baseline 4 weeks after supplementation ceased (Wang et al., 2014).

Finally, probiotics may influence neural function via interactions with immunological pathways. Probiotics have been associated with improved gut barrier integrity and reduced permeability (van Hemert et al., 2013), thought to occur as a result of increased mucin expression and occludin to improve tight-junction stability, protecting the epithelial barrier (Mennigen & Bruewer., 2009; Stoidis et al., 2011). As a result, probiotic intervention may reduce endotoxemia and therefore levels of inflammation. In addition, probiotics may offer an opportunity to attenuate the damaging effects of pro-inflammatory cytokines on the gut barrier, both by reducing proinflammatory and increasing anti-inflammatory responses. For example, in humans, chronic supplementation with *L. salivarius* has been associated with a significant reduction in serum concentrations of inflammatory markers such as high sensitivity C-reactive protein (hs-CRP), interleukin (IL) 6, IL-1b, and tumour necrosis factor alpha (TNF- α) (Rajkumar et al., 2015). These findings were echoed in a recent review which discussed frequent reports of a significant reduction in serum concentrations of proinflammatory markers, particularly TNF- α and C-reactive protein, in addition to less frequent reports of increased anti-inflammatory markers following probiotic intervention (Maia et al., 2019). However, the mechanisms responsible for changes in inflammatory response are less clear. One suggestion is that introduction of probiotic bacteria can alter the signalling for inflammatory cytokine activation. For example, *in vitro* work has demonstrated that *L. rhamnosus* GG reduced the effects of pro-inflammatory cytokines on epithelial barrier integrity, in part, through inhibition of NF-kB signalling (Donato et al., 2010).

As such, by exploiting the gut-brain axis, probiotics present an opportunity for modulation of the CNS. In particular, the potential for probiotic interventions to beneficially affect cognitive function has gained attention over recent years. Increasingly, probiotics are being investigated for their potential to reduce cognitive deficits as well as enhance cognition in the absence of clinical impairment. Studies in rodents have consistently reported positive effects of both single and multi-strain probiotics on spatial and non-spatial memory (Wang et al., 2016), and reversal of cognitive deficits have been reported in animal models of diabetes (Davari et al., 2013), anxiety (Savignac et al., 2015), Parkinson's (Castelli et al., 2020), and AD (Naomi et al., 2022) to name a few. The current

evidence from human trials is reviewed in detail in chapter 2, the conclusions from which informing the research aims of this thesis (chapter 2, section 5) and the work in chapter 5.

1.4 An *in vitro* approach to studying the effect of probiotics on the microbiota-gut-brain axis

Within recent years there has been a surge of *in vivo* human trials exploring the effect of probiotics on various aspects of host health and behaviour. While intervention studies in humans, particularly randomised control trials, are considered a gold standard methodology for establishing causal relationships between dietary interventions and human health outcomes (Lichtenstein et al., 2021), they are expensive to conduct, with complex host-environment factors to consider and limited capacity to explore underlying mechanisms of actions. This is the case with existing work exploring the effect of probiotics on cognition, where the behavioural data indicates a promising effect of probiotics but the mechanism(s) behind such effects remain unclear.

In vitro models provide an alternative methodology for exploring how a dietary intervention, such as probiotics, interacts with commensal microbes. Although not a replacement for human trials, they provide a cost-effective tool for testing specific hypotheses about host-diet-microbe interactions and discerning the mechanistic effect of probiotics on potential gut-brain pathways (Gibbons et al., 2022).

1.4.1 Batch cultures

Batch culture fermentation models provide the simplest form of *in vitro* model to study the human microbiota (Figure 1.2). Typically, batch culture fermentations are conducted in bioreactor vessels containing basal media to support the bacterial community, to which the faecal microbiota and the substrate of interest is added. Batch cultures are typically only run for short periods of up to 72 hours, as these systems result in a build-up of waste products and the limited nutrient supply is utilised within this timeframe. During this time the vessels can be monitored and sampled while maintaining control over the temperature, pH, medium composition, and anaerobic atmosphere to mimic the human gut (Wang and Gibson., 1993). As such, changes in microbial composition as well as functional activity can be assessed during this period. Due to the tightly controlled conditions, changes in the functional microbial community can be attributed to individual host responses to the substrate of interest. This *in vitro* approach has been used to study the effect of dietary flavanols (Tzounis et al., 2011; Sánchez-Patán et al., 2012), minerals (Poveda et al., 2020), pre- and probiotics

(Liu, Gibson & Walton., 2016; Likotrafiti et al., 2014; Jackson et al., 2022) on microbial composition and metabolite production. Metabolite production *in vitro* have also been matched to that found in human faecal samples, indicating translation from batch cultures to human trials with some degree of accuracy (Baxter et al., 2019). However, lack of human tissue absorption or interaction is a clear limitation of this model, as this can lead to the accumulation of metabolites beyond normal physiologic levels which impact interpretation of data from these models. Having said this, artificial build-up in *in vitro* models, particularly batch culture systems, does allow for the identification of substrates that lead to increased production of metabolites such as SCFA, whereas monitoring metabolite production in faeces would not provide this information.

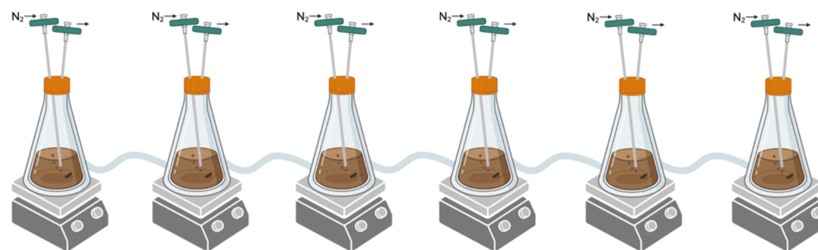


Figure 1.2 - A schematic representation of pH controlled, anaerobic, stirred batch culture fermentation model. Vessels containing culture medium, faecal microbiota and a magnetic stirring flea are placed on magnetic stirrers. Temperature is controlled and maintained by connecting vessels to a circulating water bath which floods the outer cavity of the vessels. Anaerobic conditions are maintained using a steady flow of N_2, and pH is maintained using pH controllers connected to solutions of HCL and NaOH (illustration made with BioRender.com).

1.4.2 Continuous culture models

Continuous culture models provide a step up from batch culture fermentation models, such that they more closely mimic the human colonic environment with a steady influx of nutrients and an efflux of waste (Williams et al., 2015). Additionally, models such as the three-stage continuous model (Gibson et al., 1988) allow for the modelling of different regions of the human colon from the acidic proximal colon to the transverse and finally the more neutral distal colon (Figure 1.3). Nutrients are pumped into the first, proximal vessel at a desired rate to provide new nutrients and enable stability of bacteria over time. Each region is then modelled as a separate vessel and connected to allow for gravitational feed from the proximal through to the transverse then distal, mimicking the typical transit through the human colon. As such, experiments using this type of *in vitro* gut model are typically run for longer timescales, allowing bacteria to reach a steady state

adapting to the rich nutrients available (typically around 8 turnovers – run though of media through the system over 16 days) before initiating treatment and reassessing after a second steady state is reached. To that end, these models also allow for repeat dosing over consecutive days, mimicking a daily dietary intervention *in vivo*. As with the simpler batch culture experiments, continuous stage gut models share the disadvantage of not incorporating human tissues. However, they have been validated against *in vivo* work, and again appear to provide a reliable tool for modelling the human colon (McFarlane et al., 1998; Walton et al. 2012).

Ultimately, understanding the complexity of the human microbiota and host-diet-microbiota interactions will be best served by a combination of *in silico*, *in vitro* and *in vivo* work. While *in vitro* models are not a replacement for human trials, they do provide a cost-effective compromise. As such, in vitro models provide a useful tool for proof-of-concept studies, prior to *in vivo* trials (Petrof et al., 2013).

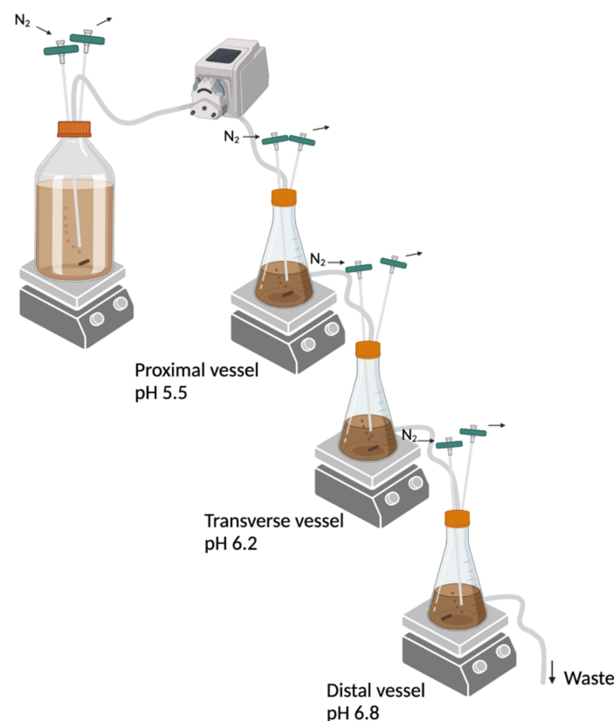


Figure 1.3 - A schematic representation of a three-stage continuous colonic model system (Gibson et al., 1998). Vessels containing culture medium, faecal microbiota and a magnetic stirring flea are placed on magnetic stirrers. Temperature is controlled and maintained by connecting vessels to a circulating water bath which floods the outer cavity of the vessels. Anaerobic conditions are maintained using a steady flow of N_2, and pH is maintained using pH controllers connected to solutions of HCL and NaOH. A continuous flow of media is pumped into the proximal vessel via a peristaltic pump. Each region is then modelled as a separate vessel and connected to allow for gravitational feed from the proximal through to the transverse then distal and finally a waste vessel, mimicking the typical transit through the human colon (illustration made with BioRender.com).

1.5 Thesis objectives and specific research questions

1.5.1 Objectives

It is clear that the GM plays a role in influencing neural function and behaviour, with growing evidence for an effect of probiotics on cognitive function. However, the literature to date in humans is yet to be systematically reviewed in its entirety. Animal and *in vitro* studies have illuminated several potential pathways via which these benefits to cognition may occur, of which microbially derived metabolites is one. Despite claims within the literature that bacteria produce neurotransmitters, the evidence for this is equivocal, especially under conditions relevant to the human GIT. As such, the objective of this PhD thesis is three-fold: firstly, to review the existing evidence for an effect of probiotic supplementation on cognitive function, secondly, to assess the potential for enteric bacteria to produce neurotransmitters under physiologically relevant conditions, and finally to assess whether probiotic intervention in healthy older adults may improve cognitive function and mood.

1.5.2 Research questions

The specific research questions addressed in this thesis are as follows:

1. **What is the current evidence for probiotics as a dietary supplement to support cognitive function?**
   Rationale and hypotheses: The last decade in particular has seen a surge in the number of trials exploring whether probiotic supplements may beneficially impact cognitive function. While the limited evidence within sub-populations, such as those with dementia, has been reviewed, the full extent of the literature is yet to be systematically reviewed. Doing so would provide clarity on whether probiotic supplementation may improve cognitive function, and for whom this approach may be beneficial. Additionally, reviewing the full scope of the literature provides an opportunity to assess the quality of the research and highlight any pervasive limitations that may compromise the ability to assess probiotic efficacy.

2. **Can microbes produce neurotransmitters under physiologically relevant conditions *in vitro* (addressed in chapters 3 & 4)?**

   Rationale and hypotheses: It is now widely accepted that bacteria found in the GIT have the capacity to produce, or directly influence the production, of various neurotransmitters. However, previous *in vitro* work assessing the neuroactive capacity of select bacteria has been performed using simplistic models under physiologically irrelevant conditions, meaning it is unclear whether reported production of neuroactive compounds could translate to the human GIT. As such, robust *in vitro* models were utilised in this thesis to assess potential neurotransmitter production under physiologically relevant conditions. Based on previous research it was hypothesised that GABA would be produced by microbes over the fermentation period, but production of other neurotransmitters remained exploratory.

3. **How does the addition of probiotic bacteria influence bacterially derived neuroactive metabolites *in vitro* (addressed in chapters 3 & 4)?**

   Rationale and hypotheses: Probiotic supplementation may act through a number of gut-brain axis pathways to enhance cognitive function. Altered faecal SCFAs have been reported following probiotic intervention, but to date it is still unclear how probiotic bacteria interact with commensal microbes to influence metabolite production. Addition of select probiotic strains in the current in *vitro* work therefore allowed for exploration into how probiotic bacteria may affect SCFA and neurotransmitter synthesis under physiologically relevant conditions. It was hypothesised that the addition of probiotic bacteria to faecal microbiota could enhance SCFA synthesis, and, based on recent work from Liu and colleagues (2021), it was hypothesised that probiotic bacteria may enhance the production of neurotransmitters such as GABA.

4. **Following a chronic multi-strain probiotic intervention, is there a beneficial effect on cognitive function in healthy older adults, and, if so, what changes in the gut microbiota are associated with this improvement (addressed in chapter 5)?**

   Rationale and hypotheses: Age-related shifts in the gut microbiota such as a reduction in overall diversity and dysbiosis are commonly reported in older adults. Additionally, cognitive decline is a common characteristic of ageing, even in the absence of age-related disorders.

Evidence from the literature suggests that probiotics may be beneficial for attenuating cognitive decline in individuals with mild cognitive impairment and Alzheimer's disease, but the research in healthy older adults is more limited. The final chapter of this thesis therefore employed a well-controlled cross-over trial to explore the effect of a multispecies probiotic supplement on cognitive function and mood in healthy ageing adults. It was hypothesised that probiotic supplementation would result in improvements to memory and executive function, as well as reducing negative mood. 16s rRNA sequencing of stool samples was also conducted pre- and post- intervention to explore potential underlying mechanisms, although changes in the microbiota community were not necessarily expected, and improved cognition was anticipated even in the absence of microbial change.

Chapter 2 – the effect of probiotics on cognitive function across the human lifespan: A systematic review

An earlier version of this chapter has been published at Eastwood, J., Walton, G., Van Hemert, S., Williams, C., & Lamport, D. (2021). The effect of probiotics on cognitive function across the human lifespan: A systematic review. *Neuroscience & Biobehavioral Reviews*, *128*, 311-327.

2.1 Introduction

As outlined in chapter 1, the gut microbiota (GM) is implicated in neural function and behaviour via several microbiota-gut-brain (MGB) pathways, including immune and neuroendocrine systems, the vagus nerve, and microbially derived metabolites. In particular, accumulating evidence from both animal and human studies highlights a role for the GM in mediating effects on cognitive function (Gareau., 2014). Dietary probiotic supplements – bacteria which when administered in adequate amounts confer a health benefit to the host (Guarner et al., 2011) – appear to interact with these MGB pathways. As such, probiotics are increasingly being explored for their potential to attenuate cognitive decline in clinical populations and improve cognitive function in the absence of clinical impairment.

Studies in rodents have consistently reported positive effects of both single and multi-strain probiotics on spatial and non-spatial memory (Wang et al., 2016). Reversal of cognitive deficits have also been reported in animal models of diabetes (Davari et al., 2013), anxiety (Savignac et al., 2015) and Parkinson's (Castelli et al., 2020), to name a few. Experimental trials in humans, largely published within the last decade, have also explored this potential benefit across a variety of clinical and non-clinical populations. A preliminary search for reviews of these experimental trials, across a range of resources including Google Scholar, JBI COnNECT+, Prospero and Cochrane Library, finds a small number of existing reviews. The literature in ageing populations experiencing Mild Cognitive Impairment (MCI) and AD was recently reviewed in a meta-analysis by Deng et al (2020), who concluded that the preliminary evidence for a beneficial effect of probiotics on cognition was promising in both MCI and AD. Age related cognitive decline may be particularly susceptive to improvement via probiotic intervention as concurrent declines in both GM diversity and cognitive

function are a hallmark of ageing, even in the absence of age related disease such as MCI and AD (Deary et al., 2009; Walrath et al, 2021; Pellanda et al., 2021). Conversely, a review into the impact of early probiotic intervention on subsequent neurocognitive development in infants and children up to age 13 found the evidence to be less compelling, with only one study reporting positive results in the form of a reduced risk of developing Attention Deficit Hyperactive Disorder (ADHD) or Autism Spectrum Disorders (ASD) (Rianda et al., 2019). A recent meta-analysis from Lv and colleagues (2021), who included 11 animal and 7 human trials in healthy and cognitively impaired populations across a range of ages, found the overall effect of probiotic intervention on cognition was non-significant in both animal and human studies when supplementing healthy populations. In populations with cognitive impairment, however, interventions in animals had a large effect size regardless of whether a single or multi-strain probiotic intervention was used, while the effect in human studies was small and showed greater efficacy following single strain interventions rather than multi-strain. Interestingly, the results appear to show a 'capping effect' of the length of intervention, where significant effects were only reported in studies of <12 weeks. Marx and colleagues (2020) concluded, following a meta-analysis including 7 human trials, that the evidence was not sufficient to support the use of probiotic supplementation for cognitive outcomes, suggesting that a greater number of well-designed, adequately powered studies are needed.

Although a number of studies have now considered cognitive outcomes following probiotic intervention, heterogeneity within the methodologies employed makes navigating this literature and interpreting the results challenging. Where previous reviews have focused on the effects of supplementation within specific populations or age groups, and therefore only ever included a small number of human trials, this review aims to collate the full extent of the current human literature. This is important as interest in the field begins to grow, not only to consider the populations for whom probiotics may provide a beneficial tool in the improvement of cognitive function, but to begin to discuss in what context an intervention might be successful with regards to probiotic strain(s), the length of supplementation and the cognitive domain(s) beneficially effected by probiotic treatment. Additionally, this review provides a unique opportunity to look at the overall quality of the existing literature and identify where future studies might improve upon this to further our understanding of how probiotics could enhance cognition.

As such, the aim of this chapter is to systematically review a broad range of experimental trials in human subjects to address the question of whether probiotic supplementation may improve cognitive function, and for whom this approach may be beneficial. Additionally, reviewing the full

scope of the literature provides an opportunity to assess the quality of the research and highlight any pervasive limitations that may compromise the ability to assess probiotic efficacy.

2.2 Method

Methods for conducting this review were pre-specified in a registered protocol on PROSPERO (registration number CRD42020164820).

Experimental human trials, recruiting participants of any age, gender or ethnicity, were eligible for inclusion if they supplemented participants with at least one live probiotic strain. With a view to including as many studies as possible, no restrictions were placed on type, quantity or length of probiotic intervention, and studies using probiotic supplements in conjunction with other interventions were also included. To that end, studies without a comparator, such as a placebo control group, were also included. To be eligible for inclusion, studies were also required to include at least one cognitive outcome measuring performance in a cognitive domain such as memory, executive function or attention. Studies that did not include a behavioural measure on a cognitive task were excluded. As such, studies solely measuring cognitive reactivity or cognitive control via use of questionnaires were not included, as these were not deemed standardised behavioural measures of cognitive performance. Studies using resting state functional Magnetic Resonance Imaging (fMRI) with no cognitive task included in the experimental design were also excluded.

A search of the databases PsychINFO, Web of Science, PubMed and Google Scholar was originally performed between December 2019 and January 2020 to identify formally published experimental trials in humans published in the English language. These searches have since been performed again to identify any additional papers meeting inclusion criteria published between January 2020 and October 2022, with a view to providing an up-to-date overview of the literature for this thesis. Reference lists of relevant studies, including review papers, were also checked, and Scholar was used primarily for this purpose. As this review focused on formally published papers, grey literature databases were not searched. Each database was systematically searched using the following terms: probiotic*** AND** gut **AND** brain **AND** axis, probiotic*** AND** clinical **AND** trial, probiotic*** AND** cognit*****, probiotic*** AND** neuro*****, probiotic*** AND** brain, probiotic* **AND** (memory **OR** learning **OR** attention), Lactobacill* **AND** cognit*, Lactobacill* **AND** (memory **OR** learning **OR** attention), Bifidobacteri* **AND** cognit*, Bifidobacteri* **AND** cognit*, Bifidobacteri* **AND** (memory **OR** learning **OR** attention). In

PubMed and PsychINFO, each search was run through ‘all fields’, including title, abstract, keywords and Medical Subject Headings (MeSH), using the advanced search feature. For Web of Science, each term was searched using ‘topic’ search fields, which includes title, abstract, author keywords and keywords plus. No other filters or descriptors were used except for in PubMed, where searches were restricted to ‘clinical’ and ‘human’ due to the larger volume of animal and *in vitro* papers available.

Initially, papers were excluded based on the title if it was evident that the research fell outside of the inclusion criteria specified- e.g. animal studies. All studies of potential interest were then shortlisted before reading the full publications to decipher eligibility for inclusion. Where database searches flagged up relevant conference abstracts or study protocols, authors were contacted to enquire whether this data had since been published (Owen et al., 2014; Noorwali et al., 2017; Bloemendaal et al., 2019; Rieger et al., 2019). This was not the case for any of the research studies in question and therefore these were not included in this review.

Studies selected for inclusion were assessed for overall quality of methodology and the potential risk of bias using the Evidence Analysis Manual Quality Criteria Checklist (QCC) from the Academy of Nutrition and Dietetics (2016) (Appendix 1). Potential areas of bias included selection and randomisation procedures, use of blinding, and funding. As one of the aims of this review was to explore the quality of the existing literature and highlight current limitations, all eligible papers were included regardless of methodological quality.

Data extraction was conducted following the Evidence Analysis Manual Data Extraction Template from the Academy of Nutrition and Dietetics (2016) (Appendix 2). This allowed systematic extraction of key information regarding design, sample characteristics, intervention/ exposure/ compliance, outcome measures and reported results. For the purpose of this review, only data relevant to cognitive outcomes was extracted for analysis, although some papers also explored physical and psychological outcome measures. Those including biochemical outcomes to explore potential underlying mechanisms for cognitive improvement are discussed in section 4.6.

With regards to data synthesis, extracted data were handled in tabular form in order to aid comparison of study characteristics and guide the grouping of studies for narrative synthesis. Due to the heterogeneity in key study characteristics, namely population, intervention and cognitive outcome, statistical synthesis of study findings was not performed.

2.3 Results

2.3.1 Study characteristics

Initial searches flagged a total of 7871 citations. After screening out 95 duplicates and 7437 papers based on titles and abstracts, a further 305 papers were rejected following more in-depth review resulting in a total of 30 studies that met the inclusion criteria described (Figure 2.1). An additional 19 studies were reviewed in October 2022, of which 8 met the inclusion criteria resulting in a final total of 38 studies (Table 2.1).

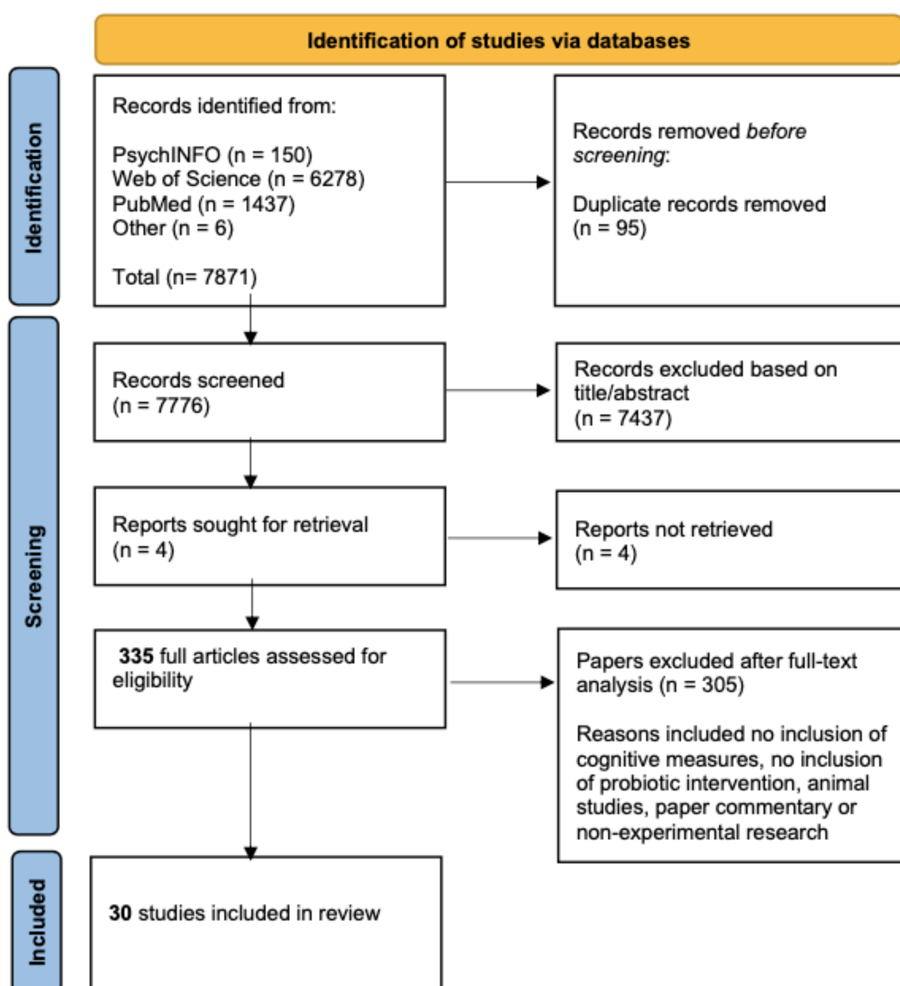


Figure 2.1 – PRISMA flow diagram illustrating the identification of studies for inclusion.

Table 2.1 - Key characteristics of included experimental trials.

Citation	Design	Participants			Intervention		Cognitive measures	Significant cognitive outcomes
		No.	Age	Clinical population	Length	Probiotic strain(s)		
Adikari et al. (2020)	Double-blind RCT	19	m = 19	N/A	8 weeks	*Lactobacillus casei* Shirota (3×10^{10} CFU/day)	DVT	Significantly improved reaction time but not accuracy on the DVT at 8 weeks compared to the placebo group
Agahi et al. (2018)	Double-blind RCT	48	m = 80 (assumed mean)	AD	12 weeks	*Lactobacillus fermentum, Lactobacillus plantarum, Bifidobacterium lactis, Lactobacillus acidophilus, Bifidobacterium bifidum*, and *Bifidobacterium longum* (3×10^{9} CFU/day)	TYM	No effect of intervention on cognition
Akar et al. (2017)	RCT with prospective follow-up	249	m = 28 weeks gestation (assumed mean)	VLBW preterm infants	Supplemented from first feed until discharge Followed up at between 18-24 months	*Lactobacillus reuteri* (1×10^{8} CFU/day)	BSID-II	No effect of intervention on cognitive development
Akbari et al. (2016)	Double-blind RCT	52	m = 79	AD	12 weeks	200 ml/day probiotic milk containing *Lactobacillus acidophilus, Lactobacillus*	MMSE	Significant improvement in MMSE score in the probiotic group following 12 weeks of supplementation compared to placebo

						casei, Bifidobacterium bifidum, and *Lactobacillus fermentum* (2 × 10^9 CFU/day)		
Allen et al. (2016)	Non-randomised crossover (no blinding)	27 (all male)	m = 25	N/A	4 weeks of placebo 4 weeks of probiotic + 2-week follow-up	*Bifidobacterium longum* 1714 (1 × 10^9 CFU/day)	PAL RVIP Emotional recognition task Emotional Stroop task	Significantly less errors in PAL following probiotics compared to baseline. Similar improvement seen following placebo
Asaoka et al. (2022)	Double-blind RCT	115	m = 78	MCI	6 months	*Bifidobacterium breve* MCC1274 (2×10^{10} CFU/day)	ADAS-Jcog MMSE	Improvement in overall mMMSE score and orientation subscales in those with a baseline MMSE of <25 following probiotic supplementation compared with placebo
Bagga et al. (2018)	Double-blind RCT	45	m = 27	N/A	4 weeks	*Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Bifidobacterium lactis* W51, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, *Bifidobacterium lactis* W52, *Lactobacillus plantarum* W62 and *Bifidobacterium bifidum* W23. (7.5x10^6 CFU/day)	Emotional decision task Emotional recognition task	Significantly less decision change for unpleasant stimuli following probiotics compared with placebo controls (improved emotional attention). Also, a significant increase in response accuracy to unpleasant stimuli in the recognition task

Bajaj et al. (2014)	Double-blind RCT	30	m = 57 (assumed mean)	Cirrhosis	8 weeks	*Lactobacillus* GG AT strain 53103	NCT-A NCT-B DST[a] BDT	No effect of intervention on cognition
Benton et al. (2007)	Double-blind RCT	124	48 - 79 m = 61	N/A	3 weeks	Yoghurt drink with *Lactobacillus casei* 6.5×10^9	WMS Logical memory Recall of capital cities Verbal fluency task	No effect of intervention on cognition
Ceccarelli et al. (2017a)	Single-arm pilot (no blinding)	10 (all male)	22 - 53 med = 42	HIV-1	6 months	*Lactobacillus plantarum* DSM 24730, *Streptococcus thermophilus* DSM 24731, *Bifidobacterium breve* DSM 24732, *Lactobacillus paracasei* DSM 24733, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734, *Lactobacillus acidophilus* DSM 24735, *Bifidobacterium longum* DSM 24736, and *Bifidobacterium infantis* DSM 24737 (1.8×10^{12} CFU/day)	ROCF RAVLT Verbal fluency CBTT VST TMT STEP PVF/SVF RCPM	Significant improvement from baseline in immediate and delayed recall of RAVLT and immediate and delayed copying in ROCF. Also, significant improvements in PVF, STEP and CBTT test scores.
Ceccarelli et al. (2017b)	Non-randomised control trial (no blinding)	35	IQR 38 - 54 med = 48	HIV-1	6 months	*Lactobacillus plantarum* DSM 24730, *Streptococcus thermophilus* DSM 24731, *Bifidobacterium breve* DSM 24732, *Lactobacillus paracasei* DSM 24733, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734, *Lactobacillus acidophilus* DSM 24735,	ROCF RAVLT Verbal fluency CBTT VST TMT STEP	Significant improvement from baseline in immediate and delayed recall of RAVLT and immediate and delayed copying of ROCF in the probiotic group. Also, significant improvements in STEP, PVF, TMT-A and CBTT test scores.

						Bifidobacterium longum DSM 24736, and *Bifidobacterium infantis* DSM 24737 (9 x 10^{11} CFU/day)	PVF/SVF RCPM	
Chou et al. (2010)	RCT with prospective follow-up	301	m = 28 weeks gestation (assumed mean)	VLBW preterm infants	Supplemented from 7 days old until discharge Followed up at 3 years CA	*Lactobacillus acidophilus* and *Bifidobacterium infantis* (2 x 10^9 CFU/day)	BSID-II	No effect of intervention on cognitive development
Chung et al. (2014)	Double-blind RCT	36	m = 65 (assumed mean)	N/A	12 weeks	fermented milk with *Lactobacillus helveticus* IDCC380	DST[b] Story recall test VLT RVIP Stroop task Serial 3/7s	Significant improvement from baseline in Stroop accuracy and serial 3/7s in probiotic group. Significantly higher accuracy following probiotics compared to placebo in RVIP and Stroop task.
Czajeczny et al. (2021)	Single-blind RCT	38 (all female)	M = 23	N/A	6 weeks	*Bifidobacterium lactis* BS01 (2x10^9 CFU/day) and *Lactobacillus acidophilus* LA02 (2 x10^9 CFU/day)	RAVLT FAS Stroop Task LDT WCST	Significant reduction in errors and non-pervasive errors, and an increase in correct responses in the WCST following probiotic but not placebo treatment. Significant improvements following placebo in RAVLT, FAS, LD and Stroop.
Firmansyah et al. (2011)	Double-blind RCT	290	m =377 days	N/A	12 months	*Bifidobacterium longum* BL999, *Lactobacillus rhamnosus* LRR + inulin, fructo-oligosaccharides and	BSID-III	No effect of intervention on cognitive development

						Long-chain polyunsaturated fatty acids (~ 1.7 x 10^{7} CFU/day)		
Hwang et al. (2019)	Double-blind RCT	92	m = 68	MCI	12 weeks	*Lactobacillus plantarum* C29 (1.25 x 10^{10} CFU/day) + fermented soybean powder	VLT ACPT DST[b]	Significantly greater improvement in composite score following probiotics than placebo, which appears to be driven by improvement in ACPT
Inoue et al. (2018)	Double-blind RCT	38	m = 70	N/A	12 weeks	*Bifidobacterium longum* subsp. *longum* BB536, *Bifidobacterium longum* subsp. *infantis* M-63, *Bifidobacterium breve* M-16V and *Bifidobacterium breve* B-3 (1.25×10^{10} CFU/day) + resistance training	MoCA Modified Flanker task	Significant improvement in composite score of both groups
Jacobs et al. (2017)	Double-blind RCT	664	m = 27 weeks gestation	VLBW preterm infants	Supplemented from first feed until discharge Followed up at 2 - 5 years	*Bifidobacterium infantis* BB–02 96579, *Streptococcus thermophilus* TH–4 15957 and *Bifidobacterium lactis* BB-12 15954 (1x10^{9} CFU/day)	BSID-III	No effect of intervention on cognitive development
Kelly et al. (2017)	Cross-over RCT (no blinding)	29 (all male)	20 - 33 m = 24	N/A	8 weeks	*Lactobacillus rhamnosus* (1x10^{9} CFU/day)	MOT PAL AST RVIP Emotional recognition task Emotional Stroop task	No effect of intervention on cognition

Kim et al. (2021)	Double-blind RCT	53	m = 72	N/A	12 weeks	*Bifidobacterium bifidum* BGN4 & *Bifidobacterium longum* BORI (total of 1×10^9 CFU/day) in soybean oil	CERAD-K	Significant improvement in mental flexibility following 12 weeks of probiotic but not placebo
Kobayashi et al. (2019a)	Open-label single-arm pilot	27	m = 82	MCI	6 months	*Bifidobacterium breve* A1 (2x10^{10} CFU/day)	MMSE DSST (WAIS III)	Significant improvement in MMSE score following probiotic supplementation
Kobayashi et al. (2019b)	Double-blind RCT	117	m = 61	MCI	12 weeks	*Bifidobacterium breve* A1 (2x10^{10} CFU/day)	RBANS MMSE	Significant improvement in delayed memory score (MMSE) in 'low scorers' at baseline. Also, significant improvement following both probiotic and placebo treatment in language and attention (RBANS) in 'low scorers' at baseline
Lew et al. (2019)	Double-blind RCT	103	m = 31 (assumed mean)	N/A	12 weeks	*Lactobacillus planturum* P8 (2 x 10^{10} CFU/day)	CBB	Significantly greater social emotional cognition in women and greater recognition memory in men following probiotic intervention compared to a placebo
Lunia et al. (2014)	RCT (no blinding)	160	m = 48 (assumed mean)	Cirrhosis	3 months	*Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus,* and *Streptococcus thermophilus* (3.3 x 10^{11} CFU/day)	PHES	Significant improvement in PHES score following probiotic intervention
Malaguarnera et al. (2010)	Double-blind RCT	125	m = 50 (assumed, for control	Cirrhosis/mild HE	60 days	*Bifidobacterium* + fructo-oligosaccharides	TMT SDMT BDT	Significant improvement from baseline in all 3 tasks following probiotic intervention. Similar improvements seen in control group taking lactulose

			group only)					
Nobile et al. (2022)	Unclear – suspected open-label single-arm pilot	30	18 - 30	N/A	4 weeks	*Lactobacillus reuteri* PBS072 (2×10^9 CFU/day) and *Bifidobacterium breve* BB077 (2×10^9 CFU/day) + FOS, inulin, folic acid, vitamin B12 & vitamin B6	Short-term memory test WCST Divided attentional performance test	Improved short-term memory accuracy and improved accuracy in the divided attention task after 4 weeks
Ohsawa et al. (2018)	Double-blind RCT	60	M = 58	N/A	8 weeks	Fermented milk with *Lactobacillus helveticus* CM4	RBANS	Significant improvement from baseline in total score, delayed recall and attention following the fermented milk. Difference between placebo and intervention group was significant post-intervention for attention.
Papalini et al. (2019)	Double-blind RCT	58 (all female)	m = 21	N/A	4 weeks	*Bifidobacterium bifidum* W23*, Bifido-bacterium lactis* W51*, Bifidobacterium lactis* W52*, L. acidophilus* W37*,Lactobacillus brevis* W63*, Lactobacillus casei* W56*, Lactobacillus salivarius* W24*, Lactococcus lactis* W19 and *Lactococcus lactis* W58 (5×10^9 CFU/day)	Emotional face matching paradigm Emotional Stroop task Stroop task DST-backwards	Working memory performance maintained in DST under acute stress following probiotic but not placebo treatment. Probiotics associated with a 'buffering effect' against stress
Roman et al. (2018)	Double-blind RCT (pilot)	31	m = 52	Fibromyalgia	8 weeks	*Lactobacillus rhamnosus* GG® *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifdobacterium bifdus* (1.2×10^7CFU/day)	Two-choice task Iowa gambling task MMSE	Significantly reduced number of impulsive choices following probiotic treatment

Román et al. (2019)	Double-blind RCT	34	m = 64	Cirrhosis	12 weeks	*Streptococcus thermophilus* DSM 24731, *Bifidobacterium longum* DSM 24736, *Bifidobacterium infantis* DSM 24737, *Lactobacillus paracasei* DSM 24733, *Lactobacillus acidophilus* DSM 24735, *Lactobacillus delbrueckii* subsp *bulgaricus* DSM 24734, and *Lactobacillus plantarum* DSM 24730 *Bifidobacterium breve* DSM 24732 (9×10^{11} CFU/day)	PHES	Significant improvement in PHES score after probiotic treatment.
Rudzki et al. (2019)	Double-blind RCT	60	m= 39	MDD	8 weeks	SSRI + *Lactobacillus plantarum* 299v 10×10^{9} CFU/day	APT Stroop task TMT AVLT RFFT	Significant improvement in work speed (APT) and total AVLT recall in probiotic group compared to placebo
Sanborn et al. (2020)	Double-blind RCT	145	52 – 75 (m = 64)	N/A	3 months	*Lactobacillus rhamnosus* GG (1×10^{10} CFU/day)	NIH toolbox cognition battery	Improvement in total cognition score following probiotics only in those with impaired cognitive function at baseline
Slykerman et al. (2018)	Single-blind RCT	342	no data	N/A	From 35 weeks gestation until six months if breastfeeding and their infants the same treatment from birth to two years.	*Lactobacillus rhamnosus* HN001 (6×10^{9} CFU/day) or *Bifidobacterium animalis* subsp. *lactis* HN019 (9×10^{9} CFU/day)	WISC -IV AST SWM OTS	No significant effect of either probiotic treatment on neurocognitive outcomes.

Tamtaji et al. (2018)	Double-blind RCT	79	m = 77	AD	12 weeks	*Lactobacillus acidophilus, Bifidobacterium bifidum* and *Bifidobacterium longum* + 200 mg of selenium (6 x 10^9 CFU/day)	MMSE	Significantly greater improvement in MMSE score in the probiotic + selenium group than selenium alone or control groups.
Tillisch et al. (2013)	Double-blind RCT	27 (all female)	18 - 53 (m = 30)	N/A	4 weeks	Fermented milk with *Bifidobacterium animalis* subsp *lactis (*I-2494)*, Streptococcus thermophilus* (I-1630)*, Lactobacillus bulgaricus* (I-1632 and I-1519) and *Lactococcus lactis* subsp *lactis* (I-1631) (~2.9 x 10^{10} CFU/day)	Emotional decision task and matched control	FMPP associated with decreased activity in widely distributed brain network during emotional task, particularly in the somatosensory cortices and insula.
Ton et al. (2020)	Open-label single-arm pilot	13	m = 78	AD	3 months	Kefir fermented milk using the species: *Acetobacter aceti*, *Acetobacter* spp.*, Lactobacillus delbrueckii delbrueckii, Lactobacillus fermentum, Lactobacillus fructivorans, Enterococcus faecium, Leuconostoc* spp.*, Lactobacillus kefiranofaciens, Candida famata*, and *Candida krusei*	MMSE Immediate & delayed recall CTPT Similarity test BNT Verbal fluency test TMT Clock drawing	Improvement in global MMSE score, immediate and delayed memory, visuo-spatial abilities as assessed by the similarity test and cookie theft picture test, and executive and language functions measured using the BNT and verbal fluency task.
Wallis et al. (2018)	Open-label single-arm pilot	44	16 - 85 (m = 44)	CFS	6 weeks	Combined antibiotic and probiotic therapy on alternate weeks: Erythromycin (800 mg) during weeks 2 and 4 and *Lactobacillus rhamnosus* (2.5 × 10^{10} CFU/day), *Bifidobacterium lactis* (1.5 × 10^{10} CFU/day), *Bifidobacterium breve* (5 × 10^6	RVIP AST SWM PAL RAVLT Logical Memory	Large treatment effects suggested for attention, processing speed, cognitive flexibility, story memory and verbal fluency.

						CFU/day), *Bifidobacterium longum* (5 × 10^6 CFU/day) weeks 3 and 5.	(WMS-IV) COWAT	
Xiao et al. (2020)	Double-blind RCT	80	m = 61	MCI	16 weeks	*Bifidobacterium breve* A1 (1 x 10^{10} CFU/day)	RBANS JMCIS	Improvements in total RBANS score and immediate memory, delayed memory, visuo-spatial, language and attention subscales in both probiotic and placebo groups, but this was significantly greater for total score, memory and visuospatial ability following probiotic compared to placebo.

DVT, Digit Vigilance Test; TYM, Test Your Memory; BSDI, Bayley Scales of Infant Development; MMSE, Mini Mental State Examination; PAL, Paired Associated Learning; RVIP, Rapid Visual Information processing; ADAS-Jcog, Alzheimer's Disease Assessment Score – cognitive subscales; NCT, Number Connection Test; DST[a], Digit Symbol Test; BDT, Block Design Test; WMS, Wechsler Memory Scale; ROCF, Rey-Osterrieth Complex Figure Test; (RA)VLT; (Rey Auditory) Verbal Learning Task; FAS, verbal fluency test; LDT, Lexical Decision Task; WCST, Wisconsin Card Sorting Taks; CBTT, Corsi Block-Tapping Test; VST, Visual Search Task; TMT, Trail Making Task; STEP, Time and Weight Estimation Test; PVF, Phonological Verbal Fluency; SVF, Semantic Verbal Fluency; RCPM, Ravens Coloured Progressive Matrices; DST[b]; Digit-Span Test; ACPT, Auditory Continuous Performance Test; MoCA, Montreal Cognitive Assessment; MOT, Motor Screening Test; AST, Attention Switching Task; CERAD-K, Korean version of the Consortium to Establish a Registry for Alzheimer's Disease; DSST, Digit Symbol Substitution Task; WAIS, Wechsler Adult Intelligence Scale; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; CBB, CogState Brief Battery; PHES, Psychometric Hepatic Encephalopathy Score; SDMT, Symbol Digit Modalities Test; DST-backwards, DST[b]-backwards; APT, Attention and Perceptivity Test; RFFT, Ruff Figural Fluency Test; WISC, Wechsler Intelligence Scale for Children; CTPT, Cookie Theft Picture Task; BNT, Boston Naming Task; SWM, Spatial Working Memory; OTS, One Touch Stockings; COWAT, Controlled Oral Word Association Task; JMCIS, Japanese Mild Cognitive Impairment score.

Selected papers included Randomised Control Trials (RCTs), single-arm Pilot Studies, a Non-Randomised Control Trial and one Non-Randomised Cross-over Trial published between 2007 and 2022 in a total of 19 countries. Of the 38 papers included, only 7 explicitly report the age range of participants (Benton et al., 2007; Tillisch et al., 2013; Ceccarelli et al., 2017a; Kelly et al., 2017; Ohsawa et al., 2018; Wallis et al., 2018; Sanborn et al., 2020) and many are unclear as to whether they are reporting mean or median and standard deviation or standard error (Malaguarnera et al 2010; Tillisch et al., 2013; Bajaj et al., 2014; Chung et al., 2014; Lunia et al., 2014; Agahi et al., 2018; Slykerman et al., 2018; Lew et al., 2019) of the sample. Based on the mean ages reported, these papers collectively included individuals from 27-weeks gestation to 82 years, although this may not reflect the full range of ages studied. Five papers studied infants and children (Chou et al., 2010; Firmansyah et al., 2011; Akar et al., 2017; Jacobs et al., 2017; Slykerman et al., 2018), 21 focused on a general adult population and 12 specifically on ageing adults (Chung et al., 2014; Akbari et al., 2016; Agahi et al., 2018; Inoue et al., 2018; Hwang et al., 2019; Kobayashi et al., 2019a; Kobayashi et al., 2019b; Tamtaji et al., 2019; Kim et al., 2021; Sanborn et al., 2020; Ton et al., 2020; Xiao et al., 2021). Across these age groups there were a number of clinical populations targeted for probiotic intervention, including very low birth weight (VLBW) preterm infants (Chou et al., 2010; Akar et al., 2017; Jacobs et al., 2017), Human Immunodeficiency Virus-1 (HIV-1) (Ceccarelli et al., 2017a; Ceccarelli et al., 2017b), Cirrhosis (Malaguarnera et al., 2010; Bajaj et al., 2014; Lunia et al., 2014; Román et al., 2019), Fibromyalgia (Roman et al., 2018), Major Depressive Disorder (MDD) (Rudzki et al., 2019), Chronic Fatigue Syndrome (CFS) (Wallis et al., 2018), Mild Cognitive Impairment (MCI) (Hwang et al., 2019; Kobayashi et al., 2019a; Kobayashi et al., 2019b; Xiao et al., 2020; Asaoka et al., 2022) and Alzheimer's Disease (AD) (Akbari et al., 2016; Agahi et al., 2018; Tamtaji et al., 2019; Ton et al., 2020) with a further 17 studies carried out in 'healthy' individuals. As such, outcome measures were often clinically relevant to the population studied, with only 31 papers stating a primary focus on cognition.

The majority of studies assessed cognitive outcomes at baseline and post-intervention, with the exception of those studying infants and one other (Lew et al., 2019). Data were reported across a number of cognitive domains, as defined by Lezak and colleagues (2012), using a combination of 56 different composite and individual task measures (see Table 2.2). Choice of measure(s) was often guided by age of the population, such as frequent use of the Bayley Scales of Infant Development for studies in infants and the Mini Mental State Examination for those in ageing adults, or by medical condition, where cognitive ability was measured using assessment tools rather than standard cognitive tasks.

Table 2.*2* - Number of studies reporting a significant positive effect of probiotic intervention versus no effect of probiotic intervention (effect/no effect) on cognitive tasks, and the respective cognitive function(s) targeted.

Cognitive function	**Tasks used**
Attention/vigilance (8/6)	Attention Switching task (1/2)
	Rapid Visual Information Processing (2/2)
	Digit Symbol Substitution Task/ Symbol Digit Modalities Test (1/2)
	Attention and Perceptivity Test (1/0)
	Auditory Continuous Performance Test (1/0)
	Divided attention task (1/0)
	Digit Vigilance Test (1/0)
Working memory (3/4)	Digit span (1/3)
	Serial 3/7s (1/0)
	Spatial Working Memory (1/1)
Immediate spatial memory (2/0)	Corsi-blocks (2/0)
Verbal memory (immediate) (7/6)	(Rey Auditory) Verbal Learning Task (4/3)
	Paired Associated Learning (1/2)
	Wechsler Memory Scale logical memory (1/1)
	Immediate recall test (1/0)
Verbal memory (delayed) (6/3)	(Rey Auditory) Verbal Learning Task (4/1)
	Weschler Memory Scale logical memory (1/1)
	Story recall (0/1)
	Delayed recall test (1/0)
Visuo-spatial memory (3/0)	Rey-Osterrieth Complex Figure Test (2/0)
	Cookie Theft Picture Test (1/0)
Episodic memory (0/1)	Capital city recall (0/1)
Psychomotor skill (3/4)	Trail Making Test A/B (2/2)
	Motor Screening Test (0/1)

	Number Connection Test A/B (0/1)
	Digit Vigilance Test (1/0)
Executive function (11/19)	Stroop task (classic) (1/4)
	Controlled Oral Word Association Task (1/0)
	Block Design Test (1/1)
	Phonemic Verbal Fluency (2/0)
	Ruff Figural Fluency Test (0/1)
	Semantic Verbal Fluency (0/2)
	Stroop task (emotional) (0/2)
	Verbal Fluency Task (1/4)
	One Touch Stockings (CANTAB) (0/1)
	Flanker task (0/1)
	Iowa Gambling Task (0/1)
	Wisconsin Card Sorting Task (1/0)
	Number Connection Test B (0/1)
	Two-choice task (1/0)
	Emotional decision task (2/0)
	Boston Naming Task (1/0)
	Lexical decision task (0/1)
Affective processing (3/4)	Stroop task (emotional) (0/2)
	Emotional recognition task (1/2)
	Emotional decision task (2/0)
Composite measures (17/7)	Mini Mental State Examination (6/1)
	Montreal Cognitive Assessment (1/0)
	Repeatable Battery for the Assessment of Neuropsychological Status (3/0)
	CogState Brief Battery (1/0)
	Bayley Scales of Infant Development II/III (0/4)

	Psychometric Hepatic Encephalopathy Score (2/0)
	Test Your Memory (0/1)
	Wechsler Intelligence Scale for Children -IV (0/1)
	NIH toolbox cognitive battery (1/0)
	Japanese version of the MCI score (1/0)
	Alzheimer Disease Assessment Scale (Japanese version) cognitive subscale (1/0)
	Consortium to Establish a Registry for Alzheimer's Disease Assessment packet (Korean) (1/0)
Fluid intelligence (0/2)	Ravens Coloured Progressive Matrices (0/2)

Cognitive outcomes were assessed following a variety of probiotic interventions. Most papers provided details of the exact probiotic strain(s) administered, while 9 only described the specie(s) (Chou et al, 2010; Lunia et al., 2014; Akbari et al., 2016; Akar et al., 2017; Agahi et al., 2018; Roman et al., 2018; Wallis et al., 2018; Tamtaji et al., 2019; Ton et al., 2020) and 1 just the genus (Malaguarnera et al., 2010). Eighteen studies utilised a single strain intervention, 20 a multi-strain intervention and 8 administered the probiotic intervention in conjunction with an additional treatment for a combined intervention. These included medicines (Wallis et al., 2018; Rudzki et al., 2019), exercise (Inoue at al., 2018), and other dietary supplements (Malaguarnera et al., 2010; Firmansyah et al., 2011; Hwang et al., 2019; Tamtaji et al., 2019; Nobile et al., 2022). Key study information regarding population, intervention used, and significant cognitive findings are summarised in Table 1.

Using the QCC, the quality of all studies was assessed as ‘neutral’, with a small number demonstrating a stronger methodology and bordering a positive rating (Firmansyah et al., 2011; Ohsawa et al., 2018; Roman et al., 2018; Papalini et al., 2019; Rudzki et al., 2019; Adikari et al., 2020; Kim et al., 2021; Sanborn et al., 2020; Xiao et al., 2020; Asaoka et al., 2022) (Appendix 3). Generally, the risk of bias across studies from sources of funding and use of blinding was low, but subject selection and randomisation procedures presented a higher risk for bias. Implications of this are discussed below in section 2.7.

2.3.2 Infants and children

Three RCTs used a prospective follow-up to assess the impact of early probiotic intervention on neurodevelopment in VLBW preterm infants (gestational age ≤ 32 weeks or birth weight ≤ 1500g). In each case neurodevelopment was assessed using the Bayley Scales of Infant Development (BSID) II (Chou et al., 2010; Akar et al., 2017), or III (Jacobs et al., 2017), with one study also using the Wechsler Preschool and Primary Scale of Intelligence III as an alternative for children who were followed up over the age of 42 months (Jacobs et al., 2017). Infants were supplemented with a mixture of *Lactobacillus reuteri* (Akar et al., 2017), *Lactobacillus acidophilus* and *Bifidobacterium infantis* (Chou et al., 2010), or *B. infantis, Streptococcus thermophilus* and *Bifidobacterium lactis* (Jacobs et al., 2017) from when first able to feed until discharged from hospital. All three studies reported no significant effects on neurodevelopment, as compared to non-treated or a placebo control group. Similarly, studies in full-term infants reported no positive effect of intervention on cognitive development, either when supplemented from a gestational age of 37 weeks until 2 years with *Lactobacillus rhamnosus* and *B. animalis* subsp. *Lactis* (Slykerman et al., 2018), or with a combined supplement of *Bifidobacterium longum* and *Lactobacillus rhamnosus*, prebiotics inulin and fructo-oligosaccharide and long-chain polyunsaturated fatty acids AA and DHA from 12 months until 24 months of age (Firmansyah et al., 2011). Cognitive outcomes were assessed at 11 years and 24 months, respectively.

2.3.3 Young and middle-aged adults

Given that cognition in young and middle-aged adults is not affected by natural confounders such as aberration in development during early life or deterioration of cognitive function in later life, studies in this age group typically focus on populations with specific pathologies known to affect cognitive function. Hepatic Encephalopathy (HE) is a severe complication of cirrhosis resulting in brain dysfunction due to a build-up of toxins in the blood stream. A number of papers explored how probiotic intervention may reduce the incidence of HE in cirrhosis patients. One study in patients evidencing HE found a positive effect of a combined *Bifidobacterium* and fructo-oligosaccharide supplement on tasks measuring visuospatial awareness, processing speed and psychomotor and executive functions (Malaguarnera et al., 2010). This improvement in performance was evident after 30 days of intervention, which was similar to the improvement reported in the comparison group

taking lactulose (a common treatment in HE). A further 3 studies focused on cirrhosis patients with no evidence of overt HE. An improvement in PHES score (a composite assessment of cognitive impairment common in HE) was reported in 2 studies following multi-strain interventions for 12 weeks (Lunia et al., 2014; Román et al., 2019), while the other reported no significant effect of 8 weeks of *L. rhamnosus* GG on a selection of tasks from the PHES (Bajaj et al., 2014).

The cognitive functioning of individuals with HIV-1 was also a target for probiotic intervention, with the authors producing an initial pilot study (Ceccarelli et al., 2017a) followed by a larger placebo-controlled trial (Ceccarelli et al., 2017b). In both studies, HIV-1 infected adults were supplemented with the same multi-strain probiotic (*Lactobacillus plantarum*, *S. thermophilus*, *Bifidobacterium breve*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *B. longum* and *B. infantis*) for six months before change in cognition was assessed using a large battery of standardised tests covering memory, executive functions and fluid intelligence. In both studies, significant improvements relative to baseline and controls were reported in immediate and delayed memory, visuospatial working memory and verbal fluency, with additional improvement in executive function and psychomotor speed reported in the latter trial. It should be noted that, in the controlled trial, assignment to condition was not random but based on cerebral spinal fluid (CSF) neopterin levels at baseline, with only those who demonstrated higher levels assigned to take the probiotic treatment, presumably because higher levels of neopterin were correlated with lower cognitive performance at baseline, therefore providing greater potential for improvement. As a result, only 9 subjects were studied for change in cognitive function following probiotic treatment, compared to 26 control subjects.

Probiotic interventions may also positively affect cognitive status in adults with other clinical conditions associated with altered gut microbiota composition. One pilot study explored this potential in individuals with fibromyalgia (Roman et al., 2018). Following 8 weeks of *L. rhamnosus* GG*, Lactobacillus casei, L. acidophilus*, and *Bifidobacterium bifidus*, those who received probiotic treatment displayed a significantly reduced number of impulsive choices in a reward based decision-making task. In another study combining antibiotic (erythromycin) and probiotic treatment (*L. rhamnosus*, *B. lactis*, *B. breve* and *B. longum*) over the course of four weeks (Wallis et al., 2018), moderate treatment effects were observed for attention, processing speed, cognitive flexibility, story memory and verbal fluency in subjects with CFS. However, this was a single-arm pilot study, making it difficult to attribute these effects specifically to the intervention. Finally, one study explored the use of *L. plantarum* in combination with selective serotonin reuptake inhibitor

treatment (SSRI) for MDD (Rudzki et al., 2019). After 8 weeks of supplementation, those taking the combined treatment as opposed to just SSRIs demonstrated improved visual search and short-term memory function, but no effect on other executive functions including inhibition and verbal fluency.

A number of studies also focused on the potential for improved cognition in clinically healthy adults. Two assessed cognition in adults with high perceived stress. In the first, adults with self-reported high stress levels during exam season demonstrated improved short-term memory and accuracy during a divided attention task following 4 weeks of a *L. reuteri* and *B. breve* intervention, but as this was an open-label single-arm study with a supplement including additional nutrients that have the potential to impact cognition, no real conclusion can be drawn as to the efficacy of the probiotic supplement. In the second, 12 weeks of *L. plantarum* in moderately stressed adults, pre-determined by the Cohen's Perceived Stress Scale, was associated with significantly faster emotional processing in women and greater verbal memory in men compared to a placebo. However, no baseline data was recorded, and analysing by gender resulted in smaller samples than the authors' calculations suggested necessary for sufficient statistical power, particularly in male subjects (Lew et al., 2019). An effect on emotional processing was also reported in a study employing emotional decision and recognition tasks during fMRI (Bagga et al., 2018). In this study, a significant increase in response accuracy during the recognition task, significantly less decision change to unpleasant stimuli in the decision task, and concurrent changes in brain activation in the anterior cingulum (a region of the brain implicated in emotional processing) was found following a multi-strain intervention in healthy adults. These findings indicate that probiotic supplementation was associated with improved emotional attention compared to a placebo or non-intervention group. Tillisch and colleagues (2013) also report altered activity in widely distributed brain network during an emotional task, particularly in the somatosensory cortices and insula, following supplementation with fermented milk (*Bifidobacterium animalis* subsp *lactis, S. thermophilus, L. bulgaricus* & *L. lactis*) in healthy women compared with placebo and non-intervention groups. However, the implications on cognitive performance are unclear, as behavioural data is not reported. A positive effect on emotional processing was not consistently reported across studies. One study utilising a range of tasks measuring attention, memory, learning and emotional processing only reported an improvement in visual memory and learning following four weeks of *B. longum*, and such improvements were also seen in the control group (Allen et al., 2016). Additionally, a study in female subjects found no effect of a four-week multi-strain intervention (*B. bifidum, B. lactis, L. acidophilus, L. brevis, L. casei, Lactobacillus salivarius & Lactococcus lactis*) on tasks focused on emotional processing or executive function, but did find that the probiotic intervention provided a 'buffer' of sorts against the negative

effects of an acute physiological stressor on working memory (Papalini et al., 2019). One study, recruiting healthy middle-aged adults with self-reported forgetfulness, also found using a standardised composite measure of cognitive function that total cognitive score, attention and delayed recall abilities significantly improved following 8 weeks of *Lactobacillus helveticus* fermented milk product. Attention scores were also significantly greater in the active group compared with the placebo group post-intervention. On the other hand, two studies in healthy adults that supplemented with *L. casei* and *L. rhamnosus* for 3 and 8 weeks, respectively, reported no significant effect of probiotic intervention on any of the cognitive domains assessed including memory, verbal fluency, attention, motor speed, learning, executive function, information processing and emotional cognition (Benton et al., 2007; Kelly et al., 2017). Similarly, results from Czajeczny and colleagues (2021) provide very little support for a positive effect of *B. lactis* and *L. acidophilus* in healthy adult females.

2.3.4 Ageing adults

Five studies explored the efficacy of probiotic interventions for improving cognitive outcomes in ageing adults with MCI. Two of these were published in succession as an initial single-arm pilot study (Kobayashi et al., 2019a) followed by a larger placebo-controlled trial (Kobayashi et al., 2019b). Both studies explored the effects of *B. breve* over 24 weeks and 12 weeks, respectively, and used the MMSE to assess cognitive status and a digit symbol substitution task. The latter trial also included a larger task battery comprising of 11 other sub-tests to assess multiple facets of memory, language and executive function. In the pilot study, MMSE composite score significantly improved after 24 weeks of supplementation. In the latter trial, MMSE composite score significantly improved after 12 weeks, but this was true of both the active and placebo group. The probiotic group evidenced an improvement in delayed recall memory in both the MMSE and cognitive battery, but only in those with lower MMSE scores at baseline. Similarly, improvements in language and attention sub-tests were seen only in those with lower baseline scores, although once again the same improvements were also reported in the placebo group taking matched placebo capsules. *B. breve* supplementation for 16 weeks (Xiao et al., 2020) and 6 months (Asaoka et al., 2022) was also found to improve total cognition score, memory and visuo-spatial abilities (RBANS) and global cognition score and orientation subscale (MMSE) in subjects with MCI, respectively. Interestingly, this was only true of subjects with an MMSE score < 25 at baseline in the latter, which corresponds to abnormal cognition and indicates possible cognitive impairment. The final study assessed change in the composite z

score of three tasks measuring memory and attention following 12 weeks of *L. plantarum* and fermented soybean powder, finding a significant improvement in composite score driven by improvement in sustained attention (Hwang et al., 2019).

A further three studies using a similar dose of probiotic species explored the effects of 12-week probiotic supplementation in those with diagnosed AD. Using the MMSE as a sole measure of cognitive status, two studies found a significant improvement in total score following supplementation with *L. acidophilus, L. casei, Lactobacillus fermentum and Bifidobacterium bifidum* (Akbari et al., 2016), and with *L. acidophilus*, *B. bifidum* and *B. longum* administered in combination with selenium (Tamtaji et al., 2019). The third study utilised an alternative measure known as 'Test Your Memory' (TYM) to assess the potential efficacy of a multi-strain intervention (*L. fermentum, L. plantarum, B. lactis, L. acidophilus, B. bifidum* & *B. longum*) (Agahi et al., 2018). The TYM is a short paper-based assessment comprising 10 cognitive tasks comparable to those found in the MMSE, and was designed as a screening tool for AD. The TYM has been validated against the MMSE for detecting mild AD in memory clinic outpatients (Brown et al., 2009), but is less widely used. In this study, Agahi and colleagues (2018) found no significant effect of probiotic intervention on cognition. An additional study reported improvements in cognitive function across a wide range of domains in patients with AD following a 3-month kefir fermented milk intervention, although this was an open-label single arm study with very few subjects.

Alongside those with age-related disorders, two studies utilised probiotic interventions in generally healthy ageing adults with no chronic medical disease, mental health disorders or evidence of cognitive decline. One study aimed to explore the efficacy of a 12-week intervention with *L. helveticus* in improving performance, particularly during cognitive fatigue (Chung et al., 2014). Cognitive measures of information processing, executive function and sustained attention were administered consecutively and repeated four times to induce cognitive fatigue, while additional tasks assessed aspects of memory. Subjects on probiotic treatment showed significantly improved information processing and higher accuracy in a task of executive function compared with placebo-matched control subjects. The second combined 12 weeks of multi-strain (*B. longum, B. infantis* & *B. breve*) probiotic supplementation with moderate resistance training to explore the impact on cognitive function using a standardised battery of cognitive assessments covering memory, attention, language, executive function and visuospatial processing (Inoue et al., 2018). Both the active and control group (just resistance training) demonstrated a significant increase in composite score with no difference between groups, suggesting a significant effect of resistance training only.

Finally, Sanborn and colleagues (2020) conducted a larger scale trial in a group of community dwelling adults with a mean age of 64 years. As no exclusion criteria regarding cognitive function was enforced, the final sample included both healthy individuals and those who demonstrated impaired cognitive function at baseline, as defined by one or more scores below 35 on the NIH toolbox tests. Interestingly, an improvement in global cognition as measured by the NIH toolbox cognitive battery was found following 3 months of *L. rhamnosus* GG supplementation, but only in the impaired cognition subgroup. This finding is comparable to that of Asaoka and colleagues in subjects with suspected MCI, where only those with lower cognitive scores at baseline benefited from the probiotic intervention. While neither take potential confounding factors into account, these findings indicate that older adults with more progressed cognitive ageing may be particularly susceptible to attenuations in decline through probiotic supplementation.

2.4 Discussion

Overall, the evidence in this review provides some support for the use of probiotics to enhance cognition, with 29/38 of the included studies reporting an improvement in at least one cognitive measure. When only considering the strongest methodological studies, i.e., those employing a double-blind randomised control trial that did not find similar improvements across both control and active probiotic groups, this figure shifts to 17/22 studies. Studies exploring early supplementation in infants consistently reported no effect on subsequent neurocognitive development up to 11 years of age, regardless of whether supplementing VLBW and premature infants or those who reached full-term. It may be that that the development during this period is too rapid to see any effect of a probiotic intervention. Additionally, studying infants brings with it a greater number of challenges. Most looked to supplement infants from when first able to feed until discharged from hospital; factors which are unique to the individual and therefore resulted in heterogeneity in supplementation length within studies. Due to personal circumstances or preferences, the vehicle for administration of the probiotic was also inconsistent for a number of these studies, with some parents using breast milk (a natural prebiotic), others formula, and some studies not disclosing the method of administration, making the nutrient content of the intervention itself a potential confounder (Deoni et al., 2018).

If we exclude the two double-blind RCTs in infants, 17/20 of studies providing the best evidence report a positive effect of probiotic intervention on cognition. The evidence suggests that probiotics

may provide a useful therapeutic adjunct to those with a variety of conditions leading to impaired cognitive functioning. In young and middle-aged adults, improved cognition was reported in those with HIV-1 (Ceccarelli et al., 2017a; Ceccarelli et al., 2017b), MDD (Rudzki et al., 2019), fibromyalgia (Roman et al., 2018) and CFS (Wallis et al., 2018), although it is important to note that these effects were explored in singular studies (with the exception of Ceceralli and colleagues who ran a follow up to their pilot study in HIV-1 patients), some of which being open-label and not randomised control trials. Reports of improved cognition were more consistent in studies exploring supplementation in cirrhosis patients, with three of four randomised control trials reporting improvement in PHES composite score (Lunia et al., 2014; Román et al., 2019) and similar sub-tests (Malaguarnera et al., 2010). While the aforementioned methodological issues need to be taken into consideration, the existing evidence in these clinical populations is positive and suggests a need for further study.

In older clinical populations, improved cognition was consistently reported in those with MCI following probiotic supplementation. Interestingly, findings of significant improvements to MMSE score were often reported only in subjects who had a lower score (poorer performance) at baseline (Kobayashi et al., 2019b; Asoaka et al., 2022), suggesting that disease progression influences the efficacy of the intervention. Two studies utilising the MMSE to assess cognition in those with AD both reported improvement following probiotic intervention compared to placebo or alternative therapy (Akbari et al., 2016; Tamtaji et al., 2019), while a third reported improvement in global MMSE score in addition to improvements in memory and visuo-spatial abilities following a kefir fermented milk intervention (Ton et al., 2020). Only one study reported no significant effect, where the TYM was used to assess cognition (Agahi et al., 2018). Again, lack of detail regarding exact probiotic strains, comprehensive demographic data and comparative groups (Tamtaji et al., 2019; Ton et al., 2020) make it more challenging to integrate findings across studies. While the preliminary evidence is positive, more trials are needed to make informed conclusions. In particular, the clinical field would benefit from RCTs longer than 12 weeks to follow the progression of these conditions, and to explore more thoroughly whether probiotics are more effective during earlier stages of AD and MCI, or whether subjects respond better when cognitive impairment is more severe.

The evidence for enhancing cognitive function in ‘healthy’ subjects is more parsimonious. Two studies report a positive effect on emotional cognition (Bagga et al., 2018; Lew et al., 2019) – the latter only finding so in women. An earlier fMRI study appears to support these findings, being the first to demonstrate modulation of cortical activity across a widely distributed brain network during an emotional decision task following supplementation with a probiotic fermented milk (Tillisch et al.,

2013). However, the descriptive results for task performance were not provided here, making it difficult to infer the effect of the probiotic intervention on cognitive performance itself. Unfortunately, this study also used an all-female sample, providing no further opportunity to assess whether the effect could be more pertinent in females than in males. While there is some indication that affective cognition may be a domain for improvement through probiotic supplementation, improved performance was not consistently reported (Allen et al., 2016; Kelly et al., 2017; Papalini et al., 2019). In other studies, a beneficial effect of probiotic intervention on attention and memory domains is arguably overinterpreted, as similar improvements were reported in the placebo group, and the placebo group demonstrated a greater improvement across other cognitive domains post-intervention than the probiotic group (Ohsawa et al., 2018; Czajeczny et al., 2021). As such, the authors recognise these findings may represent a learning effect across test visits (Ohsawa et al., 2018).

Despite seeing no improvement in cognitive performance across the task battery, Papalini and colleagues did find probiotic supplementation to be associated with maintenance of working memory performance under conditions of acute stress (induced by the socially evaluated cold-pressor test; SECPT) where it was otherwise hindered, suggesting a buffering effect against the negative impact of stress on cognitive performance. Similar findings were reported by Allen and colleagues where total cortisol output following exposure to acute stress, again induced using the SECPT, was significantly lower following probiotic intervention, as were reported daily stress levels. Additionally, a greater improvement in conditional learning was observed in the latter following probiotic supplementation compared to the placebo. However, the stepwise improvement in learning appears to be consistent with practice effects and, given that the study employed a non-randomised design with no blinding, it is difficult to ascertain whether any of these results were affected by subject bias. Additionally, the authors included both an emotional recognition task and an emotional Stroop task but report no effect of intervention on either task, further adding to the inconsistency of findings in these heathy populations.

Looking specifically at studies in healthy ageing adults, only four studies have explored the impact of probiotic supplementation on cognition to date, so conclusions are necessarily limited. One study reported improvements in executive function, working memory and sustained attention (Chung et al., 2014). Others reported an improvement in composite score of the MoCA (Inuoe et al., 2018) and in mental flexibility assessed via the CERAD-K (Kim et al., 2021), although, much like the MMSE, these are screening tools for the assessment of MCI and dementia and therefore may not provide an

appropriate measure of cognition in a healthy adult population. Additionally, probiotics were only administered in combination with resistance training in the former, and since the control subjects engaged solely in the resistance training programme demonstrated similar improvements, we can only assume that there is no additional effect of the probiotic supplement to that of the training. Finally, Sanborn and colleagues' (2020) reported that, in a general community-based sample, only those with impaired cognition (as identified by scores on the NIH-toolbox cognitive battery) at baseline demonstrated an improvement in global cognition following probiotic supplementation, suggesting that even in the absence of diagnosed MCI or AD age related cognitive decline varies between individuals, and this degree of cognitive decline may interact with the efficacy of a probiotic intervention for improving cognitive function.

Overall, the evidence in this review suggests that, for healthy young and middle-aged adults, there may be a protective effect against stress-induced declines in cognition and a potential to enhance cognitive function when processing emotional stimuli, but it is difficult to draw firm conclusions from the current literature and further well-controlled randomised trials are needed. In ageing adults, the current literature provides some consistent evidence for improvement in cognitive performance in those with MCI and AD and more limited evidence in healthy ageing adults, with conclusions from studies in both cohorts being hampered by methodological limitations. Importantly, it should also be noted that no adverse effects on cognition were reported in any of the studies discussed here, including those in infants and children.

2.4.1 Single versus multi-strain supplements

Eighteen studies provided single strain supplements and 20 provided multi-strain supplements of between two and nine different strains. Of these, 13 papers report a positive effect on at least 1 cognitive measure following a single-strain intervention, and 15 report a beneficial effect following a combination of strains. Additionally, positive effects were reported across a range of both healthy and clinical populations, in younger, middle-aged and older adults. When comparing the efficacy of single- versus multi-strain interventions it is important to do so based on exact strains, taking into account the specific population being supplemented (McFarland, 2021). Unfortunately, there are too few studies incorporating the same strains into single and multi-strain supplements to draw such comparisons at present. As such, there does not appear to be any clear evidence for use of one supplement type over the other, regardless of age, population or cognitive domain being targeted. This is consistent with findings from a recent review which found that, in most cases, multi-strain

interventions were no more effective than single strain interventions in relieving a range of medical conditions, despite speculation that multi-strain products would potentially cover a wider range of mechanisms of action or result in synergistic effects between the strains (McFarland, 2020).

2.4.2 Species/strains

Studies included species of both *Lactobacillus* and *Bifidobacterium* as single-strain supplements, and *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus* species in various combinations as multi-strain supplements. Three separate studies utilised *L. plantarum* as a single-strain intervention (Bagga et al., 2018; Hwang et al., 2019; Lew et al., 2019), although it was used in combination with fermented soybean powder and SSRIs in two of these, and each study used a different *L. plantarum* strain. Despite exploring the effects in very different populations, all three report a significant positive effect of supplementation following a double-blind RCT, particularly in the domain of sustained attention (Bagga et al., 2018; Hwang et al., 2019). *L. plantarum* has demonstrated good survival and colonisation rates in the human GI tract compared to other lactobacilli species (De Vries et al., 2006) and previous work has reported anti-inflammatory properties, reducing the permeability of the intestinal barrier (White et al., 2006; Wang et al., 2018), increasing SCFA levels (Wang et al., 2014) and restoring brain-derived neurotropic factor (BDNF) levels in cognitively impaired participants (Jeong et al., 2015). The effect of *Bifidobacterium breve* A1 has also been explored in 1 open-label pilot study and 2 double-blind RCTs from the same lab group looking to attenuate cognitive decline in individuals with MCI (Kobayashi et al., 2019a; Kobayashi et al., 2019b; Xiao et al., 2020). All 3 reported a significant positive impact on cognitive function as measured by the MMSE or RBANS following 10 - 20 billion CFU/day for between 3 and 6 months, although in one RCT this was only true for individuals with a lower baseline RBANS score, and in the latter RCT, subjects in the placebo group also demonstrated an improvement across multiple cognitive domains suggesting practice effects. While this improvement was significantly greater in the probiotic group, it does raise the likely issue of learning effects, despite alternative versions of the tests being used pre- and post-intervention. Although limited in number, these initial studies indicate that *B. breve* A1 may provide a useful therapeutic tool for those with MCI, but further longer-term studies with appropriate cognitive measures should be conducted to explore this potential further. In general, a greater number of double-blind RCTs, preferably selecting strains that have demonstrated relevant neuroactive potential and ensuring a probiotic only group, are needed to establish whether certain probiotic strains are more effective in altering cognitive performance than others.

The variety of multi-strain supplements and lack of detail regarding exact strains that were included in any intervention makes it challenging to explore whether there may be particular combinations of strains that are consistently effective at improving cognitive performance. Competition between strains is often quoted as a possible reason for reduced efficacy of multi-strain probiotic supplements, although such literature does not yet exist in relation to cognitive outcomes (Joseph & Law., 2019). However, even when strains are found to have inhibitory effects on each other in a mixed environment, efficacy is not always reduced, and in some cases these combinations outperform the strains individually (Chapman, Gibson & Rowland., 2012). While the complex nature of host/probiotic interactions reduces the likelihood of a 'one size fits all' product, understanding more about the individual mechanisms of action and how stains may interact with, enhance or inhibit one another will be important for ensuring maximum efficacy of probiotic interventions for cognitive health.

2.4.3 Dose

Specified doses ranged from 7.5×10^{6} - 1.8×10^{12} colony forming units (CFU) per day, with 3 studies not disclosing exact quantities (Malaguarnera et al., 2010; Bajaj et al., 2014; Chung et al., 2014). The evidence presented in this review suggests there is currently little consensus regarding an 'optimum' dose, with studies reporting positive effect across the full range of doses. While all trials reporting no significant effect of intervention on cognitive outcomes used a daily dose of below 10^{10} CFU, positive effects on cognition were reported following consumption of 10^{9} CFU/day and lower. Additionally, trials reporting no significant effect of intervention did so across a range of clinical conditions, ages, single and multi-strain interventions. To the best of my knowledge there are currently no dose-response studies for probiotic intervention in cognition.

2.4.4 Length of intervention period

Regarding length of intervention, the current literature comprises of studies ranging from 3 weeks to 6 months. A significant positive effect was consistently reported in studies between and including 4 weeks to 6 months. As there is only one three-week study reviewed here it is not possible to draw any conclusions as to whether this length of supplementation could be sufficient to see an effect on cognition, but, given that other health benefits have been reported following 3 or fewer weeks of

intervention (Nixon et al., 2012) and consistent effects on cognition were reported at 4 weeks, there is perhaps no mechanistic rationale to think not.

2.4.5 Areas of cognition

Despite the number of studies that have now focused on change in cognitive performance following probiotic intervention, heterogeneity in cognitive tasks and common design issues such as randomisation procedures, lack of blinding and the potential for practice effects makes it inherently difficult to identify whether there are particular cognitive domains that are more sensitive to probiotic interventions than others. As described previously, there does appear to be some consistent findings regarding emotionally loaded cognitive tasks (Tillisch et al., 2013; Bagga et al., 2018; Lew et al., 2019), although further research is needed to explore this. A recent review (Long-smith et al., 2020) highlights the mounting support for the use of probiotics in the treatment of psychological disorders, with a number of studies reporting amelioration of affective symptoms and changes in mood. It is also well established that mood affects cognitive function, both in terms of valence and information processing (Forgas, 2017). In particular, studies have demonstrated a robust effect of mood on the processing of face stimuli, both in clinically depressed (Gilboa-Schechtman et al., 2002; Leppänen et al., 2004) and healthy subjects (Van Honk et al., 2003; Curby et al., 2012). This interplay between affect and cognition is perhaps one reason why these emotional decision and recognition tasks may be sensitive to the effects of probiotic intervention.

While not a direct effect on cognitive performance itself, the limited research currently available indicates that probiotics may provide a buffering effect against stress, meaning that cognitive performance is maintained where it would otherwise be negatively affected (Staal et al., 2008). Similar findings have previously been reported following supplementation with milk-based phospholipids, where reaction times in an attention switching task following the SECPT were improved post-intervention compared to pre-intervention performance. Studies in this review employing the SECPT to induce psychological and physiological stress have reported maintenance of working memory performance (Papalini et al., 2019) and lower cortisol output (Allen et al., 2016) following probiotic interventions compared to that of placebos. The effects of probiotics on stress and anxiety are well documented, with animal studies consistently reporting behavioural and biochemical alterations following supplementation, not only in models of physiological stress, but also in those of social and chronic stress (Zareie et al., 2006; Mackos et al., 2016). Additionally, a

recent human trial found altered neural activity following supplementation during a game designed to induce social stress in adults (Wang et al., 2019). While further research is needed to ascertain the legitimacy of this buffering effect following probiotic intervention, future work may wish to establish whether the protective effects extend not only to other cognitive domains, but whether there is a potential to improve cognitive function in individuals facing chronic or perceived stress, as opposed to acute, physiological stress.

2.4.6 Mechanisms of action

Of the studies included in this review, only a handful looked to explore potential mechanisms behind change to cognition, all of which supplemented clinical or sub-clinical populations bar one (Kim et al., 2021). In Kim et al (2021), significant improvement in the mental flexibility subtest of the CERAD-K following combined *B. bifidum* and *B. longum* was associated with an increase in serum levels of BDNF in the probiotic group only. Bajaj and colleagues found following supplementation with *L. rhamnosus* GG that subjects with HE displayed a significant decrease in endotoxemia and TNF- α, in addition to various changes to serum and urine metabolites including amino acids, secondary bile acid and vitamins (Bajaj et al., 2014). However, it should be noted that these metabolic changes were found in the absence of change to cognitive performance. Lew and colleagues describe similar findings following *L. plantarum* intervention in mildly stressed adults, where better emotional cognition and recognition memory were associated with a significant reduction in pro-inflammatory cytokines interferon gamma (IFN- γ) and TNF-α (Lew et al., 2019). Similarly, Ton and colleagues reported a reduction in pro-inflammatory cytokines and oxidative stress markers following kefir fermented milk, which was coupled with significant improvements across multiple cognitive domains in participants with AD. Two studies supplementing patients with AD with multi-strain *Lactobacillus* and *Bifidobacterium* interventions reported similar changes in metabolic outcomes, including reduced serum hs-CRP, triglycerides and a decrease in insulin resistance and increase in insulin sensitivity (Akbari et al., 2016; Tamtaji et al., 2019). Additionally, Tamtaji and colleagues report a downregulation in gene expression of TNF-α and a concurrent upregulation in genes associated with maintenance of low cholesterol and energy homeostasis (low-density lipoprotein receptor and peroxisome proliferator-activated receptor gamma, respectively). In this study subjects were supplemented with a combination of selenium and probiotic strains and, while these effects were greater than in those just taking selenium, no probiotic alone group was included. In subjects with MCI, improvement in cognitive function, particularly sustained attention, following consumption of

L. plantarum was associated with increased serum BDNF levels (Hwang et al., 2019) – an important protein for neural health and one that is heavily implicated in learning and memory processes (Cunha et al., 2010). Finally, combined supplementation of SSRIs and *L. plantarum* was found to be associated with a decrease in kynurenine concentration, which may affect cognition via a number of mechanisms (Rudzki et al., 2019). While it seems *L. plantarum* supplementation is associated with different metabolic changes in each study that it was used, it is important to note that each of these studies focused on different biochemical outcomes and therefore common mechanisms of action cannot be ruled out.

By altering the gut microbiota, probiotic interventions may affect neural function and thus cognition via one or a combination of mechanisms. The current literature provides some evidence for improved cognition in clinical populations via modulation of immunological pathways and reduction in systemic inflammation, but these effects are inherently linked to physiological changes in the clinical parameters of interest, and there is little understanding regarding potential mechanisms in healthy subjects. Further research is required to elucidate precise mechanisms and factors that may influence these, such as host age, health status and microbiota composition.

2.4.7 Limitations and considerations for future work

While this area of research is gaining traction, this review highlights a number of recurring limitations to study designs which impede our ability to integrate the studies and draw reliable inferences for the effect of probiotics on cognitive function. While many studies employed RCT designs, a number of these were not carried out double-blind to the intervention and a small number used alternative single-arm or non-randomised designs. The QCC also highlighted a general lack of clarity regarding participant demographics across the studies in this review, with many not providing basic information such as an explicit age range and mean, or not indicating gender splits. Additionally, a number of studies did not include any form of power calculation to determine sample size, and those who did often did not reach this quota for all cognitive measures. Going forward it is important that studies are well powered, particularly as nutrition interventions do not typically have large effects and require a sensitive design (Flanagan et al., 2020).

As this review aimed to incorporate as many experimental trials as possible, this led to the inclusion of a number of studies that used probiotics in combination with additional treatments for cognitive

impairment or a particular clinical condition. While important to explore combined effects, not all included a comparison group only taking the probiotic supplement or the additional treatment. As such, it is difficult to extrapolate reliably the effect of the probiotic supplement relative to the other. It would be helpful in future studies wishing to explore combined effects to include a comparison group for each treatment component separately, in order to better understand both the individual and combined treatment effects.

One of the key limitations in the current literature is a lack of explicit detail regarding the probiotic interventions themselves, particularly in neglecting to specifically identify the strain(s). This is increasingly important as research suggests that effects are frequently strain specific (Savignac et al., 2014; Kekkonen et al., 2018). Additionally, despite investigating how alterations to the GM might affect cognitive function, few studies performed faecal analysis to assess microbiota composition post-intervention and none to date have collected pre-intervention samples. Assessing both pre- and post-intervention faecal community allows insights into how the intervention may have altered the composition of the resident microbiota. While this data is useful to have, current research actually suggests that probiotic interventions are unlikely to result in observable changes to the composition, particularly in healthy populations, both in terms of diversity and richness (Kristensen et al., 2016). Instead, it may be of greater insight to explore how probiotics help to stabilise and reinforce the microbiota, as opposed to numerically changing the composition (Sanders, 2016). Additionally, faecal samples provide an opportunity to explore how probiotics may enhance neurotransmitter synthesis through changes to metabolite production, which may also be crucial to understanding the mechanisms behind change in cognitive function following supplementation. Due to the complex nature of the human gut, the same probiotic intervention will inherently affect different hosts in a multitude of different ways (Wieërs et al., 2020). For example, baseline microbiota composition and diet have been identified as factors that may influence the efficacy of a dietary supplement such as probiotics for the host (Mobini et al., 2017; Volokh et al., 2019). As such, it may be of greater importance for future studies to collect baseline faecal samples to see for whom certain probiotics may be more effective. To this end it may be useful to collect information regarding habitual diet, too.

While the majority of studies in this review utilised standardised cognitive tasks with clear outcome measures, very few indicated whether parallel task versions had been used where appropriate in order to avoid practice effects. In addition, few, if any, provided subjects with practice in the cognitive tasks prior to beginning the experimental trial. Including such practice allows subjects to

become comfortable with the task(s) and perform towards the ceiling of their natural capacity at baseline, therefore helping to remove practice as a confound for improved performance (Bell et al., 2018). Finally, factors such as time-of-day effects were rarely acknowledged. There is strong evidence for the existence of time-of-day effects in cognitive testing, where an individual's performance on a range of cognitive tasks can differ depending on the time of day that it is being tested (Schmidt et al., 2007). The same is true of meals, where exacerbated time-of-day effects known as post-prandial dips can be seen in cognitive performance following food intake, particularly after lunch (Craig, 1986; Rogers & Lloyd., 1994). Again, this phenomenon was rarely acknowledged in the current literature, with very few stating what time in the day cognitive performance was measured, whether participants were provided with a standardised meal prior to cognitive testing and whether time of testing remained consistent both within and between participants. These are therefore important considerations going forwards in order to strengthen the design of studies exploring probiotic effects on cognition.

To progress this field of work, future studies should be properly randomised, placebo-controlled trials with sufficient sample sizes to detect an effect of probiotic supplementation on the primary outcome measure. Cognitive measures should be appropriate to the population of interest, with hypotheses and intended statistical analysis specified a priori. Additionally, researchers should consider designing trials in a way that mitigates potential confounding factors such as diet, practice and time-of-day effects. Finally, where possible, researchers should take the opportunity to further explore potential underlying mechanisms of action through collection and analysis of faecal microbiota community, serum and urine metabolites related to GBA, and neuroimaging.

2.4.8 Conclusions

The evidence thus far provides some support for enhancing cognition through probiotic intervention. Studies in infants and children find very little benefit of early probiotic supplementation to enhance subsequent neurocognitive development. However, studies in young and middle-aged adults do provide some support for supplementary probiotics, particularly in clinical populations where cognitive function may be impaired. Affective cognition and cognition under stress may be two aspects of cognitive function that are particularly sensitive to any effect of probiotics at this age. Similarly, studies in older adults provide some consistent evidence for a beneficial effect of probiotics, particularly on memory processes. However, this review has

highlighted a number of consistent methodological issues within the current literature that make interpretation of data challenging. A greater number of well-controlled RCTs with a primary focus on cognitive performance and potential mechanisms of action are needed in order to clarify how effective probiotic interventions are for improving cognitive function, and which cognitive functions, within specific populations. Such research may then inform exciting opportunities for both clinical and individual practice for those who might see a benefit of supplemental probiotics on cognitive function.

Chapter 3 – The effect of probiotic bacteria on the composition and metabolite production of faecal microbiota using in vitro batch cultures

A version of this chapter has been submitted to Nutrients and is currently under review.

3.1 Introduction

Given the MGB pathways outlined in chapter 1, there is growing interest in the role of microbiota-derived metabolites in the gut brain axis, and, in particular, how neuroactive metabolites produced in the gut may influence cognitive function (Connell et al., 2022). A recent study found distinct faecal microbiota metabolite profiles in those with MCI, AD, and healthy controls (HC), where AD was associated with reduced levels of 5-Hydroxytryptophan (5-HTP – precursor to serotonin) and several short-chain fatty acids (SCFAs) (Wu et al., 2021). Importantly, these decreases in metabolites were most profound in those with AD but still evident to a lesser extent in MCI compared to HC. Additionally, degree of cognitive impairment, as measured by the Montreal Cognitive Assessment (MoCA), positively correlated with the reduction in serotonin and SCFAs. Faecal metabolites have been excreted, making it difficult to infer whether lower faecal concentrations reflect higher absorption *in vivo*, or lower levels of microbially derived metabolites in the gut lumen. Additionally, cause and effect is unclear, as lower levels of microbially derived metabolites may contribute to cognitive dysfunction, or cognitive dysfunction may affect the synthesis of metabolites in the gut. Having said this, the progressive reduction in faecal metabolite concentration with disease severity does indicates a clear association between these microbially derived metabolites and cognitive dysfunction. Metagenome-wide association has also highlighted functional differences in the microbiota of medication-free schizophrenia patients compared to healthy controls, once again including altered SCFA and tryptophan metabolism, as well as the synthesis/degradation of neurotransmitters (Zhu et al., 2020). Transplantation of a *Streptococcus vestibularis* strain – a bacteria predicted through gut-brain module analysis of faecal metagenomes to influence glutamate synthesis, GABA degradation and isovaleric acid synthesis, and found to be widely present in the schizophrenia patients – into antibiotic depleted mice resulted in reduced serum levels of dopamine and GABA, alongside decreased levels of tryptophan in the prefrontal cortex and altered social

behaviour, again providing support for the effect of microbially derived metabolites on brain function.

Despite growing evidence for a role of microbially derived metabolites on the GBA, the largely accepted opinion that bacteria produce neurotransmitters is perhaps less clear-cut than suggested (Cryan & Dinan, 2012; Strandwitz, 2018). *In silico* methods show predicted changes in the abundance of gut-derived neurotransmitters such as γ-aminobutyric acid (GABA) following probiotic supplementation (Ma et al., 2021), and genome-based analyses have allowed for the cataloguing of neuroactive potential of various bacterial strains to synthesise and utilise metabolites relevant to the gut-brain axis (Valles-Colomer et al., 2019; Kaur, Bose & Mande., 2019; Zhu et al., 2020). This neuroactive potential has been explored *in vitro* for several promising strains, with the production of GABA (Cho et al., 2007; Barrett et al., 2012), dopamine (Ozogul et al, 2012), serotonin (Ozogul et al., 2012), histamine (Landete et al., 2007) norepinephrine (Tsavkelova et al., 2000) and acetylcholine (Girvin et al., 1954) from bacteria of various genera including *Lactobacillus*, *Bifidobacterium* and *Lactococcus* being reported. However, in many of these studies, probiotic bacteria were cultured in irrelevant conditions optimised for strain growth and neurotransmitter synthesis, such as modified culture mediums and optimum pH and temperature, resulting in conditions that do not translate to the human GIT. While this suggests these strains are capable of producing neurotransmitters, it is less clear to what extent this may occur under physiologically relevant conditions. The presence of several neurotransmitters was recently reported in one *in vitro* study utilising three-stage continuous gut models to explore the impact of a pre or probiotic intervention on metabolite production under conditions reflective of anorexia nervosa (Liu et al., 2021). Here, relatively low concentrations of NTs were detected following a restricted nutrient phase, and provision of pre- and probiotics modulated metabolite synthesis to resemble that of a healthy diet.

In addition to neurotransmitters, gut microbes produce SCFAs as a result of polysaccharide fermentation (Tan et al., 2014). SCFAs such as butyrate, acetate and propionate regulate the expression of precursors tryptophan 5-hydroxylase and tyrosine hydroxylase, which in turn influence the synthesis of serotonin and biosynthesis of catecholamines dopamine, epinephrine, and norepinephrine, respectively (Reigstad et al., 2015). As such, where probiotic bacteria may not directly produce neurotransmitters under physiologically relevant conditions, production of neuroactive compounds may instead be modulated as a result of increased SCFA synthesis. Further to their role in neurotransmitter synthesis, SCFAs appear to be important in the production of brain-derived neurotropic factor (BDNF), blood-brain-barrier integrity, gut permeability and regulation of

neuroinflammation, all of which have a significant effect on cognitive and psychological function (Dalile et al., 2019). Although largely established through animal research, the introduction of probiotics has been found to both modulate the number of SCFA-producing bacteria and increase concentrations of SCFAs in lumen and serum samples (Cheng, Liu & Ling., 2022; Markowiak-Kopeć et al., 2020). In the limited human GBA literature, probiotic supplementation has been associated with increases in faecal concentrations of acetate, propionate and butyrate in volunteers ranging from infants to older adults (Wang et al., 2014; Kim et al., 2015), although an effect of probiotic supplementation on faecal SCFAs is not always found (Larsen et al., 2013).

Despite accumulating evidence, it is unclear at present to what extent metabolites synthesised in the gut may directly affect neural function (Caspani & Swann., 2019). It is believed that most circulating neurotransmitters are unable to cross the blood brain barrier (BBB) due to insufficient transport systems, and instead interaction with other host systems, such as the vagus nerve, may be necessary to alter the expression of neurotransmitters in the brain (Bravo et al., 2011). On the other hand, smaller molecules such as the amino acids serving as precursors to neurotransmitters do appear to have the capacity to cross the BBB (Inazu., 2019; Zaragoza., 2020), as do SCFAs (Frost et al., 2014; Logsdon et al., 2018). Nevertheless, there is consistent evidence that altering the GM through probiotic intervention results in an alteration of circulating neurotransmitters, as intervention studies in both animals and humans have reported associated increases in lumen, serum and neural concentrations of neurotransmitters and their precursors following chronic probiotic supplementation (Pokusaeva et al., 2017; Cao et al., 2019; Leblhuber et al., 2018; Wang et al., 2020).

Based on previous metagenomic data (Valles-Colomer et al., 2019), six probiotic strains (*Lactobacillus rhamnosus* W198, *Lactobacillus reuteri* W192, *Bacillus coagulans* W64, *Propionibacterium freudenreichii* W200, *Lactococcus lactis* W58 & *Bacillus subtilis* W201) were identified as having high neuroactive potential – that is, expressing identified microbial pathways that metabolise molecules that have the potential to interact with the human nervous system – for various metabolic pathways including tryptophan synthesis and degradation, glutamate and GABA synthesis and degradation, quinolinic acid degradation, dopamine synthesis and synthesis of various SCFAs.

In vitro batch culture fermentation provides a means to screen these probiotic strains and build upon previous work by exploring metabolite production under physiologically relevant conditions, allowing for control of anaerobic conditions, pH and temperature to mimic the environment of the

proximal colon. As such, this work employed faecal batch culture fermentation with the primary aim of assessing the production of neuroactive metabolites in human faecal microbiota under conditions relevant to the human GIT. Secondly, this work aimed to explore how the selected probiotic strains may affect bacterial composition and the synthesis of both SCFAs and neurotransmitters under relevant conditions.

3.2 Method

3.2.1 Preparation of probiotic strains

Prior to performing the batch cultures, calibration curves in Man Rogosa Sharpe broth (Sigma-Aldrich, Kent UK) for *L. rhamnosus* W198, *L. reuteri* W192, *B. coagulans* W64, *Propionibacterium freudenreichii* W200, and *L. lactis* W58 and General Nutrient Broth (Sigma-Aldrich) for *B. subtilis* W201 were conducted in triplicate for each strain, in order to identify the correlation between optical density (OD_{600nm}) (Thermo Scientific Orion AquaMate 8000) and bacterial numbers in colony forming units (CFU).

In preparation for batch cultures Hungate tubes containing the appropriate anaerobic broth (detailed above) were inoculated with a colony of bacteria, these were incubated overnight at 37°C, overnight cultures were measured for OD and this was adjusted to yield 5x10^{8} CFU/mL per strain for inoculation. Plating of cultures was conducted to confirm inoculation concentration.

3.2.2 Faecal sample preparation

Fresh faecal samples were collected and placed in an anaerobic jar using Thermo Scientific AnaeroGen 2.5 L anaerobic sachets (Oxiod, Basingstoke UK). Samples were used for inoculation within 2 hours of production. 15 g of weighed faecal sample was homogenised with 135 mL of anaerobic PBS for 2 minutes using a stomacher (Stomacher 400, Seward, West Sussex, UK) at 240 paddle beats/min to form a 10% faecal slurry (w/v).

3.2.3 Batch culture fermentation

pH controlled, anaerobic, stirred batch cultures were performed in triplicate. 135 mL of standard basal nutrient medium (Tzounis et al., 2008) with additional 0.1% tryptone (0.15g) and 0.2% lactose (0.3g) for bacteria growth was steamed and aseptically added to autoclaved 300 mL vessels. Vessels were then left to gas overnight using N_2 at a rate of 15 mL/min to achieve anaerobic conditions.

Vessels were maintained at a temperature of 37◦C using a circulating water bath. The media was adjusted to pH 5.5 and subsequently maintained between 5.4 and 5.6 using pH controllers (Electrolab, Tewkesbury UK) connected to 0.5 M solutions of HCL and NaOH. This pH was selected in order to mimic conditions of the proximal colon, under which GABA synthesis has previously been reported (Otaru et al., 2021). Immediately prior to faecal inoculation, overnight probiotic cultures, were added to vessels to provide estimated concentration of $5x10^8$ CFU. In addition, each fermentation run included a negative control vessel, to which only the faecal slurry was added, and a positive control vessel, to which inulin (synergy 1, Beneo, Belgium) (1.5g) was added as an additional substrate. Inulin has a known bifidogenic effect, where, due to the bacteria's preference for inulin type fructans, provision of inulin under physiologically relevant conditions results in increased bacterial numbers of *Bifidobacterium* and subsequent production of SCFAs, particularly acetate (Meyer & Stasse-Wolthuis., 2009; Ahmed & Rashid., 2019). Given that exploration into the effect of the selected probiotic strains is novel under these conditions, it was important to include a vessel where the effect of fermentation could be predicted. Demonstration of this bifidogenic effect within the positive control vessel would therefore provide evidence that the fermentation model was functioning as intended and increase confidence in the results within the probiotic vessels.

All vessels were inoculated with 15 mL of faecal slurry (10% w/v) to give a final concentration of 1% faeces (w/v). Baseline samples were taken immediately post-inoculation, and further samples were collected at 4, 8, 24 and 48 hours while a stable pH and anaerobic conditions were maintained throughout.

3.2.4 Preparation of samples

1 mL, 1.5 mL and 0.75 mL of sample was aliquoted to Eppendorfs for Liquid Chromatography – Mass Spectroscopy (neurotransmitters), Gas Chromatography (short-chain fatty acids) and Fluorescence *in*

situ Hybridisation (enumeration of bacteria), respectively. 1 mL samples were immediately stored at -20°C. For GC, samples were centrifuged at 11, 600 *g* for 10 minutes, before transferring the supernatant and storing the pellet at -20°C (Cunningham et al., 2020). For FISH, samples were centrifuged at 11, 600 *g* for 5 minutes. After removing the supernatant, the pellet was resuspended in 375 µL of PBS before adding 1125 µL of 4% paraformaldehyde. These samples were then stored at 4°C for 4-8 hours before being washed twice with 1 mL of PBS and resuspending the pellet in 150 µL of PBS. Finally, 150 µL of ethanol was added, the samples were vortexed to homogenise, and stored at -20°C.

3.2.5 Fluorescence in situ hybridisation with flow cytometry (flow-FISH)

Preparation of samples followed the protocol of Grimaldi et al (2017). Briefly, samples were removed from storage at -20°C and vortexed to redisperse. 75 µL of sample was suspended in 500 µL of PBS before vortexing and centrifuging for 3 minutes at 11, 600 *g* (consistent for all centrifuging during this process). For permeabilisation of the bacterial cell wall, supernatant was discarded, and the pellet resuspended in TE-FISH containing lysozyme (1 mg/ml) and incubated in the dark for 10 minutes at room temperature. Samples were then re-centrifuged and washed using 500 µL PBS. For *in situ* hybridisation, pellets were resuspended in 150 µL of hybridisation buffer (0.9 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% sodium dodecyl sulphate, 30% formamide), centrifuged, and resuspended again in 1 mL. 50 µL of this solution was added to each Eppendorf containing 4 µL of the oligonucleotide probe solutions, which were vortexed and incubated overnight at 36°C using heating blocks. Following incubation, 125 µL of hybridisation buffer was added, and Eppendorfs were vortexed and centrifuged as standard. After discarding the supernatant, pellets were resuspended in 175 µL of washing buffer (0.064 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate), vortexed to homogenise and incubated at 35°C for 30 minutes in the heating block. The washed pellets were then centrifuged once again, resuspended in 300 µL of PBS, vortexed and stored in the dark at 4°C ready for flow cytometry. Enumeration of bacteria was conducted using the Accuri C6 flow cytometer and analysed using the Accuri CFlow Sampler software.

Ten oligonucleotide probes (Table 3.1) were selected for inclusion, targeting a range of functionally relevant bacterial populations. Additionally, a mixed 338EUB probe was used to enumerate total bacteria.

Table 3.1 - Oligonucleotide probe sequences and corresponding target species.

Probe	Sequence	Target species	Reference
Non-Eub	ACTCCTAGGGAGGCAGA	Control probe for EUB338	Wallner et al (1993)
Eub338I+	GCTGCCTCCCGTAGGAGT	Most bacteria	Daims et al (1999)
Eub338II+	GCAGCCACCCGTAGGTGT	*Planctomycetales*	Daims et al (1999)
Eub338III+	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	Daims et al (1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	Langendijk et al (1995)
Lab158	GGTATTAGCAYCTGTTTGGA	*Lactobacillus* and *Enterococcus*	Harmsen et al (1999)
Bac303	CCAATGTGGGGGACCTT	*Bacteroidaceae*, *Prevotellaceae*	Manz et al (1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group	Franks et al (1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia*	Walker et al (2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	Harmsen et al (2000)
Prop853	ATTGCGTTAACTCCGGCAC	*Clostridium* cluster IX	Walker et al (2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	Hold et al (2003)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Devereux et al (1992)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group	Franks et al (1998)

3.2.6 Gas Chromatography

Preparation of samples for GC was carried out in line with the method previously described by Richardson and colleagues (1989). Samples were defrosted, vortexed, and 1 mL transferred to 100 mm x 16 mm glass vials, in addition to 50 µL internal standard (0.1M 2-ethylbutyric acid) 0.5 mL concentrated HCl and 2 mL diethyl ether. Vials were vortexed for 1 minute and centrifuged for 10 minutes at 2000 *g* (Eppendorf 5804 R). The upper diethyl ether layer was extracted and transferred to new vials, from which 400 µL was taken and added to a screwcap HPLC vial with 50 µL of

MTBSTFA. The vials were protected from light and stored at room temperature for 72 hours prior to analysis to allow for all SCFAs, including lactate, to derivatise.

Samples were analysed using a 5690 series Gas Chromatograph (Hewlett Packard, UK) with HP-5ms column (L × I.D. 30 m × 0.25 mm, 0.25 μm film thickness) coating of crosslinked (5%-phenyl)-methylpolysiloxane (Hewlett Packard, UK)). 1 μL of each sample was injected with a run time of 17.7 min. Injector and detector temperatures were 275°C and the column temperature programmed from 63°C to 190°C by 5°C and held at 190°C for 30 min. Helium was used as the carrier gas at a flow rate of 1.7 mL/min (head pressure, 133 KPa). The external standard solution included: acetic acid (30 mM); propionic acid (20 mM); n-butyric acid (20 mM); n-valeric acid (5 mM); iso-butyric acid (5 mM); iso-valeric acid (5 mM) (all Sigma-Aldrich). Quality control (QC) samples of external standard solution were included between donors to maintain accurate calibration. Peak integration was performed using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK), and quantification of each SCFA (mM) was calculated using internal response factors as described by Liu (2016).

3.2.7 Liquid Chromatography Gas Spectroscopy

Samples were first removed from storage at -20°C and centrifuged for 5 minutes at 2000 *g*. 10 μL of supernatant was added to 9.99 mL of HPLC water to form a 1:1000 dilution, which was then filtered using 0.22 μm syringe filters. 1 mL was added to a screwcap HPLC vial for analysis. In addition, 1 mL of batch culture medium was prepared in the same manor to be analysed as a control. Choice of metabolites was guided by previous *in vitro* (Tsavkelova et al., 2000; Özugul et al., 2012; Barrett et al., 2012; Kaur et al., 2019) and metagenomic work (Valles-Colomer et al., 2019), suggesting bacterial capacity to produce or influence the metabolism of these compounds under optimal conditions, as well as considering neuroactive metabolites implicated in cognitive function, particularly those known to be affected in ageing (Szalardy et al., 2012; Vazey et al., 2012; Mattson & Arumugam., 2018; Schmidt-Wilcke et al., 2018). Individual stock solutions were prepared using analytical standards powders of dopamine hydrochloride (99%, Alfa Aesa), serotonin (Sigma-Aldrich), tryptophan (98%, Sigma-Aldrich), GABA (99%, Sigma-Aldrich), L(-)-epinephrine (99%, Acros Organics), L-noradrenaline (98%, Alfa Aesa) and kynurenic acid (98%, Sigma-Aldrich), each at 10000 ng/mL. A mixed standard solution was then prepared from the individual stock solutions and used to create a 7 level calibration series with the following dilutions: 10, 5, 1, 0.5, 0.25, 0.125, 0.0625 ng/mL. Additionally, a 1 ng/mL standard was run every 20 samples as a QC.

Samples were analysed by liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) using an Agilent 1200 HPLC system with a 6410 triple-quadrupole mass spectrometer with electrospray ion source in positive ion mode. A gradient separation was carried out using a 150 × 2.1 mm Discovery HS F5 – 3 column, with a 2 × 2.1 mm Discovery C18 Supelguard precolumn (both 3 µm particle size; Supelco, Dorset, UK). The column was maintained at 40 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The column flow rate was maintained at 0.4 mL/min. The timetable was as follows: 0–2 min, 100% A; 5 min, 75% A; 11 min, 65% A; 15–20 min 5% A; 20.1–30 min, 100% A. The injection volume was 25 µL. The eluant from the column was run to waste from 0 to 1 min, and data were collected from 1 to 18 min. Data were acquired in dynamic MRM mode. The transitions studied and voltages used are shown in Table 3.2. Two transitions were acquired for each compound.

Table 3.2 - LC-MS/MS conditions used for quantification in faecal supernatant.

Compound	Retention time (min)	Retention time window (min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision energy (V)	Classification
GABA	1.90	3	104	87	50	4	Organic acid
		3	104	45	50	20	
Norepinephrine	2.50	3	152	107	116	16	Catecholamine
			152	77	116	30	
Epinephrine	4.60	3	184	166	70	8	Catecholamine
			184	107	70	24	
Dopamine	7.00	3	154	137	75	8	Catecholamine
			154	91	75	28	
Serotonin	9.70	3	177	160	45	4	Amino acid derivative
			177	115	45	30	
Kynurenic acid	9.77	3	190	144	100	16	Organic acid
			190	172	100	4	
Tryptophan	10.20	3	205	188	78	4	Amino acid
			205	146	78	20	

3.2.8 Statistical analysis

All statistical analyses were performed using R statistical software (R Core Team., 2022). The effect of Time (0, 8 and 24 hours of fermentation) and Vessel (negative control, positive control (inulin), *B. coagulans, B. subtilis, L. reuteri, Lc. lactis, L. rhamnosus, P. freudenreichii)* on specific bacterial groups, SCFAs and neurotransmitters was assessed using repeated-measures two-way ANOVAs with post-hoc pairwise comparisons (Bonferroni corrected) using the R Stats package (Chambers, Freeny and Heiberger., 1992). Samples taken at 4 and 48 hours were not analysed, as a lack of HCL and NaOH usage across all vessels at these timepoints indicated limited bacterial activity. As inulin is known to affect SCFA production, particularly acetate and lactate, it was anticipated that change in SCFA concentration over the fermentation period would be greatest in the positive control vessel. Given that inulin was only used as a positive control substrate in this model and the effect of inulin on metabolite production is not relevant to the aims of this work, statistical analysis of SCFA concentration was run both including and excluding the positive control vessel, in case the larger known effect of inulin on SCFA concentration masked any smaller effects in the probiotic vessels of interest. Statistical significance was set to $p < 0.05$ and data is presented as mean ± between-subject standard error unless otherwise stated.

3.3 Results

3.3.1 Donors

Faecal samples were provided by 3 healthy donors (2 male, 1 female) aged 21, 23 and 23, respectively. Donors were not regular users of pre/probiotics or consumers of live yoghurt, and had not consumed antibiotics in the 3 months prior to donating.

3.3.2 Enumeration of bacteria with flow-FISH

Table 3.3 illustrates differences in bacterial groups at baseline (0), 8 and 24 hours. No significant difference in bacterial numbers was found between vessels at baseline. A significant main effect of Time was observed on total bacteria and most bacterial groups assessed, including *Clostridium coccoides–Eubacterium rectale* (EREC), *Roseburia* subcluster (RREC), *Faecalibacterium*

prausnitzii (FPRAU), *Desulfovibrio* (DSV), and *Clostridium histolyticum* (CHIS), where bacterial numbers steadily declined over the 24-hour period across all probiotic and non-probiotic vessels (all $p < 0.05$). In comparison, no main effect of Time or Vessel was observed for numbers of *Bacteroides-Prevotella* spp. (BAC) or *Clostridium* cluster IX (PROP). However, in contrast to other bacteria groups, visual inspection of the data indicates that numbers of *Bacteroides-Prevotella* spp. increased between T0 and T8 across all probiotic vessels (except *P. freudenreichii*), but not in the control vessels. Similarly, numbers of *Clostridium* cluster IX display a general increase in the probiotic vessels over the fermentation period compared to the control vessels, although these changes were non-significant ($p > 0.1$).

With regards to *Bifidobacterium* spp. (BIF), a Time x Vessel interaction was observed (F (14, 28) = 2.068, $p = 0.049$). Pairwise comparisons indicate that this was driven by a significant increase from 6.6 to 7.5 $\log_{10}$ cells/mL by T8 in the positive control vessel, following the fermentation of inulin (p = 0.021). No significant change in *Lactobacillus* spp. (LAB) or *Atopobium–Coriobacterium* spp. (ATO) was observed.

Table 3.3 - *Enumeration of bacteria by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.*(BIF), *Lactobacillus* spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), *Clostridium coccoides–Eubacterium rectale* group (EREC), *Roseburia* subcluster (RREC), *Faecalibacterium prausnitzii* (FPRAU), *Clostridium* cluster IX (PROP), *Atopobium-Coriobacterium* spp. (ATO), *Desulfovibrio* (DSV) and *Clostridium histolyticum* (CHIS). Values are presented as mean ± standard. * denotes significant difference from baseline within vessel, where p <0.05. No significant difference between the negative control and probiotic vessels was found at any timepoint.

Vessel	Time (hours)	Bacterial groups detected by flow-FISH										
		Total bacteria	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Negative control	0	7.91 ± 0.22	6.72 ± 0.19	5.86 ± 0.39	6.24 ± 0.39	7.42 ± 0.39	6.33 ± 0.81	5.88 ± 0.17	5.76 ± 0.82	7.28 ± 0.22	5.49 ± 0.50	5.70 ± 0.70
	8	7.60 ± 0.29	6.44 ± 0.41	5.03 ± 0.69	6.21 ± 0.49	6.86 ± 0.53	5.59 ± 0.76	6.04 ± 0.22	5.75 ± 0.69	6.96 ±0.23	3.90 ± 0.34	4.87 ± 0.75
	24	7.6 ± 0.20	6.49 ± 0.25	5.10 ± 0.79	6.09 ± 0.49	6.92 ± 0.35	4.83 ± 0.41	6.08 ± 0.49	5.71 ± 0.16	6.53 ± 0.51	4.09 ± 0.46	5.00 ± 0.72
Positive control	0	7.92 ± 0.28	6.63 ± 0.04	5.43 ± 0.90	6.12 ± 0.58	7.41 ± 0.49	6.19 ± 1.15	5.77 ± 0.34	5.87 ± 0.44	7.29 ± 0.26	4.94 ± 0.88	5.41 ± 0.72
	8	7.93 ± 0.15	7.51 ± 0.15*	5.12 ± 0.58	6.02 ± 0.77	6.92 ± 0.35	5.57 ± 0.37	6.68 ± 0.99	5.86 ± 0.47	6.91 ± 0.12	4.57 ± 0.20	5.41 ± 0.29
	24	7.73 ± 0.33	7.32 ± 0.61	4.95 ± 0.68	5.86 ± 1.09	6.36 ± 0.11	4.76 ± 0.82	6.30 ± 0.37	5.72 ± 0.39	6.35 ± 0.26	4.36 ± 0.78	4.31 ± 0.83
B. coagulans	0	7.80 ± 0.20	6.67 ± 0.22	5.44 ± 0.58	5.81 ± 0.83	7.24 ± 0.41	6.21 ± 0.81	5.55 ± 0.20	5.34 ± 0.62	7.16 ± 0.24	4.63 ± 1.15	4.82 ± 0.96
	8	7.57 ± 0.38	6.09 ± 0.96	4.96 ± 0.47	6.11 ± 0.58	6.76 ± 0.97	5.08 ± 1.53	5.99 ± 0.49	5.72 ±0.46	6.67 ± 0.68	3.73 ± 0.10	4.59 ± 0.27
	24	7.65 ± 0.24	6.83 ± 0.22	5.39 ± 0.51	5.72 ± 0.59	6.55 ± 1.04	5.00 ± 0.64	6.43 ± 0.58	6.18 ± 0.13	6.21 ± 0.48	4.33 ± 0.36	4.91 ± 0.42
B. subtilis	0	7.84 ± 0.19	6.65 ± 0.16	5.29 ± 0.57	5.85 ± 0.73	7.31 ± 0.43	6.15 ± 0.86	5.33 ± 0.30	5.75 ± 0.54	7.21 ± 0.20	4.96 ± 0.76	5.31 ± 0.59
	8	7.86 ± 0.03	6.78 ± 0.28	5.53 ± 0.30	6.49 ± 0.21	7.08 ± 0.59	5.85 ± 0.57	6.22 ± 0.26	6.18 ± 0.31	7.16 ± 0.14	4.54 ± 0.59	5.54 ± 0.29
	24	7.58 ± 0.11	6.66 ± 0.34	5.15 ± 0.55	5.72 ± 0.59	6.66 ± 0.55	5.07 ± 0.49	6.23 ± 0.24	5.84 ± 0.21	6.46 ± 0.32	3.84 ± 0.35	5.07 ± 0.62
L. reuteri	0	8.00 ± 0.08	6.64 ± 0.48	5.51 ± 0.22	6.15 ± 0.47	7.42 ± 0.32	6.50 ± 0.68	5.48 ± 0.46	5.85 ± 0.84	7.39 ± 0.08	5.11 ± 0.44	5.42 ± 0.68
	8	7.84 ± 0.11	6.58 ± 0.66	5.62 ± 0.21	6.64 ± 0.19	6.13 ± 1.66	6.00 ± 0.57	5.90 ± 0.66	6.00 ± 0.82	7.19 ± 0.13	4.70 ± 0.21	5.25 ± 0.46
	24	7.81 ± 0.11	6.48 ± 0.86	5.69 ± 0.42	6.15 ± 0.67	7.02 ± 0.44	5.56 ± 0.65	5.95 ± 0.43	6.34 ± 0.17	6.85 ± 0.38	4.59 ± 0.17	5.11 ± 1.04
P. freudenreichii	0	7.96 ± 0.09	6.64 ± 0.37	5.49 ± 0.46	6.04 ± 0.19	7.40 ± 0.30	6.29 ± 0.53	5.51 ± 0.26	5.68 ± 0.91	7.33 ± 0.02	4.91 ± 0.30	4.85 ± 0.82
	8	7.44 ± 0.55	6.16 ± 1.11	5.44 ± 0.91	5.41 ± 1.06	5.58 ± 0.72	5.05 ± 0.29	5.22 ± 1.57	5.07 ± 1.07	6.59 ± 0.55	3.94 ± 0.58	4.53 ± 0.70
	24	7.33 ± 0.74	5.95 ± 1.25	5.66 ± 1.01	4.90 ± 1.21	6.11 ± 1.06	4.26 ± 0.49	6.19 ± 0.50	5.89 ± 0.70	6.10 ± 0.81	4.33 ±0.31	4.55 ± 0.48
Lc. lactis	0	8.01 ± 0.07	6.75 ± 0.19	5.87 ± 0.37	5.99 ± 0.53	7.51 ± 0.26	6.36 ± 0.67	5.75 ± 0.24	5.99 ± 0.51	7.41 ± 0.04	4.86 ± 0.29	5.53 ± 0.70

	8	7.66 ± 0.23	6.67 ± 0.40	5.39 ± 0.15	6.53 ± 0.18	6.90 ± 0.21	5.58 ± 0.32	6. 02 ± 0.63	6.04 ± 0.53	6.92 ± 0.31	4.18 ± 0.70	5.74 ± 0.67
	24	7.56 ± 0.34	6.73 ± 0.53	5.01 ± 0.16	5.76 ± 0.26	6.65 ± 0.54	4.89 ± 0.40	6.07 ±0.72	6.16 ± 0.17	6.26 ± 0.71	3.92 ± 0.66	5.00 ± 0.44
L. rhamnosus	0	7.85 ± 0.07	6.59 ± 0.26	5.61 ± 0.44	5.86 ± 0.62	7.33 ± 0.28	6.27 ± 0.66	5.62 ± 0.36	5.84 ± 0.53	7.20 ± 0.09	5.23 ± 0.48	5.52 ± 0.70
	8	7.85 ± 0.16	6.79 ± 0.34	5.70 ± 0.35	6.65 ± 0.38	7.12 ± 0.41	5.88 ± 0.85	5.99 ± 1.00	5.94 ± 0.73	7.19 ± 0.29	4.38 ± 0.63	5.25 ±0.43
	24	7.74 ± 0.27	6.57 ± 0.76	5.54 ± 0.46	5.69 ± 0.64	7.02 ± 0.24	5.20 ± 0.52	5.83 ± 1.10	6.19 ± 0.83	6.56 ± 0.15	4.48 ± 0.57	4.69 ±0.75

3.3.3 Short-chain fatty acids

Figure 3.1 demonstrates change in SCFA concentration over the course of fermentation. No significant difference between vessels at baseline was found. Levels of valerate, iso-valerate and iso-butyrate were below that of minimum detection and are therefore not presented.

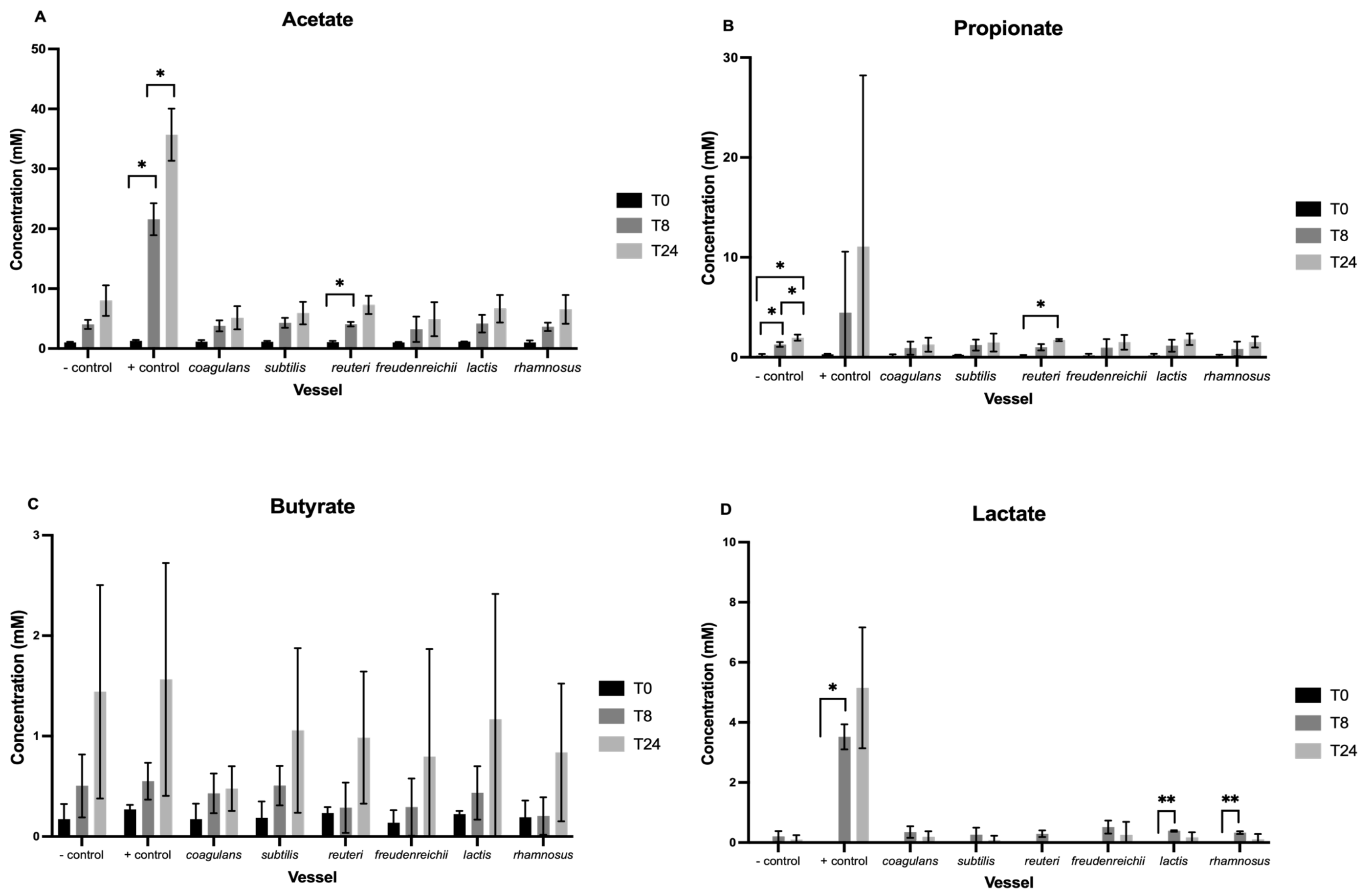


Figure 3.1 - SCFA concentrations of acetate (A), propionate (B), butyrate (C), and lactate (D) (mM) per vessel (excluding the positive control vessel) at baseline and following 8 (T8) and 24 hours (T24) of fermentation. Values are mean ± standard error (between-subject). Significant change within vessels is indicated as * $p < 0.05$ and ** $p < 0.01$. No significant difference between the negative control and other vessels was observed at any of the sampling timepoints.

Looking at acetate (Figure 3.1A), there was a significant main effect of Time ($F(1,68) = 24.66$, $p < 0.001$), Vessel ($F(1,68) = 10.2$, $p = 0.002$) and Time x Vessel interaction ($F(1,68) = 5.94$, $p = 0.017$). Pairwise comparisons highlight a significant increase from T0 to T8 ($p < 0.05$), and T0 to T24 ($p < 0.05$) in the positive control vessel, in addition to a significant increase from T0 to T8 following the addition of *L. reuteri* ($p < 0.05$). After exclusion of the positive control vessel (Figure 3.2A), only the main effect of Time was maintained, where concentration increases over the 24-hour period across all vessels ($F(2,42) = 68.36$, $p < 0.001$). No change in pairwise comparisons was observed.

For propionate a significant main effect of time was observed ($F(1,68) = 6.254$, $p = 0.015$) (Figure 3.2B). Pairwise comparisons indicate this increase in concentration is significant from T0 to T8 ($p < 0.05$), T8 to T24 ($p < 0.05$), and T0 to T24 ($p < 0.05$) in the negative control vessel. Additionally, concentration significantly increased between T0 and T24 following the addition of *L. reuteri* ($p < 0.05$). The main effect of time ($F(2,42) = 44.55$, $p < 0.001$) and all post-hoc effects were maintained when excluding the positive control vessel.

Concentration of butyrate increased over the 24-hour period across all vessels, reflected as a significant main effect of Time ($F(1,68) = 32.86$, $p < 0.001$) (Figure 3.2C). However, no main effect of Vessel or interaction was observed.

Concentration of lactate increased across all vessels by T8 and fell by T24 (Figure 3.1D). Main effects of Time ($F(1,68) = 5.13$ $p = 0.027$) and Vessel ($F(1,68) = 6.38$, $p = 0.014$) were significant, while their interaction was bordering on significant ($F(1, 68) = 3.92$, $p = 0.052$). Pairwise comparisons indicate a significant increase in concentration from T0 to T8 in the positive control vessel ($p < 0.05$) and following the addition of *Lc. lactis* ($p < 0.01$) and *L. rhamnosus (*$p < 0.05$). When excluding the positive control vessel, the main effect of Time ($F(2, 42) = 23.22$, $p < 0.001$) and pairwise comparisons remain significant (Figure 3.2D).

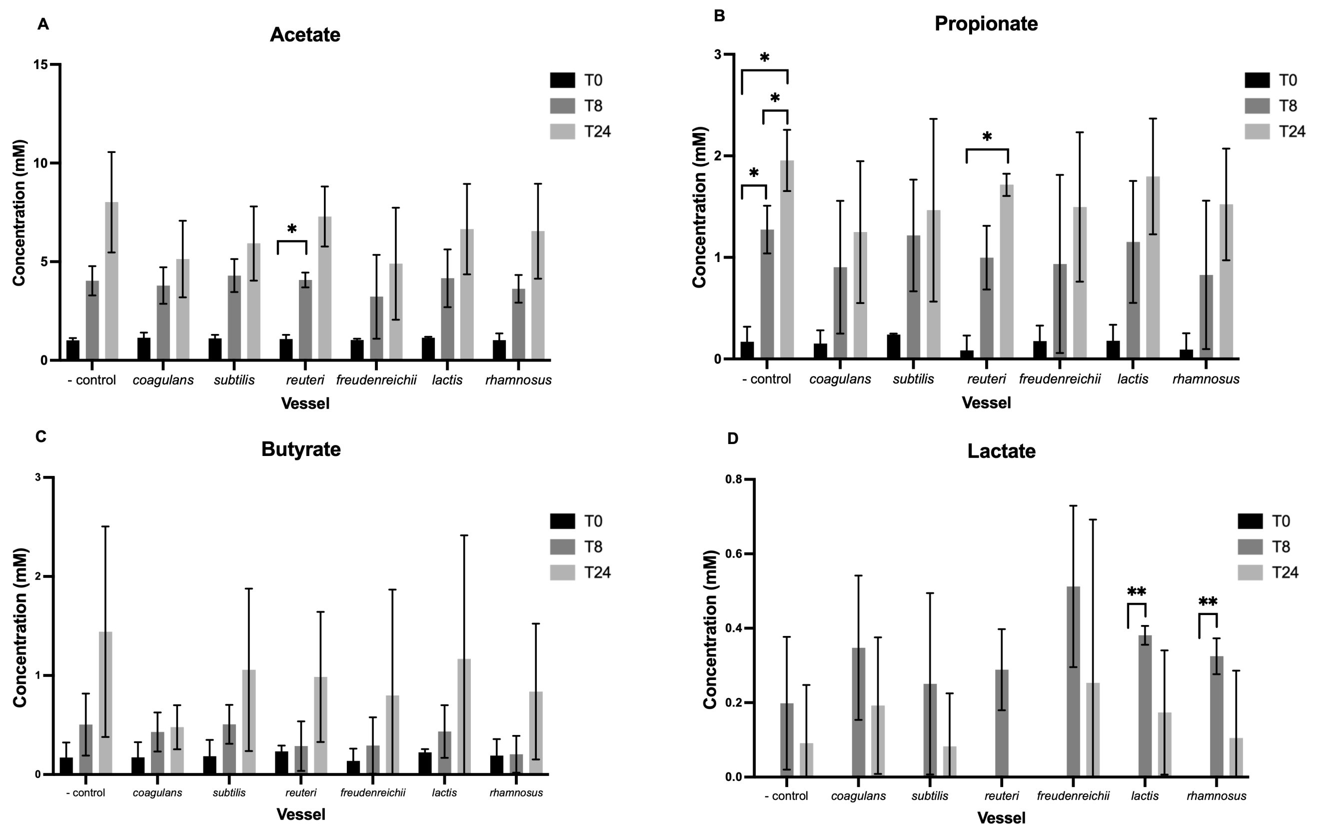


Figure 3.2 - SCFA concentrations of acetate (A), propionate (B), butyrate (C), and lactate (D) (mM) per vessel (excluding the positive control vessel) at baseline and following 8 (T8) and 24 hours (T24) of fermentation. Values are mean ± standard error (between-subject). Significant change within vessels is indicated as * $p < 0.05$ and ** $p < 0.01$. No significant difference between the negative control and other vessels was observed at any of the sampling timepoints.

3.3.4 Neurotransmitters

Changes in neurotransmitter concentrations are illustrated in Figure 3.3. No significant difference in baseline concentration was detected between vessels for each compound. Levels of epinephrine, norepinephrine and kynurenic acid were below that of minimum detection and are therefore not presented.

The fermentation process elicited a significant main effect of Time on GABA concentration ($F(1, 68) = 8.63$, $p = 0.005$). Pairwise comparisons reveal that the increase in concentration from T0 to T8 was trending towards significance following the addition of *L. reuteri* (p = 0.090), *Lc. Lactis* (p = 0.094), and *L. rhamnosus* (p = 0.094), and from T0 to T24 in the vessel with added *B. coagulans* (p = 0.064) (Figure 3.3A). No other statistically significant changes in neurotransmitter production were observed.

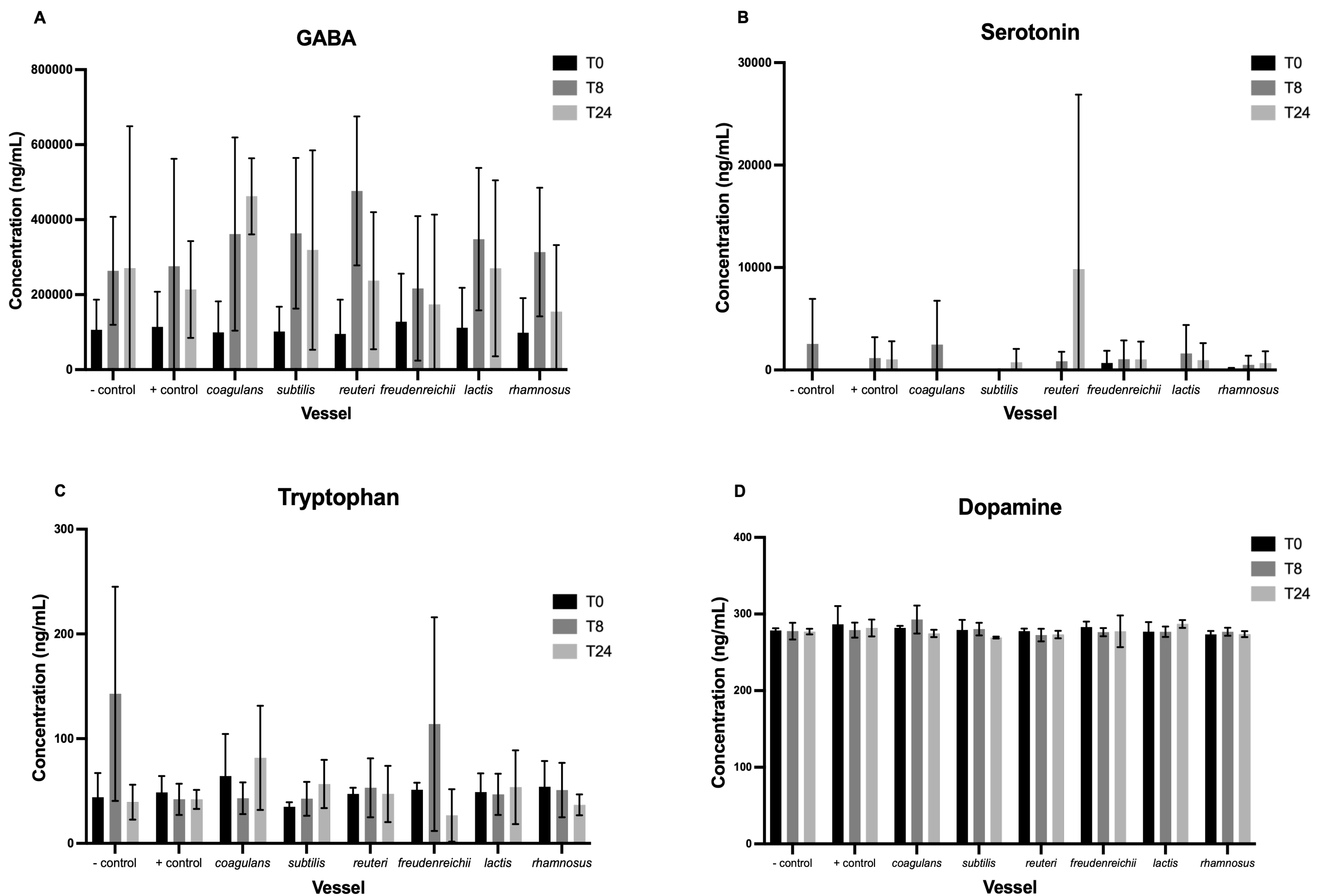


Figure 3.3 - Concentrations of GABA (A), serotonin (B), tryptophan (C), and dopamine (D) (ng/mL) per vessel (excluding the positive control vessel) at baseline and following 8 (T8) and 24 hours (T24) of fermentation. Values are mean ± standard error (between-subject). No significant difference within or between vessels was observed at any of the sampling timepoints.

3.4 Discussion

This work aimed to assess the production of neuroactive metabolites by faecal microbiota under physiologically relevant conditions, without the presence of human cells, and to explore the additional impact of several probiotic bacteria on both the faecal bacterial community and metabolite production using pH controlled, anaerobic *in vitro* batch culture models. In addition to a negative control vessel, which allowed for comparison of the probiotic vessels to the natural microbiota, inulin was included as a positive control substrate due to its known effects on *Bifidobacterium* spp. and SCFA production (Ahmed & Rashid., 2019). As expected, fermentation of inulin resulted in a substantial increase in *Bifidobacterium* spp., coupled with significantly increased concentrations of acetate and lactate over the 24-hour period. These results are in line with previous data describing a bifidogenic effect of inulin, and therefore provide evidence that the batch culture fermentation models worked as intended.

Batch culture fermentation models allowed the detection of GABA, serotonin, tryptophan, and dopamine under conditions relevant to the human GIT. Whereas previous work has typically employed optimal pH, temperature, and growth mediums when reporting the presence/production of neurotransmitters by isolated bacteria strains (Villegas et al., 2016; Li & Cao., 2010; Özoğul et al., 2012), the current work demonstrated neurotransmitter production by human faecal microbiota when under physiologically relevant conditions, using a standard basal media, in the absence of colonic cells. As such, the current data provide strong evidence for the bacterial derivation of these four metabolites under conditions relevant to the human GIT.

The detection of GABA in the absence of cells is perhaps not surprising. GABA is synthesised through the decarboxylation of l-glutamate by glutamic acid decarboxylase (GAD) – a system which has been established in several bacteria strains to provide a protective mechanism against the acidic environments, and hence GABA synthesis has been found to be highest at low pH (Otaru et al., 2021). However, the presence of tryptophan, serotonin and dopamine under these conditions is more novel, and at present is it unclear how enteric bacteria may mediate and/or produce these neuroactive metabolites. Serotonin synthesised in isolated bacterial cultures has been speculated to occur in the same manner as seen in plants, via the decarboxylation of tryptophan into tryptamine (Williams et al., 2014). The gut microbiota also appears to mediate how dietary tryptophan is metabolised into its various derivatives, such as indole, kynurenine, and serotonin (Agus et al., 2018; Hyland et al., 2022), but microbial production of tryptophan and dopamine is not yet understood. While the detection of these metabolites in the current fermentation models suggests some level of

bacterial derivation under relevant conditions, the concentrations of serotonin, tryptophan and dopamine were relatively low compared to that of GABA. This implies that while there may be bacteria with the capacity to synthesise these compounds, human intestinal cells are likely required in these production pathways to produce physiologically relevant quantities in the host. For example, gut microbiota may mediate the biosynthesis of serotonin by influencing the expression of tryptophan hydroxylase 1 (TPH1) – a rate limiting step in the synthesis of serotonin – in enterochromaffin cells, where the majority of host serotonin is located and transferred to the periphery (Yano et al., 2015). As such, while suitable for exploring levels of microbially derived GABA, batch culture fermentation models may not provide an optimal method for the exploration of other neuroactive compounds such as serotonin that likely require the provision of cells. That said, it should be noted that the present batch cultures were purposely maintained at a pH comparable to that of the proximal colon to stimulate GABA production, but this pH may not be optimal for the utilisation and production of other neurotransmitters and more alkaline pH such as that found in the transverse or distal colon may elicit different results. Modelling of the transverse and distal areas of the colon may also be beneficial when exploring neuroactive metabolite production as the vagus nerve is believed to have afferent nerve interactions with both regions, providing a potential gut-brain pathway (Wang & Powley., 2007).

In addition to assessing the potential for microbes to produce neurotransmitters in the absence of cells, this work also explored the effect of additional probiotic bacteria on both microbiota composition and metabolite production. With regards to microbiota composition, the selected probiotic bacteria did not result in a significant shift in log_{10} cells/mL for any bacteria group assessed, including *Lactobacillus* spp., over the fermentation period. This is perhaps unsurprising given the abundance of faecal bacteria relative to the quantity of probiotic bacteria added per mL ($3.3 \cdot x10^6$ CFU). As batch cultures provide a closed-loop, anaerobic environment with a limited supply of nutrients, a steady decline in bacterial numbers may be expected due to depletion of nutrients present in the basal medium. Flow FISH results indicate that this was the case for total bacteria, and across most bacteria groups assessed. In comparison, numbers of *Bacteroides-Prevotella* spp. and *Clostridium* cluster IX were maintained and appear to gradually increase following the addition of *B. coagulans*, *B. subtilis*, *L. reuteri*, *Lc. Lactis* and *L. rhamnosus* over 8 and 24 hours, respectively, compared to the control vessels. While this difference in trajectory suggests these strains may facilitate the maintenance and/or growth of these specific bacteria groups, log_{10} increases from baseline were not significant within these probiotic vessels, nor statistically different to numbers in the negative control vessel.

Concentrations of acetate, propionate and butyrate increased over the fermentation period across all vessels, while concentrations of lactate increased by 8 hours and fell once again by 24 hours. This general increase in SCFA production over the fermentation period is likely due to fermentation of the lactose and tryptone within the basal media present in all vessels. On the other hand, the fall in lactate between 8 and 24 hours is likely a reflection of important cross-feeding pathways, where certain bacteria are able to utilise lactate for the production of other SCFAs and metabolites (Louis & Flint., 2017). This fall in concentration would not be expected for other SCFAs present within this closed environment, as they are broken down less readily than lactate. With the exception of the positive control vessel (inulin), synthesis of lactate was greatest in the *Lc. lactis* and *L. rhamnosus* vessels, where concentrations significantly increased from baseline after 8 hours. Both species are known lactic acid producing bacteria (LAB), and their ability to produce lactic acid has previously been confirmed *in vitro* (Kwon et al., 2001; Song et al., 2017). The current data not only provide evidence of enhanced lactic acid production under physiologically relevant conditions, but also highlight that probiotic bacterium such as *Lc. lactis* and *L. rhamnosus* are able to interact with existing host bacteria to influence metabolite production without necessarily causing a quantitative shift in bacterial composition.

Microbially derived lactic acid has been linked to several health benefits, including lowering cholesterol, anti-inflammatory properties, and increased nutrient absorption from diet (Pessione., 2012). Additionally, as mentioned previously, lactic acid is involved in the production of other SCFAs such as acetate, butyrate, and propionate. For example, lactate can be converted to propionate via the acrylate pathway by select Firmicutes (Flint et al., 2015) or via the succinate pathway, primarily by Bacteroidetes (Louis & Flint., 2017). Many commensal species have the ability to convert lactate into acetate via acetyl-CoA (Koh et al., 2016), while select bacteria such as *Eubacterium hallii* species are able to produce butyrate through butyryl-CoA:acetate-CoA transferase route (Duncan, Louis & Flint., 2004). As such, increasing the availability of lactate may subsequently increase synthesis of other beneficial SCFAs. This may be significant in the context of the microbiota-gut-brain axis, as SCFAs play a role in the synthesis of various neuroactive metabolites and neurotransmitters (Reigstad et al., 2015; Dalile et al., 2019; Silva et al., 2020). In addition, SCFAs support gut barrier function and immune function, which in turn may improve tryptophan availability for serotonin (Xiao et al., 2022). However, previous work suggests that while pH 5.5 is supportive for the production of lactate by LAB, it does not provide an optimal environment for lactate-utilising bacteria and can lead to a detrimental accumulation of lactic acid (Louis et al., 2022; Wang et al., 2020). As the current

fermentation models were maintained at pH 5.5, a significant increase in lactate perhaps then would not be expected to be reflected as an increase in the concentrations of other SCFAs.

Although there were no statistically significant effects observed of the selected probiotic strains on neurotransmitter production, trending increases in GABA following the addition of *L. reuteri*, *Lc. Lactis*, *L. rhamnosus* and *B. coagulans* suggest these strains may lead to enhancement of GABA production. Production of GABA has typically been associated with LAB bacteria, and previous work has found species including *Lc. lactis* and *B. coagulans* to be good candidates for GABA synthesis due to the expression of GAD system genes. (Redruello et al., 2021; Tette et al., 2022). Additionally, species such as *L. rhamnosus* are being actively investigated for their potential GABAergic effect on mental and cognitive health disorders, with promising effects in animal models, particularly for depression (Tette et al., 2022). However, it is important to note that as outlined in the introduction, there is currently no evidence that gut-derived neurotransmitters cross the blood brain barrier, and there is little understanding as to the mechanisms via which gut-derived neurotransmitters may affect the brain.

The work outlined in this chapter is not without limitations. Batch culture models provide a closed system with an equal amount of nutrients for bacteria to grow on within each vessel, and use of a negative control vessel allows for undigested food sources within the faeces to be ruled out as responsible for changes over the fermentation period. As such, the results can be viewed as a true reflection of microbial fermentation, and any changes in the active vessels can be attributed to the additional pre- or probiotics. However, the three faecal donors in this study elicited substantial inter-donor variability in both bacterial composition and metabolite production (see appendix 4). As a result, the ability to observe statistically significant changes in these parameters may have been compromised, making it difficult to establish the effects of the select probiotic strains. As such, determining which microbial members are involved in these changes how different starting consortium of bacteria interact with the effect of probiotics is an important avenue of future work. With that said, *in vitro* batch cultures performed in triplicate do provide valuable data that matches well with the outcomes of intervention studies, and this is exemplified in the current experiment by the bifidogenic effect seen in the positive control vessel which is supported by the results of *in vivo* work (Bouhnik *et al.,* 2004). In addition, although the abundance of SCFAs matched that as found *in vivo* with acetate being most abundant, followed by propionate and butyrate in similar quantities, concentration of SCFAs in these models were generally lower than expected compared to

previous work using more complex media (Poveda et al., 2020). It is possible, therefore, that the lactose content in these batch cultures was too low to support greater production.

3.4.1 Conclusion

This work provides evidence for the production of several neurotransmitters in the absence of colonic cells while under physiologically relevant conditions, suggesting bacterial derivation of these neuroactive metabolites. However, relatively low concentrations of tryptophan, serotonin and dopamine, compared to GABA, suggest that bacterial synthesis may not provide a primary production pathway for these metabolites, and instead colonic cells may be required to reach physiologically relevant levels. The addition of probiotic bacteria did not lead to significant shifts in microbiota composition, although visual trends in the current data suggest they may support the growth of *Bacteroides-Prevotella* spp. and *Clostridium* cluster IX and enhance concentrations of microbially derived GABA. In addition, *Lc. lactis* and *L. rhamnosus* led to significantly increased concentrations of lactate after 8 hours of fermentation, thus enhancing metabolite production. The results in this experimental chapter therefore provide preliminary evidence for neurotransmitter synthesis under physiologically relevant conditions, and further work, perhaps using more sophisticated *in vitro* modelling will further elucidate the influence of probiotic bacteria on neuroactive metabolite production.

Chapter 4 – Exploring the effect of a multi-strain probiotic supplement on bacterial community and metabolite production in the faecal microbiota of older adults, using *in vitro* gut models

4.1 Introduction

The batch culture fermentation experiments discussed in chapter 3 provided evidence for the production of neurotransmitters by the faecal microbiota of healthy young adults under physiologically relevant conditions, in addition to the potential to increase the production of select SCFAs and NTs following the addition of lactic acid producing bacteria. While valuable as a preliminary step in understanding how probiotics may influence microbially derived metabolites and subsequently the gut-brain axis, healthy young adults arguably do not represent the best recipient for a probiotic intervention with a view to influencing cognitive function, since the bacterial community is likely already working well, and cognitive function is intact.

In contrast, older adults may find greater benefit from a probiotic supplement. Cognitive decline is a common characteristic of ageing, even in the absence of age-related diseases such as mild cognitive impairment (MCI) and Alzheimer's disease (AD). Imaging studies indicate that the brain undergoes numerous changes in older age including loss of cerebral volume, enlarged ventricles, and altered synaptic connectivity and plasticity (Anderton., 2002; Burke & Barnes., 2006). Blood brain barrier (BBB) permeability appears to increase with age, and more so in age-related disease (Farrall & Wardlaw., 2009), and levels of neurotransmitters, particularly dopamine and serotonin, appear to progressively decline with age (Morgan et al., 1987). Both animal and human research indicate that the hippocampus and prefrontal cortex (PFC) may be particularly vulnerable to age-related change, and as a result decline in cognitive functions such as learning, memory and executive function, is often observed (Burke and Barnes., 2006). Subjective memory complaints are also common in older adults, which in turn are often associated with poorer psychological wellbeing and quality of life, even in those following a healthy ageing trajectory (Steinberg et al., 2013; Montejo et al., 2011).

Alongside these neural changes, ageing is associated with several shifts in the GM. The ageing microbiome is often characterised by a reduction in overall diversity as well as an alteration in microbial species, that has sometimes been referred to as dysbiosis (Odomaki et al., 2016; Walrath et al, 2021; Pellanda et al., 2021). In healthy older adults, Shannon alpha diversity was found to significantly predict cognitive performance on learning and memory tasks, where poorer cognitive performance was associated with lower alpha diversity (Canipe III, Sioda & Cheatham., 2021). At present, it is generally accepted that a more diverse GM is advantageous for host health (Mosca, Leclerc & Hugot., 2016), although this is somewhat debated, as which organisms are there is obviously a key influencing factor. As such, it should also be noted that diversity metrices alone are perhaps not the most informative, as although diversity may be high, the bacterial species contributing to this diversity may not be beneficial to the host. Microbial changes of the GM in ageing adults are associated with increased permeability of the epithelial gut barrier, and consequently increased systemic inflammation and poorer BBB integrity (Cattaneo et al., 2017; Sochocka et al., 2019). In particular, higher abundance of *Bacteroides* has been identified as a possible marker of unhealthy ageing, whereas a depletion in *Bacteroides* and a shift towards compositional uniqueness of the GM is associated with healthy ageing (Wilmanski et al., 2021; Kim and Benayoun., 2020; Claesson et al., 2012). Observational data in those with AD support this theory, where relative decreases in Firmicutes and *Bifidobacterium* and increased Bacteroides were observed in individuals with AD compared to age and sex-matched healthy controls (Vogt et al., 2017), although this finding is not always consistent (Cattaneo et al., 2017). Having said this, there is often a large amount of variance between individuals as a result of external factors such as medication, geography and diet, making it difficult to ascertain strong associations between relative abundance of taxa and cognitive phenotypes (O'Toole & Jeffery., 2015).

Cause and effect are yet to be established, as it is unclear at present whether changes in the microbial community precede and therefore contribute to hallmarks of ageing, or vice versa. However, it is sensible to postulate that age-induced changes in the GM would subsequently lead to an imbalance in gut-brain axis mediators such as gut-derived SCFAs, hormones and other neuroactive metabolites. Studies assessing faecal SCFAs demonstrate that concentrations of acetate, propionate and butyrate progressively decline with age, leading to significantly lower levels in healthy older adults compared to healthy young adults (Salazar et al., 2019; Woodmansey et al., 2004). Although arguably a useful estimate, faecal SCFAs have been excreted and are therefore not utilised by the host, meaning they do not provide an accurate insight into rate of microbial synthesis or metabolite-host interactions. However, in a study comparing faecal SCFA profiles in individuals

with Alzheimer's to those with MCI and healthy controls (Wu et al., 2021), abundance of acetate, propionate, butyrate and valerate significantly declined in a progressive manner in MCI and AD compared to healthy controls. In addition, the same pattern was observed for indole derivatives of the tryptophan pathway metabolised by gut microbes, such as 5-Hydroxyindole, and these marked reductions in metabolite excretion were associated with reductions in Fimicutes, specifically in the order *Clostridiales* and family *Ruminococcaceae*. Furthermore, increased cognitive impairment as measured by the MoCA was positively correlated with reduced 5-HTP and SCFAs, suggesting that altered metabolite function contributes to severity of cognitive impairment.

As discussed in the literature review in chapter 2, several studies have now employed probiotic interventions to leverage the gut microbiome as a target for ameliorating cognitive decline in advancing age. Studies in populations with MCI and AD consistently report improvements in cognitive function as measured by composite screening measures such as the MMSE and RBANS following probiotic intervention (Kobayashi et al., 2019a; Kobayashi et al., 2019b; Xiao et al., 20202; Asoaka et al., 20222; Hwang et al., 2019; Akbari et al., 2016; Tamtaji et al., 2019). In the case of MCI, these improvements tend to be reported in individuals with cognitive assessment scores below the threshold for normal cognition at baseline. On the other hand, research into the potential effect on cognitive decline experienced in healthy ageing was limited, with only three studies having explored this. Of these, two indicated a potential beneficial effect on executive function (Chung et al., 2014) and global cognition (Sanborn et al., 2020), the latter once again only finding so in those with scores below the threshold for normal cognition at baseline. Only a handful of these studies explore potential underlying mechanisms, where improvement in AD population was accompanied by reduced hs-CRP triglycerides, decreased insulin resistance and a downregulation in gene expression of TNF- α (Akbari et al., 2016; Tamtaji et al., 2019), and improved cognitive function in MCI was associated with increased serum levels of BDNF (Hwang et al., 2019). None of the aforementioned studies assessed bacterial composition or concentration of SCFAs or other neuroactive metabolites (except BDNF) in faecal, serum or urine samples, and it is not apparent that metabolite production by elderly faecal microbiota following probiotics has been assessed to date *in vitro*. Given the critical role microbially derived metabolites appear to play in modulating gut barrier permeability, systemic inflammation, host production of downstream metabolites such as BDNF, and ultimately cognitive status, it is important to explore how probiotics may influence metabolite production in older adults.

As such, the current study employs a continuous culture systems mimicking the large intestine, with an aim to explore the effect of a multi-strain probiotic supplement on bacterial composition and

metabolite production of faecal microbiota from three healthy older adults. The decision to utilise a more sophisticated modelling system over batch culture fermentation allowed for more accurate representation of microbial community over time, and has the advantage of being able to model different regions of the colon where taxonomic profiles and metabolic activity may differ (Macfarlane et al., 1998). This model utilises a more complex nutritional input, so observations at the first equilibrium phase (steady state 1) can be compared to the post treatment equilibrium phase (steady state 2). Based on previous data a significant shift in microbial community following probiotic supplementation was not anticipated, but it was hypothesised that supplementation would increase the production of GABA in the lowest pH vessel, and likely increase general production of SCFAs. No prediction was made as to the effect on other neurotransmitters, given the exploratory nature of the work.

4.2 Methods

4.2.1 Continuous 3 stage model

A three-stage continuous culture system such as that described by Macfarlane et al (1998) was employed to simulate the proximal (vessel 1, 80mL, pH = 5.5), transverse (vessel 2, 100mL, pH = 6.2), and distal colon (vessel 3, 120mL, pH = 6.8). Each region, modelled using glass fermenter vessels, was connected to the next in series to allow for a continuous flow of gut model media to mimic the nutritional input to each region of the colon. Nutrients (g/L) included in the gut model media were as follows: potato starch (5g), peptone water (5g); tryptone (5g), yeast extract (4.5g), casein (4g); guar gum (1g), inulin (1g), pectin (2g), arabinogalactan (2g), xylan (2g); Potassium (KCL, 4.5g); Chloride (NaCl, 4.5g); Sodium ($NaHCO_3$, 1.5g); Magnesium ($MgSO4 \cdot 7H_2O$, 1.25g); Phosphorus (KH_2PO_4, 0.5g; K_2HPO_4, 0.5g); Calcium ($CaCl2 \cdot 6H_2O$, 0.15g); Iron (Hemin, 0.5g; FeSO4.7H2O, 0.005g); Vitamin K, 10µL. Additionally, each litre of media contained 0.8 g L-cystine HCl, 1 mL Tween 80), 4 g mucin (Porcine gastric type III), and 0.4 g bile salts, representing human secretions, and 4 mL resazurin solution (0.025 g/100 mL, pH 7) as an anaerobic indicator. As with the batch culture fermentation experiments described in chapter 3, physiologically relevant conditions were maintained throughout via continuous supply of N_2 to ensure an anaerobic environment, a circulating water bath to maintain vessels at a temperature of 37 °C, and maintenance of pH per vessel via pH controllers

(Electrolab, Gloucestershire, UK). Vessels were placed on magnetic stirrers to ensure continual homogenisation of the contents and therefore more accurate pH detection. A schematic representation of this model can be found in Figure 1.2 in chapter one.

Faecal samples were collected in anaerobic jars (AnaeroJarTM 2.5L, Basingstoke, UK, Oxoid Ltd.) with anaerobic sachets (AnaeroGen, Oxoid) and used for inoculation within 2 hours of production. To prepare the faecal sample, a 20% (wt:v) faecal slurry with PBS (anaerobic phosphate buffered saline; 0.1 mol/L; pH 7.4) was homogenised in a stomacher (Stomacher 400, Seward, West Sussex, UK) for 2 min (240 paddle beats/min). Vessels were inoculated to give a final concentration of 6% faecal slurry.

Following inoculation, the vessels were left for 24 hours to allow the faecal bacteria to grow within the new environment. After 24 hours, the flow of gut model media was initiated by connecting a 3.5L vessel of media to vessel 1 via a media pump. The flow was maintained at a retention rate to mimic the GI transit time of a healthy adult (48 h, 6.25mL/h). After 8 turnovers (384h, 2400 mL), the first equilibrium (SS1) was reached (this was established by stabilising of SCFA over 3 consecutive days (+/- 10%), and samples taken. After which, the probiotic (as detailed below) was added to vessel 1 as a supplement every morning until a second steady state (SS2) was achieved after a further 384h. As such, the effect of additional probiotic bacteria on bacterial enumeration and metabolite production could be assessed by comparing SS2 with SS1.

4.2.2 Multi-strain probiotic supplement

The probiotic intervention used in the present study was a multi-strain probiotic supplement, commercially known as Ecologic® Barrier, containing the following 9 probiotic strains: *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus salivarius* W24, *Lactobacillus casei* W56, *Bifidobacterium bifidum* W23, *Lactobacillus brevis* W63, *Lactococcus lactis* W19, *Lactococcus lactis* W58. These strains were selected due to previous work illustrating the capacity to strengthen the intestinal barrier, and previous clinical work utilising this probiotic formulation has reported positive effects on reactivity sad mood (Steenbergen et al., 2015; Chahwan et al., 2019), symptoms of anxiety and depression (Dao et al., 2021), and protection of working memory under conditions of acute stress (Papalini et al., 2019). All strains were present in approximately equal amount to total 1×10^{10} CFU, and quality of the batch utilised had been tested

every 3 months to confirm viability of the strains. The gut model utilised in the current study was one third of the size of the validation model, which was treated as a full-size representative of the human colon. As such, to provide the equivalent of consuming 4g of the supplement ($1x10^{10}$ CFU per/day), 1.3 grams of supplement was administered daily to the proximal vessel.

4.2.3 Preparation of samples

1mL, 1.5mL and 0.75mL of sample was aliquoted to Eppendorfs for Liquid Chromatography – Mass Spectroscopy (neurotransmitters), Gas Chromatography (short-chain fatty acids) and Fluorescence *in situ* Hybridisation (enumeration of bacteria), respectively. 1mL samples were immediately stored at -20°C. For GC and sequencing, samples were centrifuged at 11, 600 *g* for 10 minutes, before transferring the supernatant and storing the pellet at -20°C. For FISH, samples were centrifuged at 11, 600 *g* for 5 minutes. After removing the supernatant, the pellet was resuspended in 375 µL of PBS before adding 1125 µL of 4% paraformaldehyde. These samples were then stored at 4°C for 4-8 hours before being washed twice with 1 mL of PBS and resuspending the pellet in 150 µL of PBS. Finally, 150 µL of ethanol was added, the samples were vortexed to homogenise, and stored at -20°C.

4.2.4 Fluorescence in situ Hybridisation with Flow Cytometry (flow-FISH)

Preparation of samples followed the protocol of Grimaldi et al (2017). Briefly, samples were removed from storage at -20°C and vortexed to redisperse. 75µL of sample was suspended in 500 µL of PBS before vortexing and centrifuging for 3 minutes at 11, 600 *g* (consistent for all centrifuging during this process). For permeabilisation of the bacterial cell wall, supernatant was discarded, and the pellet resuspended in TE-FISH containing lysozyme (1 mg/ml) and incubated in the dark for 10 minutes at room temperature. Samples were then re-centrifuged and washed using 500 µL PBS. For *in situ* hybridisation, pellets were resuspended in 150 µL of hybridisation buffer (0.9 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% sodium dodecyl sulphate, 30% formamide), centrifuged, and resuspended again in 1 mL. 50 µL of this solution was added to each Eppendorf containing 4 µL of the oligonucleotide probe solutions, which were vortexed and incubated overnight at 36°C using heating blocks. Following incubation, 125 µL of hybridisation buffer was added, and Eppendorfs were vortexed and centrifuged as standard. After discarding the supernatant, pellets were resuspended in

175 µL of washing buffer (0.064 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate), vortexed to homogenise and incubated at 35°C for 30 minutes in the heating block. The washed pellets were then centrifuged once again, resuspended in 300 µL of PBS, vortexed and stored in the dark at 4°C ready for flow cytometry. Enumeration of bacteria was conducted using the Accuri C6 flow cytometer and analysed using the Accuri CFlow Sampler software.

Ten oligonucleotide probes (Table 4.1) were selected for inclusion, targeting a range of functionally relevant bacterial populations. Additionally, a mixed 338EUB probe was used to enumerate total bacteria.

Table 4.1 - Oligonucleotide probe sequences and corresponding target species.

Probe	Sequence	Target species	Reference
Non-Eub	ACTCCTAGGGAGGCAGA	Control probe for EUB338	Wallner et al (1993)
Eub338I+	GCTGCCTCCCGTAGGAGT	Most bacteria	Daims et al (1999)
Eub338II+	GCAGCCACCCGTAGGTGT	*Planctomycetales*	Daims et al (1999)
Eub338III+	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	Daims et al (1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	Langendijk et al (1995)
Lab158	GGTATTAGCAYCTGTTTGGA	*Lactobacillus* and *Enterococcus*	Harmsen et al (1999)
Bac303	CCAATGTGGGGGACCTT	*Bacteroidaceae*, *Prevotellaceae*	Manz et al (1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group	Franks et al (1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia*	Walker et al (2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	Harmsen et al (2000)
Prop853	ATTGCGTTAACTCCGGCAC	*Clostridium* cluster IX	Walker et al (2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	Hold et al (2003)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Devereux et al (1992)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group	Franks et al (1998)

4.2.5 16s rRNA sequencing

Following assessment of the microbial changes with flow-FISH, microbial groups of interest were looked at more closely with 16S sequencing with a focus on the genera found within the supplement (*Lactobacillus, Lactococcus and Bifidobacterium*) and in addition *Bacteroides*, given that higher relative abundance of *Bacteroides* may be implicated in less healthy ageing, as outlined in the introduction (Wilmanski et al., 2021; Vogt et al., 2017). Additionally, 16s sequencing was used to further explore any significant changes in microbial composition indicated by flow-FISH.

Pellets were resuspended in 300 µL of sterile H_2O before DNA extraction was performed using QIAamp PowerFecal Pro DNA kits (QIAGEN, Germany) according to manufacturer's instructions. The concentration of extracted DNA as well as purity (260/280 ratio) was measured using a Nanodrop (NanoDrop^{TM} ND-1000 Spectrometer). As per instructions, concentration was deemed acceptable if between 20 – 100 ng/µL. If greater than 100 ng/µL, additional C6 solution was added in 25 µL quantities until satisfactory.

16S rRNA gene sequencing and bioinformatics were outsourced to Microsynth AG (Schützenstrasse 15, 9436 Balgach, Switzerland). 25 uL of extracted bacterial DNA per sample was shipped on dry ice in sealed 96-well plates. To sequence the V3 and V4 regions of the bacterial 16S rDNA gene, two-step, Nextera barcoded PCR libraries using the locus specific primer pair 341F (5'- CCT ACG GGN GGC WGC AG -3') and 805R (5'- GAC TAC HVG GGT ATC TAA TCC -3') with 20 PCR cycles for the first step and 20 PCR cycles for the second step were created. Subsequently the PCR libraries were sequenced on an Illumina MiSeq platform using a v2 500 cycles kit.

Subsequent sequencing of PCR libraries was performed on an Illumina MiSeq platform using a v2 500 cyles kit (2 x 300 pb, V3-V4). The produced paired-end reads which passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina's bcl2fastq software version v2.20.0.422. The quality of the reads was checked with the software FastQC version 0.11.8 and sequencing reads that fell below an average Q-score of 20 or had any uncalled bases (N) were removed from further analysis. The locus specific primers were trimmed from the sequencing reads with the software cutadapt v3.2 and discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to in-silico reform the sequenced

molecule considering a minimum overlap of 15 bases using the software USEARCH version 11.0.667. Merged reads that contained ambiguous bases or were outliers regarding the expected amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded, to not distort the statistical analysis. The remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form operational taxonomic units (OTUs) discarding singletons and chimeras in the process. The resulting OTU abundance table was then filtered for possible barcode bleed-in contaminations using the UNCROSS algorithm. OTU sequences were compared to the reference sequences of the RDP 16S database (https://www.drive5.com/usearch/manual/sintax_downloads.html) and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH. Functional profiles were predicted by hidden state reconstruction using the software picrust2 v2.1.4-b and its integrated EC, KO, MetaCyc, COG, PFAM and TIGRFAM databases.

4.2.6 Gas Chromatography

Preparation of samples for GC was carried out in line with the method previously described by Richardson and colleagues (1989). Samples were defrosted, vortexed, and 1 mL transferred to 100 mm x 16 mm glass vials, in addition to 50 µL internal standard (0.1M 2-ethylbutyric acid) 0.5 mL concentrated HCl and 2 mL diethyl ether. Vials were vortexed for 1 minute and centrifuged for 10 minutes at 2000 *g* (Eppendorf 5804 R). The upper diethyl ether layer was extracted and transferred to new vials, from which 400 µL was taken and added to a screwcap HPLC vials with 50 µL of MTBSTFA. The vials were protected from light and stored at room temperature for 72 hours prior to analysis to allow for all SCFAs, including lactate, to derivatise.

Samples were analysed using a 5690 series Gas Chromatograph (Hewlett Packard, UK) with HP-5ms column (L × I.D. 30 m × 0.25 mm, 0.25 µm film thickness) coating of crosslinked (5%-phenyl)-methylpolysiloxane (Hewlett Packard, UK)).1 µL of each sample was injected with a run time of 17.7 min. Injector and detector temperatures were 275 $^{\circ}$C and the column temperature programmed from 63 $^{\circ}$C to 190 $^{\circ}$C by 5 $^{\circ}$C and held at 190 $^{\circ}$C for 30 min. Helium was used as the carrier gas at a flow rate of 1.7 mL/min (head pressure, 133 KPa). The external standard solution included: acetic acid (30 mM); propionic acid (20 mM); n-butyric acid (20 mM); n-valeric acid (5 mM); iso-butyric acid (5 mM); iso-valeric acid (5 mM) (all Sigma-Aldrich). Quality control (QC) samples of external standard

solution were included between donors to maintain accurate calibration. Peak integration was performed using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK), and quantification of each SCFA (mM) was calculated using internal response factors as described by Liu (2016).

4.2.7 LCMS/MS

Samples were first removed from storage at -20°C and centrifuged for 5 minutes at 2000 *g*. 100 µL of supernatant was added to 9.9 mL of HPLC water to form a 1:100 dilution, which was then filtered using 0.22 µm syringe filters. 1 mL was added to a screwcap HPLC vial for analysis. In addition, 1 mL of batch culture medium was prepared in the same manor to be analysed as a control. Individual stock solutions were prepared using analytical standards powders of dopamine hydrochloride (99%, Alfa Aesa), serotonin (Sigma-Aldrich), tryptophan (98%, Sigma-Alridch), GABA (99%, Sigma-Aldrich), L(-)-epinephrine (99%, Acros Organics), L-noradrenaline (98%, Alfa Aesa) and kynurenic acid (98%, Sigma-Aldrich), each at 1000 ng/mL. A mixed standard solution was then prepared from the individual stock solutions and used to create a 7 level calibration series with the following dilutions: 10, 5, 1, 0.5, 0.25, 0.125, 0.0625 ng/mL. Additionally, a 1 ng/mL standard was run every 20 samples as a quality control.

Samples were analysed by liquid chromatography−mass spectrometry/mass spectrometry (LC−MS/MS) using an Agilent 1200 HPLC system with a 6410 triple-quadrupole mass spectrometer with electrospray ion source in positive ion mode. A gradient separation was carried out using a 150 × 2.1 mm Discovery HS F5 – 3 column, with a 2 × 2.1 mm Discovery C18 Supelguard precolumn (both 3 µm particle size; Supelco, Dorset, UK). The column was maintained at 40 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The column flow rate was maintained at 0.4 mL/min. The timetable was as follows: 0–2 min, 100% A; 5 min, 75% A; 11 min, 65% A; 15–20 min 5% A; 20.1–30 min, 100% A. The injection volume was 25 µL. The eluant from the column was run to waste from 0 to 1 min, and data were collected from 1 to 18 min. Data were acquired in dynamic MRM mode. The transitions studied and voltages used are shown in Table 4.2. Two transitions were acquired for each compound.

Table 4.2- LC-MS/MS conditions used for quantification in faecal supernatant

Compound	Retention time (min)	Retention time window (min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision energy (V)	Classification
GABA	1.90	3	104	87	50	4	Organic acid
		3	104	45	50	20	
Norepinephrine	2.50	3	152	107	116	16	Catecholamine
			152	77	116	30	
Epinephrine	4.60	3	184	166	70	8	Catecholamine
			184	107	70	24	
Dopamine	7.00	3	154	137	75	8	Catecholamine
			154	91	75	28	
Serotonin	9.70	3	177	160	45	4	Amino acid derivative
			177	115	45	30	
Kynurenic acid	9.77	3	190	144	100	16	Organic acid
			190	172	100	4	
Tryptophan	10.20	3	205	188	78	4	Amino acid
			205	146	78	20	

4.2.8 Data analysis

All statistical analyses were performed using R statistical software (R Core Team., 2022). The effect of Time (SS1 vs SS2) and Vessel (proximal, transverse, and distal) on specific bacterial groups, relative abundance of a priori specified genera, SCFAs and neurotransmitters was assessed using repeated-measures two-way ANOVAs with post-hoc pairwise comparisons (Bonferroni corrected) using the R Stats package (Chambers, Freeny and Heiberger., 1992). Statistical significance was set to $p < 0.05$ and data is presented as mean ± between-subject standard error unless otherwise stated.

4.3 Results

4.3.1 Donors

The experiment was performed in triplicate using faecal samples from 3 healthy donors between 65 – 72 (2 female and 1 male, all white British). No antibiotics, pre- or probiotics were consumed within 3 months of sample collection.

4.3.2 Fluorescence in situ Hybridisation

Table 4.3 outlines the $\log_{10}$ bacterial numbers per mL within each functional group. No significant changes in bacterial enumeration from SS1 to SS2 were observed, although the effect of time on *Roseburia* subcluster (RREC) was trending [$F(1,1.81) = 3.83$, $p = 0.074$], where bacterial numbers were higher at SS2 following probiotic feeding than SS1 [$p = 0.074$].

4.3.3 16s rRNA Sequencing

Relative abundance of *Bifidobacterium* increased between SS1 and SS2 in all vessels while *Bacteroides* decreased in the proximal and distal vessels, but these shifts were non-significant. However, the effect of time on *Lactococcus* was significant [$f(1,12) = 9.32$, $p = 0.010$], where relative abundance of *Lactococcus* increased significantly from SS1 to SS2 [$p = 0.022$]. Pairwise comparisons indicate that this shift did not reach significance within individual vessels, only when considering the relative abundance of *Lactococcus* across the vessels at each steady state, and appears to be driven by an increase in one particular subspecies of *Lactococcus* – *Lactococcus lactis* ssp *hordinae*. Since flow-FISH highlighted a trending shift in bacterial numbers of *Roseburia*, this genus was also explored with 16s sequencing. In line with the flow-FISH data, relative abundance in *Roseburia* increased between SS1 and SS2 in all vessels, but once again this did not reach statistical significance. *Lactobacillus* was not detected in 16s rRNA gene data and therefore a significant change could not be assessed. Changes in relative abundance of each genus are illustrated visually using metagenomic Krona charts in Appendix 5.

4.3.4 Short-chain fatty acids

Figure 4.1 illustrates change in concentration for each SCFA of interest between SS1 and SS2. Generally, SCFA levels increased between SS1 and SS2 following daily probiotic feeding, particularly in the distal region. However, this was not translated into a significant main effect of time or vessel on any of the SCFAs except valerate where the main effect of time was trending [$F(1,12) = 3.67$, $p = 0.08$]. Concentrations of lactate were below that of minimum detection and are therefore not presented.

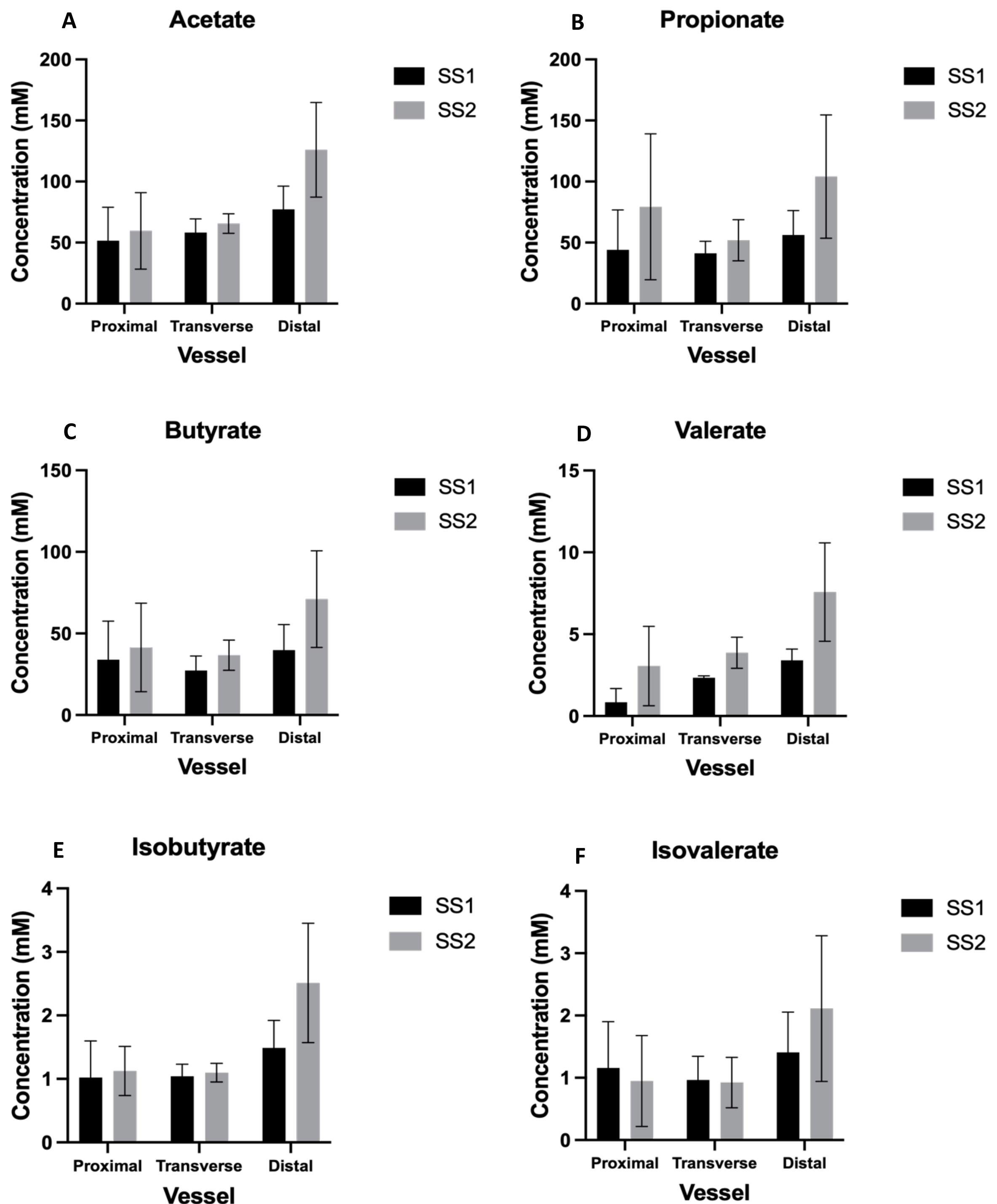


Figure 4.1 - SCFA concentrations of acetate (A), propionate (B), butyrate (C), valerate (D), isobutyrate (E), and isovalerate (F) (mM) per vessel at steady state 1 (SS1) and steady state 2 (SS2). Values are mean ± standard error (between-subject).

4.3.5 LCMS

Figure 4.2 illustrates change in concentration for each neuroactive compound of interest between SS1 and SS2. GABA, dopamine, norepinephrine, tryptophan and kynurenic acid were detected at sufficient quantities, but concentrations of epinephrine and serotonin were below that of minimum detection and are therefore not presented. As with the batch culture experiments discussed in chapter 3, quantities of GABA were far greater than that of the other metabolites, particularly at the lower pH found in the proximal vessel. Despite numerical increases in GABA, dopamine and tryptophan following probiotic feeding, no statistically significant changes in concentration were detected between SS1 and SS2 for any of the neuroactive metabolites measured. Substantially higher values for tryptophan in the proximal vessel driven by one donor (which are discussed later in the chapter) make it difficult to visualise changes in concentration between SS1 and SS2 in the other vessels. As such, a second graph for tryptophan is presented below (Figure 1F) where these extreme values have been replaced with the mean concentration of the other two donors, for the purpose improving visual clarity.

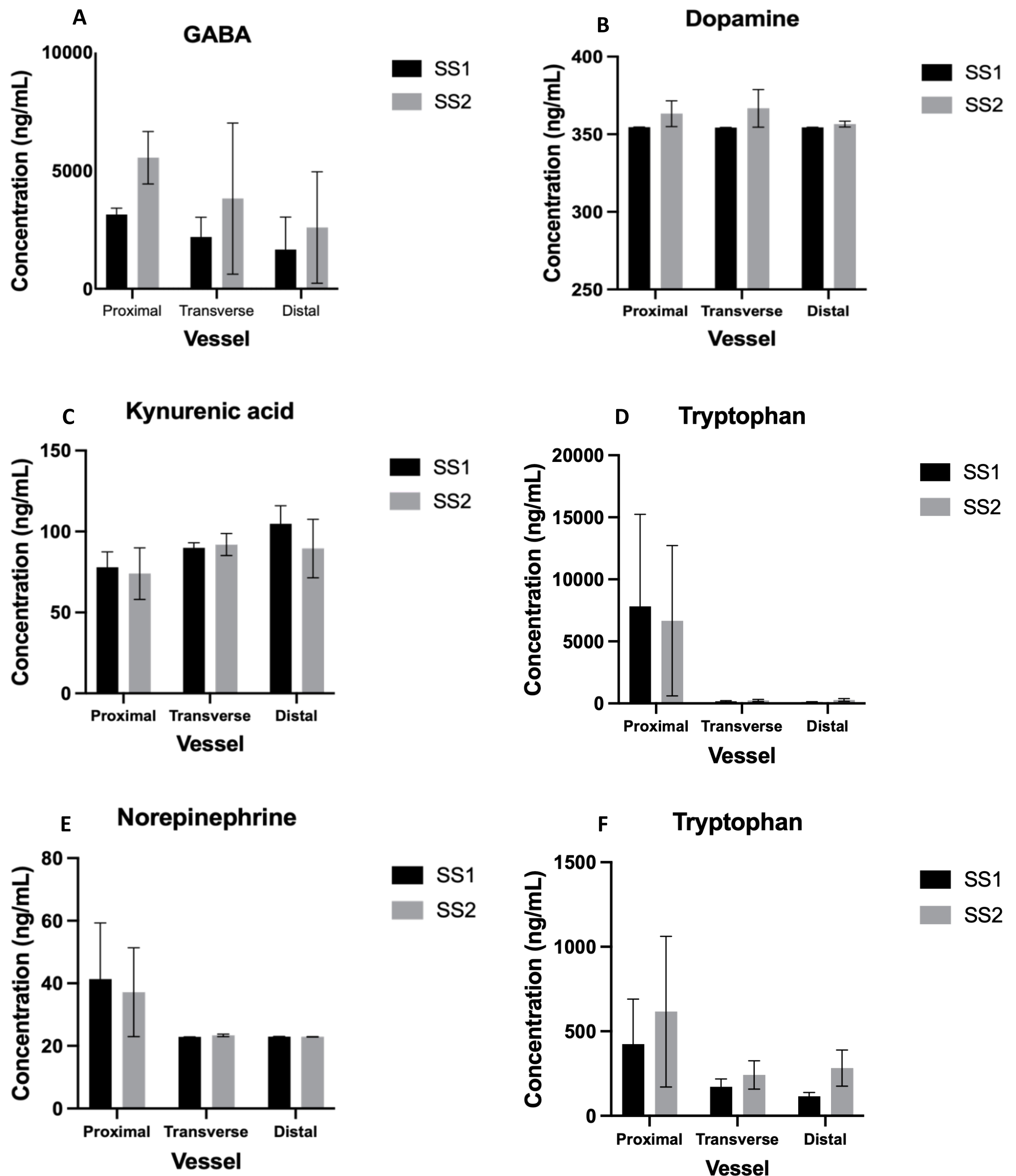


Figure 4.2 - Concentrations of GABA (A), dopamine (B), kynurenic acid (C), tryptophan (D), norepinephrine (E) and tryptophan with extreme values removed (F) (mM) per vessel at steady state 1 (SS1) and steady state 2 (SS2). Values are mean ± standard error (between-subject).

Table 4.3 - Enumeration of bacteria by Flow-FISH at steady state 1 (SS1) and steady state 2 (SS2) within the proximal, transverse, and distal vessels represented as log10 cells/mL culture. Target bacteria: *Bifidobacterium spp.*(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS). Values are presented as mean ± standard.

Bacterial groups detected by flow-FISH												
Vessel	**Time**	**Total bacteria**	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Proximal	SS1	8.55 ± 0.48	5.41 ± 1.19	6.52 ± 1.35	7.54 ± 1.64	7.09 ± 2.01	6.12 ± 0.99	5.69 ± 0.59	6.38 ± 1.72	6.29 ± 1.69	6.29 ± 1.69	6.81 ± 1.37
	SS2	8.90 ± 0.25	6.38 ± 0.91	6.99 ± 1.32	7.90 ± 1.08	7.24 ± 2.37	6.72 ± 0.75	6.22 ± 0.88	6.98 ± 0.72	6.85 ± 1.23	6.85 ± 1.23	6.77 ± 0.51
Transverse	SS1	8.60 ± 0.25	5.54 ± 0.79	7.28 ± 0.49	7.87 ± 0.06	8.06 ± 0.29	5.80 ± 0.82	5.84 ± 0.72	7.40 ± 0.17	7.06 ± 0.88	7.06 ± 0.88	6.07 ± 1.71
	SS2	8.59 ± 0.30	6.03 ± 1.28	6.52 ± 0.77	7.69 ± 0.56	8.24 ± 0.44	6.38 ± 0.42	5.75 ± 0.49	7.21 ± 0.34	6.92 ± 1.04	6.92 ± 1.04	5.61 ± 1.01
Distal	SS1	8.26 ± 0.29	5.21 ± 0.75	6.44 ± 1.70	7.23 ± 0.17	6.59 ± 1.86	5.47 ± 0.67	5.40 ± 0.86	7.00 ± 0.40	6.69 ± 0.56	6.69 ± 0.56	5.54 ± 1.81
	SS2	8.32 ± 0.78	5.73 ± 1.75	6.25 ± 1.53	6.75 ± 0.76	7.99 ± 0.73	6.20 ± 0.10	5.99 ± 0.32	7.12 ± 0.44	6.43 ± 0.81	6.43 ± 0.81	5.69 ± 1.03

4.4 Discussion

This work aimed to assess how the addition of a multi-strain probiotic supplement may affect both bacterial composition and the production of neuroactive metabolites within faecal bacterial communities from healthy older adults, using comprehensive three-stage continuous culture systems modelling the large intestine.

Fluorescence in situ hybridisation showed little change in bacterial composition between steady states, which is perhaps unsurprising given that, outside of antibiotic induced dysbiosis, there is little evidence that probiotic supplements alter the composition of the GM (Wieërs et al., 2020). Instead, the interaction between probiotic and enteric bacteria and the subsequent effect on metabolite production and support of beneficial bacteria appears to be of greater importance (Sanders et al., 2018). Having said this, numbers of *Roseburia* spp. did increase following probiotic feeding. *Roseburia* is a genus of Gram-positive bacteria belonging to the phylum Firmicutes under the family Lachnospiraceae (Stackebrandt., 2014); the genus of which gained attention for being prolific butyrate producers (Duncan et al., 2002; Nie et al., 2021). Generally, studies report lower abundance of *Roseburia* in older adults compared to younger adults (Tamanai-Schacoori et al., 2017; La-ongkham et al., 2020; Wu et al., 2021), although there are some discrepancies (Park et al., 2015), and maintenance of *Roseburia* in older adults has been associated with healthier ageing (Claesson et al., 2012). The abundance of SCFA producing bacteria such as *Faecalibacterium prausnitzii* and *Roseburia* has also been associated with health status in older adults in the NU-AGE project, where promotion of such bacteria through Mediterranean dietary intervention reduced incidence of frailty and cognitive decline (Ghosh et al., 2020). The shift in *Roseburia* evidenced here was only trending towards statistical significance, but observed via both sequencing and FISH analysis, hence the current data suggests that this probiotic supplement may have the potential to support levels of *Roseburia* in older age, which could have a beneficial effect on inflammatory status and cognitive function.

Additionally, sequencing data illustrated a significant increase in the relative abundance of *Lactococcus lactis*, specifically in the subspecies *Lc. lactis* spp *hordinae*. *Lc lactis* is a Gram-positive lactic acid producing bacterium thought to be of particular importance for immune function, with *in vitro* and animal models reporting enhanced immune response against pathogenic bacteria

(Santibañez et al., 2021), inhibition of cancer cells and proinflammatory cytokines (Han et al., 2015), and stimulation of ileal mucosal immunity (Yu et al., 2021). In the latter study, authors reported alterations in serum tryptophan and ileal GABAAα5 receptor gene expression, suggesting *Lc. lactis* may influence immune function by regulating amino acid profiles and the GABAergic system. As such, *Lc lactis* species may influence the GBA via immune pathways.

Several SCFAs were detected at SS1 and SS2, including acetate, propionate, butyrate, and valerate. Branch-chain fatty acids isobutyrate and isovalerate were also successfully detected from these continuous culture systems unlike in the batch fermentations, likely due to the higher initial bacterial load at inoculation and more nutrient-dense media. Interestingly, lactate was not detected at either sampling timepoint across any of the modelled regions. This is likely a more accurate representation of the rapid conversion of lactic acid to other SCFAs such as acetate, butyrate and propionate (Duncan et al., 2004; Flint et al., 2015; Louis & Flint., 2017) in comparison to the batch culture experiments in chapter 3 which allow for an unnatural build-up of metabolites, hence allowing lactate to be detectable despite concentrations falling following conversion to other SCFAs. SCFAs were produced in the expected relative quantities, such that acetate > propionate/butyrate > valerate. Although concentrations generally increased at SS2, particularly in the distal region, daily feeding of probiotics did not increase the amount of any of the detected SCFAs by a statistically significant margin. However, the increase in valerate in the distal region was trending towards significance. Valerate is understood to a lesser degree than the more abundant SCFAs (Perez Chaia & Olivier., 2003), but microbial synthesis has been reported *in vitro* via number of pathways by select bacteria such as *Escherichia coli*, *Prevotella copri* and *Megasphaera* (Oliphant & Allen-Vercoe., 2019; Akhtar et al., 2022; Yoshikawa et al., 2018). Reported benefits of valerate on the host include improved epithelial barrier integrity (Gao et al., 2022) and anxiolytic effects, likely through GABAergic type activity (Vishwakarma et al., 2016), meaning it is often consumed as a supplement to reduce anxiety, insomnia, and pain. Additionally, valeric acid appears to have a neuroprotective effect against pro-inflammatory cytokines and neurodegeneration in mouse models of AD and PD (Jayaraj et al., 2020; Dulla et al., 2022), and is therefore actively being investigated as a potential therapeutic for neurodegenerative disorders. Altered valerate production is often not reported following probiotic intervention, likely as it is difficult to observe *in vivo* due to being produced in relatively small quantities and absorbed. As such, use of these *in vitro* models enables production to be better mapped. The trends in the current data suggest Ecologic Barrier may enhance microbially derived valerate, which, given that older adults commonly encounter the aforementioned issues of epithelial permeability, pain and low mood, could be beneficial.

The results of this work provide further support for the bacterial derivation of neurotransmitters in human faecal microbiota when under physiologically relevant conditions, using a standard gut media, in the absence of colonic cells. Comparative to the results of batch culture fermentations in chapter 3, GABA was the most abundant metabolite produced, and concentrations were higher at the lower pH as expected (Otaru et al., 2021). In general, concentrations seen in the current gut models were lower than that seen in the batch cultures, although this might be expected as the continuous flow of liquid through to a waste outlet means metabolites do not accumulate in the same way they do in batch culture fermentations. While concentration did increase in the proximal vessel following the addition of Ecologic Barrier as expected, this change did not reach statistical significance.

The data acquired for levels of tryptophan was somewhat unexpected. One donor demonstrated substantially higher tryptophan levels under proximal conditions than the other two donors, or indeed compared to the levels elicited by that same donor in either of the other vessels (see appendix 6, figure 11). Although vastly different to the other data, high concentrations were present at both SS1 and SS2 for the same donor, within the same vessel, and chromatograph peaks were manually checked to ensure the metabolite was being correctly classified. Given the consistency of these findings across time, a decision was made that the data should not be treated as erroneous, although it remains challenging to interpret. Given that the primary source of tryptophan is diet, one possible explanation is that this donor consumes a diet higher in tryptophan. In line with this theory, levels of tryptophan were higher at baseline for this donor in comparison to the other two. Additionally, the difference in tryptophan concentration continues to grow by SS1 and SS2, suggesting this donor's microbes are more adept at metabolising tryptophan and its precursors due to receiving a higher quantity through diet.

In contrast to the findings in chapter 3, serotonin was not detected in the present experiments. This perhaps provides further evidence that synthesis of serotonin is a host-led pathway and requires the presence of cells such as enterochromaffin cells, while microbially derived levels are below that of physiological relevance. On the other hand, kynurenic acid was successfully detected, albeit at relatively low concentrations, suggesting initial conversion of tryptophan to kynurenine by enzymes indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase and kynurenine formamidase, and subsequent conversion of kynurenine into kynurenic acid through kynurenine aminotransferase (KAT) (Chen et al., 2021). No effect of probiotic administration was found, although this may once

again support the argument that bacterial derivation only provides a minor pathway, and colonic cells are required for the metabolism of tryptophan via the kynurenic or serotonin pathways. Although certain bacteria, such as *Escherichia coli*, have been shown to possess enzymes high in KAT and the ability to convert kynurenine to kynurenic acid at 37°C (Kuc et al., 2008), KAT is highly expressed in gut epithelial cells and this likely provides a primary production pathway in the gut (Wirthgen et al., 2018; Walczak et al., 2011). For example, probiotic interventions have consistently been associated with a general downregulation of the kynurenic pathway, but evidence for this is found in serum (Purton et al., 2021). Kynurenine has also been found to cross the BBB (Schwarcz et al.,2012), so it may be that conversion to the neuroprotective kynurenic acid happens here.

Collectively, the present data indicate that whilst this this multi-strain may not vastly alter bacteria on a community level, probiotic bacteria can impact metabolite production in as seen through trending increase in valerate production. Despite collecting samples from ageing volunteers, bacterial composition appears relatively healthy. As such, a lack of age related dysbiosis may have limited the scope for a potential benefit of the probiotic. Additionally, where concentrations of metabolites have changed between steady states, inter-donor variability in the production of both SCFAs and neuroactive metabolites (Appendix 6) makes it challenging to observe whether these changes are statistically significant. Again, this highlights that microbial behaviour is unique to the donor, and while performing gut models in triplicate provides valuable data regarding the capacity for bacterial synthesis of metabolites, understanding how differences in initial community and response to dietary interventions will be an important avenue for future research. Using faecal donors who are older than those utilised here, or perhaps older adults with subjective memory complaints, may better allow for an exploration into how a probiotic supplement could benefit an older adult who is experiencing age related declines in microbiota or cognition. Additionally, the nutrient content of the media could be altered in future experiments to represent a diet with less fibre and more protein, as is commonly reported in older adults (Zaragoza-Martí et al., 2020). Exploring the combination of probiotic bacteria with additional prebiotic fibre may also be advantageous in future work. Gut model media used in the present study contained a selection of dietary fibre sources designed to mimic typical availability of fermentable fibre in the human colon. However, previous *in vitro* work demonstrated a superior effect of probiotic supplementation for the improvement of gut dysbiosis in models of Anorexia Nervosa, which recent evidence suggests may contribute the pathology of the disorder (Fan et al., 2023), when administered in combination with a prebiotic, compared to when administered alone (Liu et al., 2021). Similar findings have also been reported in clinical trial outcomes for patients with ulcerative colitis (Fujimori et al., 2009). Although

the aim of this work was to understand how these probiotic strains alone may interact with commensal microbes, a synbiotic approach may be more beneficial and is something to consider going forwards. It should also be noted that the metabolites targeted here are by no means an exhaustive list of metabolites with the potential to influence gut-brain activity. Future work utilising continuous culture systems would likely benefit from looking at a wider range of metabolites, including other derivatives of the tryptophan pathway and bile acids (Connell et al., 2022), to continue expanding our understanding of GBA pathways and the role the probiotic bacteria may play. Finally, these modelling systems do not incorporate human cells, which, as is implied by the low levels of production in the current data, are likely necessary for sufficient production of neurotransmitters in the gut microbiota. Exposing the supernatant from these models to colon cells to see the impact on subsequent metabolite production, such as in enterochromaffin cells and enteroendocrine cells for example, would provide further insight into these potential pathways.

In conclusion, the trends in the current data suggest administration of this multi-strain probiotic supplement may support the prevalence of *Roseburia* and *Lactococcus* and synthesis of valerate within faecal microbiota of healthy older adults, but the effect on other SCFAs and neuroactive metabolites remains unclear. However, the data does provide further support for the microbial production of neurotransmitters and highlights that microbial production of GABA under low pH may be a particularly relevant target for the gut-brain axis. The current data suggests that replication with additional donors, expanding the remit of metabolites assessed, media alterations and complimentary use of alternative models would be warranted in future research.

Chapter 5 – the effect of a multi-strain probiotic on cognitive function and mood in healthy older adults

This trial was pre-registered at clinicaltrials.gov, identifier NCT04951687.

5.1 Introduction

As outlined in chapter 4, concurrent shifts in the gut microbiota (GM) and cognitive function are a hallmark of ageing, even in the absence of age-related disease. Accumulating epidemiological evidence supports the association between the GM and ageing and indicates a number of GM signatures linked with both longevity and neurodegeneration (Biagi et al., 2017). In fact, recent work proposes that GM profiles could be used to predict biological age based on a selection of predictive microbial taxa associated with age-related change (Galkin et al., 2020). As the role of the gut microbiome in healthy ageing becomes more apparent, attention has turned to the use of dietary approaches for the prevention of age-related neurodegeneration and support of healthy ageing, including probiotic supplements (Flanagan et al., 2020). Probiotics present a particularly exciting approach as the bacteria and their associated metabolites have the potential to interact with multiple gut-brain axis pathways, including immune function, endocrine pathways and integrity of gut and brain barriers (as summarised in chapter 1) (Oleskin & Shenderov., 2019), all of which are noted to be affected in ageing (Ratto et al., 2022). While several trials have explored the efficacy of probiotic interventions to alleviate cognitive decline in age related disorders such as mild cognitive impairment (MCI) and Alzheimer's disease (AD), few have explored the potential to attenuate cognitive decline and support healthy ageing. Given that the ageing population is growing and with it the incidence for neurodegenerative disease (Cimler et al., 2019), therapeutic dietary interventions which may help to optimise cognitive function during ageing and mitigate the incidence of age-related disorders are becoming increasingly important.

The current literature in healthy older adults provides some support for a beneficial effect of probiotics on cognitive function, but as there are only a handful of trials which present various methodological limitations, the potential for probiotic supplements to support cognitive function in this population remains unclear. In a RCT in older adults with no chronic disease or diagnosed cognitive impairment, authors report an improvement in sustained attention, working memory and the executive function of cognitive flexibility following the daily consumption of fermented milk with *Lactobacillus helveticus* for 12 weeks compared to a placebo (Chung et al., 2014). Of particular significance is that these effects were found when tasks were administered multiple times as part of a cognitive battery designed to induce cognitive fatigue, potentially indicating a beneficial effect of supplementation under conditions of high cognitive demand. Further support for an effect of supplementation on cognitive flexibility was reported by Kim et al (2021), although this was only evidenced on one sub-test of an Alzheimer's screening assessment (CERAD-K), which is perhaps not an appropriate measure of cognitive function in a healthy ageing population. Sanborn and colleagues (2020) explored the effect of cognitive function across a wide cohort of middle and older-aged (52 – 75) community dwelling adults. Supplementation with *Lactobacillus rhamnosus* for 12 weeks was associated with a significant improvement in composite cognition score as measured by the NIH toolbox – a cognitive battery designed to assess cognition in healthy samples – but this improvement was only demonstrated in individuals that met the NIH toolbox criteria for possible mild cognitive impairment at baseline, and therefore does not necessarily illustrate a benefit to healthy ageing adults. Finally, Inoue and colleagues (2018) reported no additional beneficial effect of probiotics over the effect of resistance training on the Japanese version of the Montreal cognitive assessment and a modified flanker task in healthy adults aged 66-73 following 12 weeks of a multi-strain *Bifidobacterium* supplement. Given that probiotics were not administered alone to any group of participants in this study, the effect of the probiotic supplement cannot be fully evaluated. Only one study (Kim et al., 2021) explored potential underlying mechanisms, where improved cognitive flexibility was associated with increased serum BDNF following 12 weeks of *Bifidobacterium* supplementation, which was only evident in the probiotic group.

Alongside decline in cognitive functions, research suggests that older adults are particularly vulnerable to decline in mental health due to changes in circumstance and health status (Fisk, Wetherell & Gatz., 2009; Lyons et al., 2018;). In this age group subjective memory complaints are associated with poorer psychological wellbeing even in those following a healthy ageing trajectory (Montejo et al., 2011). In addition to cognitive function, the myriad of microbiota-gut-brain pathways outlined in chapter 1 appear to be implicated in mood and mental health (Appleton., 2018; Margolis, Cryan & Mayer., 2021) indicating the potential to influence mental wellbeing via probiotic intervention. Accordingly, the current evidence from clinical trials indicates that probiotics may be beneficial in relieving depressive symptomology (Musazadeh et al., 2022), although the evidence for improvement in conditions such as

anxiety disorders and schizophrenia is less supportive (Reis et al., 2018; Ng et al., 2019; Fond et al., 2020). Three of the four studies assessing cognitive function in healthy older adults also included mood measures. Kim and colleagues (2021) administered a stress questionnaire, the positive and negative affect scale and the Geriatric Depression Scale, and reported a significant reduction in self-reported stress following probiotic supplementation but not placebo. Chung and colleagues (2014), however, found no effect of supplementation on the perceived stress scale or geriatric depression scale. Inoue et al (2018) administered the Patient Health Questionnaire (PHQ-9) and General Anxiety Disorder assessment (GAD-7) to assess depressive symptoms and anxiety, respectively, and summed the score to provide a measure of 'overall mental state'. Probiotic supplementation was associated with significantly improved overall mental state, but placebo consumption was associated with reduced anxiety, and probiotics did not significantly reduce depression or anxiety as individual measures. Although acknowledged to be an important avenue of research (Ruiz-gonzalez et al., 2022), the current evidence is limited with mixed findings.

As such, to further the existing literature, the current work employed a double-blind cross-over RCT with a view to exploring the effect of a multispecies probiotic supplement on cognitive function and mood in healthy older adults in a robust, well-controlled trial. Additionally, 16s rRNA sequencing was carried out on stool samples pre- and post-intervention to explore underlying mechanisms. It was hypothesised that probiotic supplementation would result in improvements to the primary outcome measures of verbal and visuo-spatial working memory, in addition to executive function, as well as reducing negative mood. Abundance changes in the microbiota community were not necessarily expected, and improved cognition was anticipated even in the absence of microbial change.

5.2 Method

5.2.1 Participants

5.2.1.1 Recruitment

Healthy older adults aged 65-80 (M = 71.2, SD = 4.13) were recruited from the local Reading community via the internal Nutritional Psychology lab database, advertisements to local recreational groups, posters, and the Hugh Sinclair Volunteer Panel. The selected age range was based on previous research noting age related shifts in the gut microbiome from age 65 (O'Toole & Claesson., 2010). Inclusion was capped at 80 years old to ensure subjects were capable of the cognitive tasks.

5.2.1.2 Screening

All potential participants were emailed a study information document outlining the study protocol and inclusion/exclusion criteria to read prior to the screening visit (Appendix 7). To be enrolled on the study, individuals were required to be between the age of 65-80 and free from coeliac disease, diabetes mellitus (type 1 and 2), epilepsy, gastrointestinal disorders including irritable bowel disease and irritable bowel syndrome, allergy to any treatment ingredients, and diagnosis of and/or receiving treatment for mental health illness. Being a regular smoker, regular consumer of pre- or probiotics (including probiotic yogurt) or having antibiotic treatment within 3 months of enrolment would also result in being ineligible. Individuals taking regular medications for hypertension or cholesterol were accepted proving the medication was not altered during the study, but medications acting on the gut, such as proton pump inhibitors, were not accepted due to potential effects on the microbiota which could interact with the probiotic intervention. If the participant felt they met the requirements and were willing to commit to the study, the inclusion/exclusion criteria were checked for a second time at the screening visit to ensure suitability before proceeding.

5.2.1.3 Informed consent

Prior to study enrolment I completed the National Institute for Health and Care Research (NIHR) Good Clinical Practice (GCP) training programme on informed consent, as is required when handling Human Tissue Act (HTA) samples. If, after reading the study information document and having the chance to ask questions, participants were happy to continue, they were then required to sign the consent form at the start of the screening visit (Appendix 8). This study was performed according to the guidelines laid down in the Declaration of Helsinki following Good Clinical Practice and approved by The University of Reading Research Ethics Committee (UREC 20/17) (Appendix 9).

5.2.2 Design

This trial employed a crossover, randomised placebo-controlled design with both acute and chronic measures and a 4-week washout period (illustrated in Figure 5.1). Acute data was collected one day after baseline data, specifically 23-hours after consumption of the intervention at baseline in order to keep the time of cognitive testing consistent across visits. Chronic supplementation was then administered for 8 weeks, based on previous research suggesting 8 weeks is sufficient to see an effect of probiotic intervention on our primary cognitive outcomes (Ohsawa et al., 2018; Rudzki et al., 2019;

Nobile et al., 2022). A crossover design was selected over a parallel design due to the advantage that each subject acts as their own control, and fewer participants are required. Although resulting in a longer trial duration which increases the chance of attrition, several effects were made to reduce this possibility, and no compromise was necessary on the length of intervention as 8 weeks was possible as a crossover trial.

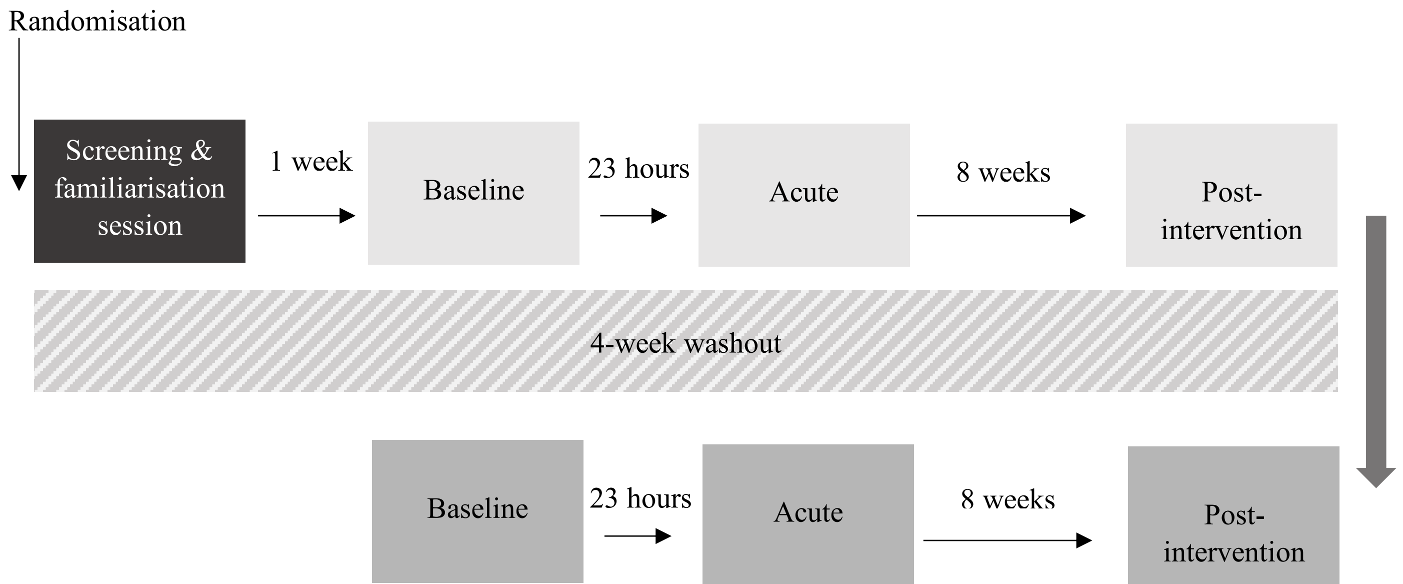


Figure 5.1 – schematic representation of the study design including 7 visits to the Nutritional Psychology lab at the University of Reading. Participants attended an initial screening and familiarisation session. One week later, baseline data was collected and one serving of the intervention administered. 23 hours after administration, acute data was collected. Subjects then consumed the intervention as instructed for 8 weeks before returning for the post-intervention session. Following a 4-week washout period, the protocol was repeated.

5.2.2.1 Intervention

The probiotic intervention used in the present study was a multi-strain probiotic supplement, commercially known as Ecologic® Barrier, containing maize starch, maltodextrin, vegetable protein, and potassium chloride, in addition to the following 9 probiotic strains: *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus salivarius* W24, *Lactobacillus casei* W56, *Bifidobacterium bifidum* W23, *Lactobacillus brevis* W63, *Lactococcus lactis* W19, *Lactococcus lactis* W58. These strains are identical to those used in the gut model experiments discussed in chapter four. The product was administered as a powder in individual sachets, with each 2g sachet providing a daily dose of 5 x 10^9 CFU. All strains were present in approximately equal amount, and quality of the

study batch utilised had been tested every 3 months to confirm viability of the strains. 60 sachets were included per box to provide one sachet per day plus extras in case of rescheduling beyond 8 weeks. Participants were instructed to mix the powder into 90 mL of lukewarm water until dissolved and consume immediately before breakfast to minimise any possible incidence of gastrointestinal symptoms. The placebo was identical to the active intervention with the bacteria strains removed, meaning it was matched for packaging, visual appearance, smell, and taste.

5.2.2.2 Randomisation

Each participant received treatment box A in the first arm and box B in the second arm. The contents of boxes A and B per participant was pre-randomised by Winclove, such that per each block of 10 participants an equal number would receive the probiotic or placebo in box A, and the alternative treatment in box B. As such, by assigning a participant number upon enrolment, subjects were automatically randomised to receive the probiotic or placebo treatment in the first arm. A copy of the randomisation schedule can be found in Appendix 10.

5.2.3 Procedure

Participants attended the lab for a total of 7 visits over a 6-month period, including an initial screening and familiarisation session, followed by a baseline, acute and post-intervention visit per study arm. During the screening visit, participants would sign the consent form, complete a demographics questionnaire (Appendix 11), and complete the Montreal Cognitive Assessment (MoCA) to give an indication of baseline cognitive function. A copy of the MoCA can be found in Appendix 12. Following this, participants completed 2 practice versions of the cognitive battery. These were carried out back-to-back with a short break between, and scores were checked following each practice to ensure the participant had understood the tasks. Two practice batteries were included upon recommendation from previous research illustrating strong practice effects in cognitive data, particularly between the first and second iterations, which could incorrectly be reported as a significant effect of intervention (Bell et al., 2018).

The basic procedure for each test visit was identical. Sessions began at 08:00 or 08:45 at the preference of the participant, and this remained consistent within-participant for all study visits. Participants fasted for 12 hours before each session and were instructed not to consume alcohol the evening before a study visit. Upon arrival they would be given a standardised breakfast of two croissants and 5g of unsalted butter to be consumed within 10 minutes, followed by a 15-minute interval to allow for

digestion. Croissants were selected as an appropriate breakfast due to the low nutrient content, as any phytonutrients consumed at breakfast could influence cognitive performance and confound the ability to assess the effect of the probiotic intervention. During this digestion window, participants completed the mood questionnaires (outlined in section 5.2.4.2) on paper. Once complete, participants then carried out the cognitive battery. This was carried out in individual test cubicles to allow for maximum concentration, and the cubicle was kept consistent for each participant across sessions as best as possible. The task battery was self-paced but took roughly 45 minutes to complete. On baseline visits, participants consumed one sachet of their allocated treatment for that arm at the end of the session. The reason for this was two-fold: firstly, to provide a demonstration on how to consume the product at home, and secondly to provide a single dose of the treatment in order to assess the acute 23-hour effect the following day. On acute visits, subjects were given their box of sachets to begin taking the same day and a compliance diary to fill out each day for 8 weeks (Appendix 13). On the final visit, participants completed a short end of study questionnaire (Appendix 14) and received a copy of the study debrief (Appendix 15).

5.2.4 Outcome measures

All cognitive tasks were programmed in E-Prime 3.0 (Psychology Software Tools, Inc.). Cognitive tasks were selected for inclusion based on findings from the literature review in chapter two, where either the specific tasks or the underlying cognitive domains were most consistently associated with a positive effect of probiotic intervention, particularly in older adult populations. The E-Prime battery was completed in the following fixed order: Positive & Negative Affect Schedule (extended), Rey Auditory Verbal Learning Task (RAVLT), Corsi Block Tapping Task, Task Switching Task, Go/No-Go task, and delayed recall and word recognition components of the RAVLT. As participants completed the cognitive battery a total of 8 times across the screening and test visits, alternate matched versions of tasks were used where appropriate to attenuate learning effects (Bell et al., 2018). These alternate forms have previously been tested in our lab and were found to be well matched, and cognitive battery versions were counterbalanced across participant sessions using a balanced Latin square method (Appendix 16).

5.2.4.1 Cognitive outcomes

Rey Auditory Verbal Learning Test (RAVLT)

Verbal memory performance declines with age (Simensky & Abeles., 2002) and is evident in healthy ageing in the absence of dementia (Weaver Cargin et al. 2007). The RAVLT (Rey, 1964) is a widely used task designed to measure verbal learning and memory. Using headphones, participants were presented with a list of 15 nouns (list A) at a rate of one word per second. After the list was presented, participants were given one minute to recall out loud as many words as possible, not worrying about the order in which they recalled the words. This procedure was repeated five times (trials 1-5), and each recall was recorded. Following this, participants were presented with a second 15-word list (list B), and once again asked to recall as many words as possible from that list (trial 6). Immediately after recalling list B, participants were asked to think back to list A and, without hearing it again, recall as many words as possible (trial 7). After completing the remaining cognitive tasks (approximately 30 minutes), participants revisited this task to perform a delayed recall (trial 8) and a word recognition task. During the recognition task, 50 of words were presented one at a time on screen – 15 from list A, 15 from list B and 20 distractor items, which were semantically or phonologically similar to the items in list A and B. Participants were asked to respond using one key for each word they believed to be from list A, and another key for all other words. Each cognitive battery utilised alternate words lists, which can be found in Appendix 17.

As such, the RAVLT provides the following outcome measures: immediate recall (trial 1), total acquisition (sum of trials 1-5), amount learned (trial 5 - trial 1), proactive interference (trial 1 - trial 6), retroactive interference (trial 5 - trial 7), delayed recall (trial 8), correctly identified items (list A), correctly rejected items (list B), and correctly rejected distractors, which in turn can be broken down into correctly rejected semantic and phonological distractors. All outcomes were analysed, but delayed recall was selected as a primary outcome based on previous research which suggests delayed recall in particular may be enhanced through probiotic intervention (Ceccarelli et al., 2017a; Ceccarelli et al., 2017b; Kobayashi et al., 2019b; Ohsawa et al., 2018; Ton et al., 2020; Xiao et al., 2020).

Corsi Block Tapping Task (CBTT)

The CBTT provides a measure of visuo-spatial working memory. Previous work suggests that visuo-spatial working memory may be a cognitive domain sensitive to an effect of probiotic supplementation, and this has been evidenced in both clinical and ageing populations (Ceccarelli et al., 2017a; Ceccarelli et al., 2017b; Ton et al., 2020; Xiao et al., 2020). Originally a task carried out using blocks on a wooden board, the CBTT is now often administered digitally with similar error rates to the original version (Robinson & Brewer., 2016). In this version, 9 white blocks were arranged in fixed positions across the screen. On each trial, the blocks lit up red one by one in a random sequence anywhere between 2 and 9 blocks in length, where each block would illuminate for 1s before returning to white and the next block

in the sequence illuminating. Sequence lengths were presented at random, as opposed to starting with 2-block sequences and increasing sequentially. This decision was made such that it increased the difficulty of the task, as subjects could not pre-empt the number of blocks they were looking for. Difficulty of the task also increased with the number of blocks in the sequence, and lower accuracy is therefore expected at the higher sequence lengths. Importantly, the cursor was not available to participants during this time, so they were unable to track the sequence in this manner to aid working memory. Once the full sequence was complete, participants used the mouse to click on the blocks in the order in which they believed they were illuminated. Each version of the task contained 32 main trials and two practice trials. To attenuate possible learning effects, the position of the blocks differed in each version of the task battery. Outcome measures included the percentage of correctly tapped sequences and the percentage of trials in which participants tapped the correct blocks but not in the correct order. Based on previous trials assessing the efficacy for probiotic intervention to improve spatial working memory, percentage of correct sequences was selected as a primary outcome in this trial.

Task Switching Task (TST)

The TST employed here was a modified version of the task described in Miller, Hamilton, Joseph and Shukitt-Hale (2018) used to measure cognitive flexibility, and has been successfully used within our lab across a range of ages, including older adults (Rutledge et al., 2021; Whyte et al., 2019). Participants viewed eight equally spaced radii of a circle displayed in such a way that there are eight equally spaced segments; four above a central bold line and four below. A single digit randomly selected from between 1–9 (excluding 5) appeared in each segment in turn in a clockwise direction. Each digit was displayed for a duration of 3000 ms, or until the participant responded. The inter-stimulus interval was 500ms. Participants were required to respond while the digit was present on screen, otherwise responses were not recorded. Dependent on whether the digit was presented in a segment above or below the bold line, participants responded based on a different set of rules. If the number was above the bold line, participants responded based on whether the stimulus was odd or even by pressing the relevant response key, whereas if the number was below the bold line, participants responded based on whether the number was higher or lower than 5, once again by pressing the relevant response key. As such, the task switches between these two rules every four trials, resulting in two switch trials (the initial trial after switching to the new rule) and six non-switch trials per completed circle. Switch trials are considered more cognitively demanding with slower response time and increased errors expected compared to the non-switch trials. Outcome measures included overall accuracy as % of correct trials, and accuracy as % of correct trials on switch, non-switch trials, odd/even, and high/low trials. RT was also assessed for each of these outcome measures. Participants completed 24 trials (3 complete circles)

as practice with feedback each time they completed the battery, to refresh their memory and reacclimate to the response keys prior to the main trials.

Go/ No-Go

There is consistent evidence for a decline in inhibitory control in older adults compared to middle-aged and younger adults (Christ et al., 2001; Sweeney e al., 2001). A Go/No-Go task was therefore employed to measure both sustained attention and inhibitory control. ‘Go’ stimuli were white circles 8cm in diameter, while no-go stimuli were identical but with a small black cross present at one of two randomised locations within the circle. Participants were instructed to respond to go trials by pressing the spacebar, and withhold from pressing the space bar on no-go trials. Stimuli were presented in the centre of the screen for 250ms before a 750ms blank holding screen. Responses were recorded if elicited with 1000ms of stimulus presentation. Should participants respond within 1000ms, this would trigger the presentation of the next stimulus, otherwise the next stimulus was presented when the maximum time limit was reached. the Inter-stimulus duration was randomly varied between the limits of 400 – 800ms. Participants completed a total of 180 trials, 70% of which were go trials and 30% no-go in order to create a sufficient bias towards the go response. Outcome measures included commission errors (incorrectly responding to no-go trials), which provide an accepted measure of inhibitory control, and omission errors (not responding to a “go” trial), which are widely regarded as lapses in attention (Wright et al., 2014). Additionally, RT was measured for correct ‘go’ trials.

5.2.4.2 Mood

Positive & Negative Affect Schedule – expanded form

The PANAS-X is a well-validated 60-item questionnaire that measures general positive and negative affect, as well as 11 specific primary affects including fear, sadness, hostility, guilt, shyness, fatigue, surprise, joviality, self-assurance, attentiveness, and serenity (Watson et al., 1994). It has been validated as a measure of both state and trait mood (Watson et al., 1994), and was incorporated into the E-Prime battery here as a measure of present state mood, such that it could also be included in acute sessions. Participants were presented with 60 individual mood related adjectives and asked to rate for each adjective ‘to what extent they were feeling this way right now’ using a 5-point Likert scale with anchor points from ‘not at all/very slightly’ to ‘extremely’. Positive and negative affect were based on 10 items each and scored according to the manual by summing the responses to positive and negative items, respectively. As such, high scores indicate both high positive and negative affect. Scores for each affective sub-scale, including sadness, hostility, fear, fatigue, joviality, serenity, and self-assurance, were

determined in the same manner. Shyness and surprise were felt not to be of interest as individual subscales with regards to a potential benefit of probiotic supplementation, and therefore were not analysed.

The Centre for Epidemiological Studies Depression scale (CESD)

The CESD is a 20-item questionnaire designed to measure depressive symptomology in the general population (Radloff., 1977). It has been validated against the geriatric depression scale in older adult populations (Park and Lee., 2021) and has been found to be an accurate screening tool for clinical depression in community dwelling older adults (Lewinsohn et al., 1997). Items such as 'I felt I could not get going' are scored on a 4-point Likert scale between 'rarely or none of the time' and 'most or all of the time'. Items are scored between 0-3, with reverse scoring on four items and a possible score range of 0-60. Higher scores indicate greater depressive symptoms, and a score of 16 or higher indicates risk of clinical depression (Radloff., 1977). As this is a measure of depressive symptomology and responses are based on feeling over the previous week, this measure was only included at baseline and post-intervention visits.

State Trait Anxiety Inventory (STAI)

The STAI is a widely used measure of trait and state anxiety (Speilberger et al., 1983), and has been validated in older adult communities (Potvin et al., 2011; Bergua et al., 2012). The present study used only the state items from the STAI (STAI form Y-1) to assess the potential effect of probiotic intervention on present feelings of anxiety. The state questionnaire consists of 20-items such as 'I feel tense' which are scored on a 4-point Likert scale from 'not at all' to 'very much so'. Scores range from 20-80, with higher scores indicating higher levels of anxiety. Sores of 20-37 indicate no or low anxiety, 38-40 moderate anxiety, and 45-80 high anxiety.

Perceived Stress Scale (PSS)

The PSS is a 10-item questionnaire widely used for measuring the perception of stress (Cohen., 1983), and was designed to tap into how unpredictable, uncontrollable, and overloaded respondents perceive their lives to be. It has been validated in older adult communities (Cohen 1988; Ezzati et al., 2014), and normative data from a US cohort of 65+ is provided (Cohen., 1983). Items are scored between 0-4 with reverse scoring for positive items and summed to give a total perceived stress score. Scores range from

0 – 40, with scores between 0-13 indicating low perceived stress, 14-26 moderate perceived stress, and 27-40 high perceive stress.

Leiden Index of Depression Sensitivity-Revised (LEIDS-R)

The LEIDS-R provides a measure of cognitive reactivity to sadness (Van der Does and Williams., 2003). This can be defined as the relative ease with which negative thinking is activated by mild low mood and is a significant risk factor of depression (Van der Does and Williams., 2003). While the CESD was included to capture depressive symptomology that might exist within this pool of participants, the LEIDS-r is perhaps a more apt measure in a population with no diagnosed mental health difficulties, as it assesses the underlying sensitivity to low mood as opposed to depression itself. LEIDS-R score has been shown to predict depression vulnerability better than other measures such as the Ruminative Response Scale (Moulds et al., 2008) and predicted first-onset depression in never-depressed individuals (Kruijt et al. 2013). The revised version comprises 34 items which participants respond to using a 5-point Likert scale with anchors from 0 'not at all' to 4 'very strongly'. Importantly, participants are asked to imagine a time when they felt 'somewhat sad' but not depressed and respond to items with this in mind. Items are summed to provide a total score (ranging from 0 – 136) in addition to six sub-scales which reflect hopelessness/suicidality (HOP; maximum score of 20), acceptance/coping (ACC; maximum score 20), aggression (AGG; maximum score 24), control (CON; maximum score 24), risk aversion (RAV; maximum score 24), and rumination (RUM; maximum score 24). Higher total sand sub-scale scores indicate higher cognitive reactivity, and therefore greater risk of depression. Previous work found a positive effect of probiotic intervention on cognitive reactivity in young adults as assessed by the LEIDS-R, where participants demonstrated particularly strong reductions in the rumination and aggression sub-scales (Steenbergen et al., 2015). Similarly, individuals with mild/moderate self-report depression scores have also reported a reduction in reactivity to sad mood using the LEIDS-r following chronic probiotic intervention (Chahwan et al., 2018).

5.2.4.3 Additional measures

The Montreal Cognitive Assessment (MoCA)

Given that this experimental chapter aimed to explore the effect of a probiotic intervention on cognitive function it was important to establish the baseline level of cognitive ability within this study cohort. The MoCA is screening tool originally designed to assist health professionals in the detection of MCI and AD, assessing short-term memory, working memory, visuo-spatial abilities, executive functions, attention,

language, and orientation to time and space (Nasreddine et al.,2005). It has been well validated in adults aged 60-85 across various clinical and research settings (Kenny et al., 2013; Sachs et al., 2021), and has been used as a measure of cognitive function in several nutritional intervention studies for the mitigation of cognitive decline in older age (Rainey-Smith et al., 2016; Sakurai et al., 2021). The MoCA was selected over alternatives such as the mini mental state examination (MMSE) as it has been validated as a significantly more sensitive tool for the detection of subtle cognitive impairment (Trzepacz et al., 2015; Aiello et al., 2022). This is relevant here given that the aim was to assess cognitive function in individuals without diagnosed MCI who are instead likely to be experiencing more subtle declines in cognition within an accepted range for healthy ageing.

Epic-Norfolk Food Frequency Questionnaire (FFQ)

The Epic-Norfolk FFQ is a semi-quantitative self-report questionnaire in which participants can provide retrospective information regarding their habitual diet over the last 12 months. It was originally developed to assess dietary intake in adults following a traditional UK diet and has been well validated within this population (Bingham et al., 1997). Part one comprises 130 items adapted from the McCance and Widdowson's UK food consumption database (1991) (each with a specified serving size) for which the participants must select an appropriate frequency of consumption from nine options, ranging from 'never or less than once a month' to '6+ per day'. Part two asks for further detail regarding type and quantity of milk consumed, type of cereal consumed, and types of fats used for cooking and baking. Scoring was carried out in accordance with the FFQ guidelines. Frequency data were entered as a series of codes into a comma-separated values file, which was then analysed for nutrient data using the associated FETA tool (Mulligan et al., 2014). Briefly, FETA calculates average nutrient intake by converting the frequency category into a multiplier, which is then multiplied by portion size to obtain an average daily food weight. These weights are then multiplied by the nutrient composition per gram and summed across all food items to give the average daily nutrient intake for 46 nutrients and 14 food categories. The FFQ was administered between screening and baseline visits via the online platform Gorilla (www.gorilla.sc), although five participants were not comfortable completing it online and instead completed the paper version. Data were automatically entered for those who completed the online version, and manually entered for those who completed the paper copy. The FFQ was included as it provides an indication of baseline diet for the study cohort, which is important given that there are currently mixed views as to how habitual diet interacts with the efficacy of probiotic bacteria (Senan et al., 2015; Abildgaard et al. 2017).

Microbial DNA extraction and 16S rRNA sequencing

In order to explore how the probiotic intervention might affect the gut microbiota, stool samples were collected from participants at each baseline and post-intervention session. Fresh faecal samples were collected and placed in anaerobic jars using Thermo Scientific AnaeroGen 2.5 L anaerobic sachets (Oxiod, Basingstoke UK). Participants were instructed to collect the sample as close to the session as possible, with the ideal scenario being collection on the morning of the session prior to arrival. However, to reduce stress, samples collected within 12 hours of the session were accepted. Samples were stored on ice blocks during the session if provided beforehand, a minimum of 3g was aliquoted into a falcon tube and frozen at -80°C within 3 hours of receiving the sample.

DNA extraction was performed using QIAamp PowerFecal Pro DNA kits (QIAGEN) according to manufacturer's instructions. 0.5g of faecal sample was placed in a 10 mL falcon tube with glass beads (3mm) with phosphate buffered saline (PBS) to create a 1:10 dilution. Tubes were vortexed and centrifuged at 1500 *g* (Eppendorf 5804 R), from which 200uL of raw extract was used for DNA isolation. The concentration of extracted DNA as well as purity (260/280 ratio) was measured using a Nanodrop (NanoDrop^{TM} ND-1000 Spectrometer). As per instructions, concentration was deemed acceptable if between 20 – 100 ng/µL. If greater than 100 ng/µL, additional C6 solution was added in 25µL quantities until satisfactory.

16S rRNA gene sequencing and bioinformatics were outsourced to Microsynth AG (Schützenstrasse 15, 9436 Balgach, Switzerland). 25uL of extracted bacterial DNA per sample was shipped on dry ice in sealed 96-well plates. To sequence the V3 and V4 regions of the bacterial 16S rDNA gene, two-step, Nextera barcoded PCR libraries using the locus specific primer pair 341F (5'- CCT ACG GGN GGC WGC AG -3') and 805R (5'- GAC TAC HVG GGT ATC TAA TCC -3') with 20 PCR cycles for the first step and 20 PCR cycles for the second step were created. Subsequently the PCR libraries were sequenced on an Illumina MiSeq platform using a v2 500 cycles kit.

Subsequent sequencing of PCR libraries was performed on an Illumina MiSeq platform using a v2 500 cyles kit (2 x 300 pb, V3-V4). The produced paired-end reads which passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina's bcl2fastq software version v2.20.0.422. The quality of the reads was checked with the software FastQC version 0.11.8 and sequencing reads that fell below an average Q-score of 20 or had any uncalled bases (N) were removed from further analysis. The locus specific primers were trimmed from the sequencing reads with the software cutadapt v3.2 and discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to in-silico reform the sequenced molecule considering a

minimum overlap of 15 bases using the software USEARCH version 11.0.667. Merged reads that contained ambiguous bases or were outliers regarding the expected amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded, to not distort the statistical analysis. The remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form operational taxonomic units (OTUs) discarding singletons and chimeras in the process. The resulting OTU abundance table was then filtered for possible barcode bleed-in contaminations using the UNCROSS algorithm. OTU sequences were compared to the reference sequences of the RDP 16S database (https://www.drive5.com/usearch/manual/sintax_downloads.html) and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH. Alpha diversity was estimated using the Richness (Observed), Simpson and Shannon indices. Beta diversity was calculated using the weighted Unifrac distance method on basis of rarefied OTU abundance counts per sample. Alpha and beta diversity calculations were performed with the R software packages phyloseq v1.26.1 and vegan v2.5-5. To detect differentially abundant OTUs depending on treatment and time variables, differential OTU analysis using normalised abundance counts was performed with the R software package DESeq2 v1.26.0. Functional profiles were predicted by hidden state reconstruction using the software picrust2 v2.1.4-b and its integrated EC, KO, MetaCyc, COG, PFAM and TIGRFAM databases.

5.2.5 Data analysis

5.2.5.1 Power calculation

A priori power analysis was performed using GPower 3.1.9.6 to determine the minimum number of participants required in order to achieve a statistical power of 0.8 with an alpha level of 0.05 (Cohen, 1992). Previously, moderate effect sizes (partial eta squared) have been reported for probiotic intervention on tasks assessing aspects of memory, and moderate-large effect sizes on tasks of attention and executive function (Wallis et al., 2018). Assuming an effect size of 0.3, 30 participants were deemed sufficient to detect an effect of the probiotic supplement compared to a placebo in tasks of memory, attention, and executive function. As such, the final recruitment aim was 30 participants +10% to allow for attrition.

5.2.5.2 Outlier procedure

All raw data were screened for outliers prior to statistical analysis and calculation of mean and standard deviations. Outliers per outcome variable of interest were identified for each time x treatment condition using Tukey's interquartile range method. Values above Q3 + 1.5 x IQR or below Q1 - 1.5 x IQR were considered outliers and removed from the dataset. IQR is a valid method for removing outliers in both parametric and non-parametric data sets and equates to the removal of values that lie beyond 2.7 standard deviations from the mean (Barbato et al., 2010). Visual inspection of boxplots was used to clarify that all outliers had been successfully removed. In the case of RT data, only RT values for correct responses were screened for outliers and included in subsequent analyses. Additionally, RTs quicker than 200ms in the Go/No-Go and 250ms in the TST tasks were removed on the basis that participants of this age are unlikely to be responding at that speed given the respective cognitive demands of the tasks (Wasylyshyn, Verhaeghen & Sliwinski., 2011; Hsieh et al., 2016) and are therefore likely to be carry-over from the previous trial or made without intent.

5.2.5.3 Compliance cut-off

A degree of noncompliance is to be expected in chronic intervention trials, and it was therefore important to select a compliance cut-off that is considerate of this. Many probiotic trials to date exploring the effect on cognitive or psychological function do not overtly consider compliance, but, based on previous work where compliance was reported (Chung et al., 2014; Akkasheh et al., 2016; Romijn et al., 2017; Kazemi et al., 2019), a compliance rate of 90% was deemed acceptable for inclusion. As such, for each 8-week arm, participants were required to miss no more than 5 days, providing these 5 days were not consecutive. The maximum number of consecutive missed days accepted was two.

5.2.5.4 Linear Mixed Models

All cognitive and mood data were analysed using LMMs. LMMs are statistical models that expand on simple linear models to allow for both fixed and random effects. LMMs have several advantages for handling repeated measures data from chronic RCTs. Firstly, this type of model deals well with unbalanced data sets meaning subjects with missing data can be included in analysis, thereby retaining greater statistical power. This is a key benefit of LMMs, as missing data is common in chronic RCTs. In addition, LMMs more accurately model the true nature of such data, as it is not assumed that all data observations are independent, which is often not the case in repeated measures designs, and variance relating to both fixed and random effects can be modelled, better accounting for within-subject variance in the data (Hoffman & Rovine., 2007). For these reasons, LMMs were felt to be a better fit than alternative models such as repeated measures ANOVAs.

LMMs were run using the lme4 package in r (Bates et al., 2015). Models were estimated with restricted maximum likelihood (REML) which is typical of LMMs as is allows for unbiased estimates of variance. LMMs assume normal distribution of residuals, although data from RCTs often violates this assumption. However, simulation estimates indicate that LMMs are particularly robust to violation of distributional assumptions (Schielzeth et al., 2020). Furthermore, efforts to reduce violation and improve model fit such as non-linear transformation of data reduces the interpretability of data, and, given that the model is robust to assumptions, is arguably unnecessary (Schielzeth et al., 2020; Knief & Forstmeier., 2021). As such, LMMs were deemed to be robust to potential violation for the current data. For all analyses, subject was included as a random factor to control for non-independence of data within-subjects. In addition, partial pooling was applied, such that both the intercept and slope were varied for each level of subject factor, as this has been shown to improve model estimates (Singmann & Kellen., 2019). For each model the fixed effect of treatment (probiotic or placebo), session (baseline, acute or post-intervention) and treatment x session interactions were included, alongside fixed covariates for order (whether participants received the probiotic or placebo first), age, sex, education and MoCA score. Factors which significantly predicted the outcome variable were explored using Bonferroni corrected pairwise comparisons, as this type of correction is reported to be the most appropriate for controlling type I error (Field., 2009). Where treatment or session significantly predicted outcome, pairwise comparisons were explored at the level of interaction, even if the interaction was non-significant (Howell., 2010; Huck., 2015; Wilcox., 1987). Additionally, trending significance ($p < 0.01$) of predictors treatment and session were explored following the same procedure in order to understand patterns in the data where the analysis was perhaps underpowered. The MuMIn package in r was used to retrieve R^2 value for model fit. This package produces two R^2 values per model – a marginal value (associated with fixed effects), and a conditional value (fixed + random effects).

Significant results are reported in text with the corresponding model highlighted in brackets, and all LMM results and R^2 values are reported in Appendix 18. Where appropriate, results of interest are presented in bar charts with between-subject error bars.

5.2.5.5 Microbiome data

Alpha and beta diversity metrices will be reported per treatment x time condition. Change in relative abundance will be explored at the level of order and family and presented as stacked bar charts at the level of order for visual clarity. In addition, the effect of treatment and session on specific genera of interest, including those present in the probiotic supplement (*Lactobacillus*, *Lactococcus*, and *Bifidobacterium*) and those consistently associated with age-related change and health outcomes in older adults, such as *Bacteroides* (Wilmanski et al., 2021), *Akkermansia* (Ghosh et al., 2022) *Blautia* (Liu

et al., 2021) and *Clostridium* (Schütte et al., 2021), will be assessed. Given the shift in *Roseburia* found following daily addition of Ecologic® Barrier in the experiments detailed in chapter 4, the effect of supplementation on *Roseburia* was also assessed in the current study.

The collection of 16S rRNA sequencing data also provides an important opportunity to explore whether relative abundance of key genera is associated with cognitive performance and mood outcomes. In line with previously published work (Chahwan et al., 2018), spearman's rank correlation was used to assess the relationship between cognitive outcomes for which the probiotic intervention had a significant benefit and relative abundance of the genera administered within the probiotic (*Lactobacillus*, *Lactococcus*, and *Bifidobacterium*). Additionally, if probiotic supplementation was associated with a significant shift in the other genera of interest listed above, the same approach was applied. Significant correlations ($p < 0.05$) of weak strength and above (≥ 0.20) are reported and discussed.

5.3 Results

5.3.1 Participant demographic data

A total of 33 participants were enrolled. Two subjects declined to participate following the screening visit due to time commitment and medication, respectively. A third participant withdrew after one baseline test visit due to the decision to start taking antidepressant medication. A final participant withdrew after completing the first arm but provided no reason. Since the third participant dropped out prior to receiving either study intervention, the decision was made to exclude this subject from all analyses. Data from the fourth participant who completed one study arm was included as ITT in all analyses. Demographic details of the final sample (N = 30) are outlined below in Table 5.1. Average self-reported compliance was 99%, and all participants met the pre-determined cut-off of 90%. Rescheduling of sessions due to Covid resulted in two participants taking the placebo for an additional 7 days. All participants scored within the healthy range on the MoCA (≥ 26), indicating no evidence of dementia in this population.

Table 5.1 – demographic information per randomisation group.

Demographic information		Group 1 (Placebo – Probiotic)	Group 2 (Probiotic – placebo)
N		15	15
Gender	Male	6	4
	Female	9	11
Age (years)	M	71.73	70.73
	SD	4.45	3.88
Ethnicity	White British	14	14
	White other	1	1
BMI (kg/m^2)	M	24.01	25.83
	SD	0.92	0.92
Education	≤ 12 years	8	6
	> 12 years	7	9
MoCA	M	27.6	27.6
	Range	26 – 30	26 – 30
Compliance (%)	M	99.2	98.8
	SD	1.32	2.16

5.3.2 Missing data

Two acute visits were missed as a result of experimenter illness, where rescheduling was not possible due to the time sensitive nature of the acute timepoint. No other sessions were missed while subjects were enrolled. Technical issues with recording devices led to full missing RAVLT recordings for 10 (three baseline, five acute & two post-intervention) sessions, and partial missing data affecting the interference outcomes for one baseline session and the delayed recall for three baseline sessions. Technical issues with E-prime unfortunately resulted in loss of Go/No-Go data for a total of 23 sessions across 7 subjects. The LEIDS-r was not completed by one participant in one baseline session. Three subjects were unable to provide one of the requested stool samples and one subject was unable to provide two, resulting in a total of five missing samples. Finally, two participants declined to complete the FFQ either online or on paper. An intention to treat approach was taken with all missing data, in order to retain maximum statistical power. In this instance, the LMe4 package uses listwise deletion prior to maximum likelihood estimation.

5.3.3 Cognitive measures

RAVLT

Mean and standard deviation for all RAVLT outcome measures can be found in Table 5.2.

Regarding the primary outcome of delayed recall, session [$F(2,113.83) = 9.63$, $p< 0.001$], along with covariates MoCA score [$F(4,51.56) = 11.53$, $p < 0.001$] and age [$F(12,57.05) = 9.94$, $p < 0.001$], were significant predictors (Model 1A). Post-hoc comparisons indicated that delayed recall was significantly worse in the acute session compared to both the baseline [$p = 0.016$] and post-intervention [$p < 0.001$] sessions, and this pattern of results was evident across both treatment conditions. Regarding MoCA scores, lower MoCA scores were associated with poorer delayed recall, where participants who scored 26 [$p=0.020$], 27 [$p=0.013$], 28 [$p=0.046$] or 29 [$p=0.006$] had significantly poorer delayed recall than those who scored the maximum of 30. Again, this was consistent across treatments.

Neither treatment nor session significantly predicted immediate recall (Model 1B), number of words learned (Model 1C) or proactive interference (1E) [$p >0.1$]. Proactive interference was generally low ($M = -0.67$, $SD = 3.03$), indicating that initial recall of list B was generally better than initial recall of list A, perhaps because participants had settled into the task by this point. Proactive interference was significantly predicted by the covariate education [$F(1,9.64) = 6.92$, $p = 0.026$], such that those with less than 12 years of education demonstrated significantly more proactive interference than those who had received 12 years of education or greater [$p = 0.037$]. Session significantly predicted total acquisition [$F(2,101.26) = 4.68$, $p = 0.011$] (Model 1D), where total acquisition in the acute session was significantly lower than post-intervention [$p = 0.011$]. Further analysis indicated this was driven by changes in the placebo group, where total acquisition fell from baseline in the acute session [$p = 0.015$] and increased between the acute and post-intervention sessions [$p = 0.070$]. Generally, participants demonstrated greater retroactive interference ($M = 1.72$, $SD = 1.72$) than proactive interference. Session significantly predicted retroactive interference [$F(2,101.76) = 4.04$, $p = 0.021$] (Model 1F), which pairwise comparisons revealed was due to greater retroactive interference in the acute session compared to baseline [$p = 0.026$], and this was apparent in the probiotic group only [$p = 0.029$]. However, this is likely due to a lower incidence of retroactive interference at baseline in the probiotic condition. In addition, participants demonstrated a reduction in retroactive interference between the acute and post-intervention session following the probiotic intervention, which was trending towards significance [p =

0.099]. Finally, with regards to the number of repetitions made, both session [F(2,114.66) = 2.51, p = 0.086] and treatment x session [F(2,114.89) = 2.67, p = 0.073] were trending towards being significant predictors (Model 1G). Pairwise comparisons reveal this was due to an increase in number of repetitions in the placebo condition between baseline and post-intervention [p=0.012].

For the word recognition element of the RAVLT, neither treatment nor session significantly predicted general accuracy on the word recognition component of the RAVLT [p > 0.1] (1H). Looking specifically at correctly identified words from list A, neither session nor treatment significantly predicted accuracy, although session was trending [p = 0.079] (Model 1I). Pairwise comparisons indicate that accuracy in recognising list A words was higher at baseline compared to the acute session across treatments [p = 0.084]. In addition, sex flagged as a significant covariate in the model [F(1,10.04) = 5.20, p = 0.05], where females performed better in list A recognition than males across treatments [p = 0.065]. With regards to correctly rejected items from list B, treatment x session interaction was a significant predictor [F(2,104.48) = 3.32, p = 0.04] (Model 1J). Accuracy in rejecting list B words was significantly greater following chronic probiotic supplementation compared to placebo [p = 0.023], although accuracy did not change significantly within treatment, and this post-intervention difference is likely a reflection of the generally higher accuracy in the probiotic condition. Within this model, covariates order [F(1,9.77) = 5.01, p = 0.050] and age [F(12, 9.99) = 3.04, p = 0.044] also significantly predicted accuracy in rejecting list B words. However, pairwise comparisons weren't significant.

Finally, looking at the percentage of correctly rejected distractors, treatment nor session significantly predicted accuracy, neither when considering all distractors together (Model 1K) or as separate semantic/phonological distractor types (Model 1L &1M).

Table 5.2 – RAVLT data per outcome measure of interest as mean (M) and standard deviation (SD)

Variable	Treatment	Test session					
		Baseline		Acute		Post	
		M	SD	M	SD	M	SD
RAVLT (N words)							
Immediate recall	Placebo	6.90	1.63	6.04	2.14	6.72	2.27
	Probiotic	6.85	2.31	7.04	1.81	7.32	1.89
Amount learned	Placebo	5.5	1.55	5.63	2.04	5.69	1.89
	Probiotic	5.65	2.30	5.58	2.17	5.30	2.25
Total acquisition	Placebo	52.93	8.21	47.93†	10.02	51.76	11.14
	Probiotic	52.65	9.87	51.70	6.55	55.96	5.74

Proactive interference	Placebo	-0.28	3.10	-1.21	2.66	-1.14	2.98
	Probiotic	-0.61	3.63	-0.07	3.02	-0.69	2.82
Retroactive interference	Placebo	1.72	1.87	2.30	1.54	1.75	1.90
	Probiotic	1.07	2.00	2.12†	1.58	1.27	0.83
Delayed Recall	Placebo	10.56	3.26	8.79†	3.71	10.72$^{\#}$	2.74
	Probiotic	10.07	3.30	9.77	2.44	11.00$^{\#}$	2.46
Total repetitions	Placebo	4.04	2.68	4.92	3.95	6.73†	4.33
	Probiotic	7.00	4.11	5.27	3.56	6.00	4.83
Word recognition							
Recognition accuracy (%)	Placebo	92.73	9.32	92.16	9.72	91.98	12.25
	Probiotic	93.60	9.32	91.82	11.72	93.28	10.20
Correctly identified items (list A) (%)	Placebo	91.43	8.86	87.69	8.15	89.29	10.79
	Probiotic	91.11	8.27	87.69	11.11	89.43	12.28
Correctly rejected items (list B) (%)	Placebo	88.05	12.39	88.81	12.58	85.56	17.60
	Probiotic	93.33	8.43	88.64	14.54	92.38	10.99
Correctly rejected distractors (%)	Placebo	95.75	6.25	96.11	6.81	96.95	5.78
	Probiotic	95.03	9.68	95.60	9.08	95.86	7.66
Correctly rejected distractors (P) (%)	Placebo	95.40	6.31	95.06	7.76	97.86	4.47
	Probiotic	95.30	9.52	94.67	10.70	94.64	9.21
Correctly rejected distractors (S) (%)	Placebo	96.10	6.27	97.20	5.62	96.10	6.74
	Probiotic	94.76	10.02	96.50	7.31	97.35	5.00

Significant pairwise comparisons are represented, * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and $^{\#}$ indicates significant difference from the acute session within treatment

CBTT

Mean and standard deviation for all CBTT outcome measures can be found in Table 5.3.

Performance on the CBTT was as expected, where accuracy in correctly copying the sequence of blocks progressively declined as the number of blocks in the sequence increased, resulting in a range of average performance from 100% in 2 block trials to 9% in 9 block trials. With regards to the primary outcome of % of correct sequences, session [$F(2,2551.37) = 3.80$, $p = 0.022$] and number of blocks in the sequence [$F(7,2543.85) = 1496.67$, $p = <0.001$] significantly predicted performance (Model 2A). In addition, treatment x block [$F(7,2545.59) = 2.27$, $p = 0.027$], session x block [$F(14,2545.59) = 1.92$, $p = 0.020$] and session x treatment x block interactions were significant [$F(14,2543.02) = 1.97$, $p = 0.017$]. Performance between treatment conditions did not differ on trials with 2, 3, 6, or 7-block sequences, but the % of correct sequences was significantly higher in the placebo condition in the acute [$p=0.014$] and post-intervention [$p=0.003$ sessions on 4-block and the acute session [$p = 0.007$] on 5-block trials. Additionally, performance at baseline on 8-block trials was significantly greater in the placebo condition [$p = 0.046$] but significantly greater post-intervention in the probiotic condition [$p=0.004$], and was trending towards being significantly higher on 9-block trials post-intervention in the probiotic condition [$p = 0.061$]. Within treatment, performance following the placebo significantly improved between baseline and acute sessions on 5-block [$p = 0.015$], and was trending towards significant improvement on 4-block [$p = 0.055$]. Improved performance between baseline and post-intervention sessions was trending towards significance following placebo in 6-block [$p=0.062$] and 7-block [$p=0.077$] trials. Additionally, performance significantly declined in the placebo condition between baseline and post-intervention on 8-block [$p=0.002$] and 9-block [$p=0.006$] trials, and between acute and post-intervention sessions on 8-block [$p=0.010$] and 9-block [$p=0.020$] trials. No within-treatment changes were found under the probiotic condition. As such, better performance on 8 and 9 block trials in the probiotic condition can be attributed to a decline in performance in the placebo condition, as opposed to improved performance following the probiotic supplement.

Looking at participant ability to select the correct blocks, but not in the correct sequence, session [$F(2,2599.16)) = 7.69$, $p < 0.001$], number of blocks in the sequence [$F(7,2543.85) = 1496.67$, $p <0.001$], as well as treatment x block [$F(7,2545.59) = 2.27$, $p = 0.027$], session x block [$F(14,2542.60) = 1.92$, $p = 0.020$] and treatment x session x block [$F(14,2543.02) = 1.97$, $p = 0.017$] interactions significantly predicted % of trials on which correct blocks were selected (Model 2B). Pairwise comparisons reveal that % of correct blocks was significantly higher at in the acute [$p=0.017$] and post-intervention [$p=0.004$] sessions in the placebo condition for 4-block trials compared to the probiotic condition. Additionally, performance was significantly lower in the placebo condition at baseline on 8-block trials

[p <0.001], and trended towards being significantly lower post-intervention on 5-block [p = 0.055] and 8-block [p=0.059] trials. Within-treatment, performance significantly improved from baseline in the acute and post-intervention sessions in the placebo condition on 4-block [p = 0.042; p = 0.012] and 8-block [both p < 0.001] trials. Additionally, performance significantly fell on 5-block trials between acute and post-intervention in the placebo condition [p=0.007]. In the probiotic condition, performance improved from baseline in the post-intervention session on 8-block trials, and was trending towards significant improvement in 5-block trials [p=0.062].

Table 5.3 - CBTT data per outcome measure of interest as mean (M) and standard deviation (SD)

Variable	N blocks	Treatment	Test session					
			Baseline		Acute		Post	
			M	SD	M	SD	M	SD
% of correct sequences	2	Placebo	100.00	0.00	100.00	0.00	100.00	0.00
		Probiotic	100.00	0.00	100.00	0.00	100.00	0.00
	3	Placebo	100.00	0.00	100.00	0.00	100.00	0.00
		Probiotic	100.00	0.00	100.00	0.00	100.00	0.00
	4	Placebo	83.45	16.65	91.25	14.50	88.28	16.82
		Probiotic	81.79	20.06	83.46*	17.97	78.45*	26.06
	5	Placebo	58.67	33.75	68.45†	25.60	63.68	23.27
		Probiotic	61.21	25.60	59.29*	29.12	59.83	27.89
	6	Placebo	30.51	28.64	34.48	24.04	41.53	34.62
		Probiotic	33.62	28.31	31.73	22.75	37.07	32.86
	7	Placebo	23.56	23.34	30.88	31.33	26.10	30.26
		Probiotic	26.55	23.88	29.64	28.41	30.34	30.03
	8	Placebo	13.64	18.52	12.75	18.96	0.00†#	0.00
		Probiotic	7.41*	15.07	14.29	19.57	11.00*	16.10
	9	Placebo	11.82	16.20	10.39	18.13	0.00†#	0.00
		Probiotic	9.82	17.29	11.96	17.55	8.15	14.99
% of trials in which	2	Placebo	100.00	0.00	100.00	0.00	100.00	0.00

correct blocks were selected		Probiotic	100.00	0.00	100.00	0.00	100.00	0.00
	3	Placebo	100.00	0.00	100.00	0.00	100.00	0.00
		Probiotic	100.00	0.00	100.00	0.00	100.00	0.00
	4	Placebo	91.03	12.20	100.00†	0.00	100.00†	0.00
		Probiotic	90.36	14.40	91.25*	15.37	90.86*	15.06
	5	Placebo	85.58	18.29	90.00	15.99	79.33#	22.69
		Probiotic	81.61	17.95	89.26†	17.41	86.43*	19.19
	6	Placebo	61.21	22.74	64.51	24.20	63.33	31.30
		Probiotic	66.07	23.78	66.07	29.04	65.52	28.67
	7	Placebo	63.00	27.93	69.29	23.28	64.11	25.24
		Probiotic	68.62	23.90	68.75	32.25	64.31	26.62
	8	Placebo	44.17	28.34	60.34†	31.71	60.00†	25.09
		Probiotic	56.03*	28.86	61.61	27.62	66.38*†	30.09
	9	Placebo	100.00	0.00	100.00	0.00	100.00	0.00
		Probiotic	100.00	0.00	100.00	0.00	100.00	0.00

Significant pairwise comparisons are represented, * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment

TST

Mean and standard deviation for all TST outcome measures can be found in Table 5.4.

Neither treatment nor session were significant predictors of accuracy in the switching task [$p > 0.1$], as were none of the included covariates (Model 3A). Accuracy was affected by whether the trial was a switch trial (the first trial of a new rule) or not, where switch type was a highly significant predictor of accuracy [$F(1,592.97) = 143.61$, $p < 0.001$]. As expected, accuracy on switch trials was significantly worse than that of non-switch trials in both placebo and probiotic conditions [$p < 0.001$] and across all sessions [$P<0.001$]. There was, however, no interaction between treatment and switch type (Model 3B).

In addition to switch type, trial type – i.e., whether subjects were responding to trials based on the number being odd/even or high/low – was also a significant predictor of accuracy [F(1,592.62)= 9.14, p < 0.01], where accuracy was significantly better on odd/even than high/low trials [p < 0.01] (Model 3C). Post-hoc analysis of the significant trial type x treatment interaction revealed that this effect of trial type on accuracy was true at baseline regardless of treatment allocation [p = 0.02], but only true of the placebo group post-intervention [p = 0.03], suggesting improvement on high/low trials following probiotic intervention (Figure 5.2). Accuracy did improve across sessions on the probiotic intervention, but this improvement from baseline in acute [p = 0.10] and post-intervention [p= 0.18] sessions on high/low trials did not reach significance. Additionally, performance on high/low trials did not differ significantly between treatments post-intervention. However, performance on odd/even trials was significantly better following placebo compared to probiotic in the post-intervention session [p = 0.02].

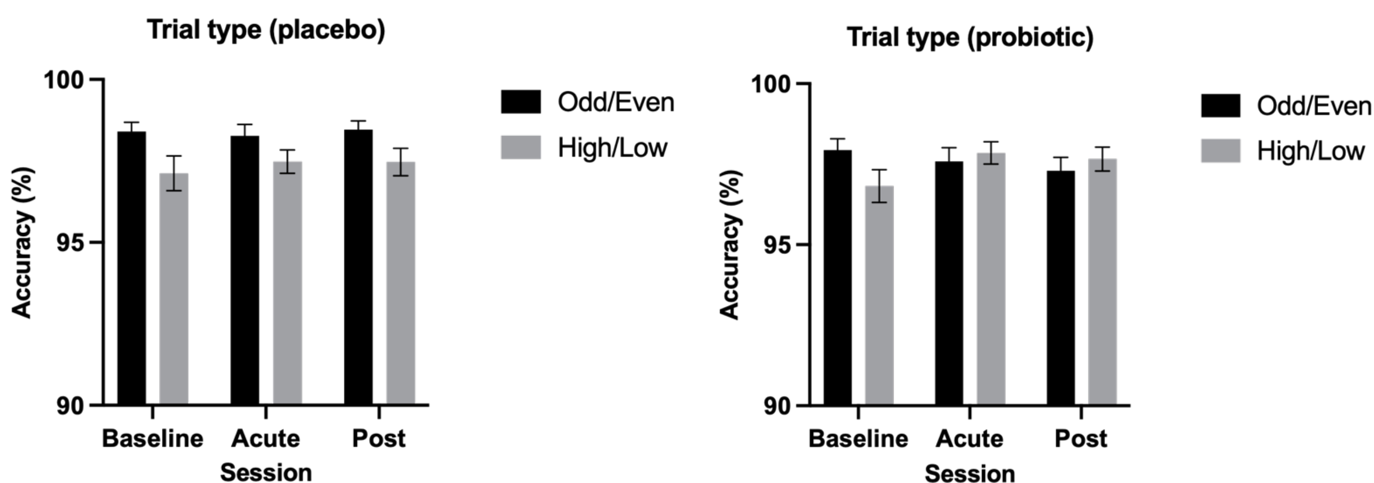


Figure 5.2 - Average TST accuracy in odd/even and high/low trials within the placebo and probiotic conditions. Values are represented as M ± SE (between-subject). * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment, p <0.05.

Session [F(2,62390) = 166.89, p < 0.001] was a significant predictor of general RT, as was the interaction between session and treatment [F(2,62390) = 9.88, p <0.001] (Model 3D). Post-hocs indicate that RTs significantly differed between all sessions [all p < 0.001] for both treatments, where RTs were slowest in the baseline session, followed by post-intervention and fastest in the acute session. Analysis of the treatment x session interaction revealed no significant difference in RT between treatments at baseline

or post-intervention, but significantly faster RTs following probiotics in the acute session compared to the placebo [p < 0.01] (Figure 5.3).

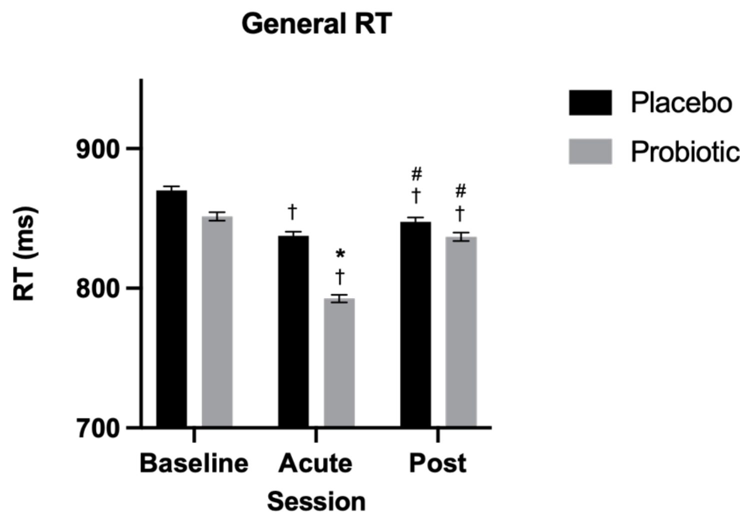


Figure 5.3 – Average TST RT across trials. Values are represented as M ± SE (between-subject). * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment, p <0.05.

As with accuracy, RT was affected by whether the trial was a switch trial or non-switch trial (Model 3E). Session [F(2,62377) = 342.37, p < 0.001] and switch type [F(1,62365) = 30055.41, p < 0.001] were significant factors in predicting RT, while treatment was trending [F(1,29) = 4.05, p = 0.053]. Interactions between treatment x session [F(2,62377) = 26.51, p < 0.001], treatment x switch type [F(1, 62366) = 8.24, p = 0.004], session x switch type [F(2, 62365) = 93.47, p < 0.001] and treatment x session x switch type [F(2, 62365) = 14.74, p < 0.001] were also significant. As expected, RTs were quicker on non-switch than switch trials, and RTs follow the same trend on switch trials as they did in general, with quickest RTs in the acute session. Further inspection of these interactions highlights that RTs on non-switch trials were significantly faster at baseline for the probiotic arm compared to the placebo [p = 0.006]. At the acute timepoint, probiotic intervention was associated with significantly faster RTs on switch [p < 0.001] and non-switch [p =0.041] trials compared to the placebo, but no significant difference between treatments was evident post-intervention, regardless of switch type (Figures 5.4C & D).

When considering the effect of trial type, session [F(2,62384) = 166.87, p < 0.001], treatment x session [F(2,62384) = 9.87, p < 0.001] and treatment x session x trial type [F(62366 = 3.94, p =0.019] all significantly predicted RT, while treatment alone was trending [F(2,62366) = 3.94 , p = 0.10] (Model 3F). For both treatment groups, RTs were significantly quicker in the acute session for both trial types [all p's

< 0.001] and increased again between the acute and post-intervention sessions [all p's <0.01]. Between treatments, pairwise comparisons reveal that RT on odd/even trials was significantly faster at baseline on the probiotic arm than the placebo [p = 0.045]. In addition, RTs were significantly faster in the acute session following probiotic treatment compared to placebo in both odd/even [p = 0.015] and high/low trials [p = 0.004]. No significant difference between treatments was found post-intervention on either trial type (Figures 5.4A & B).

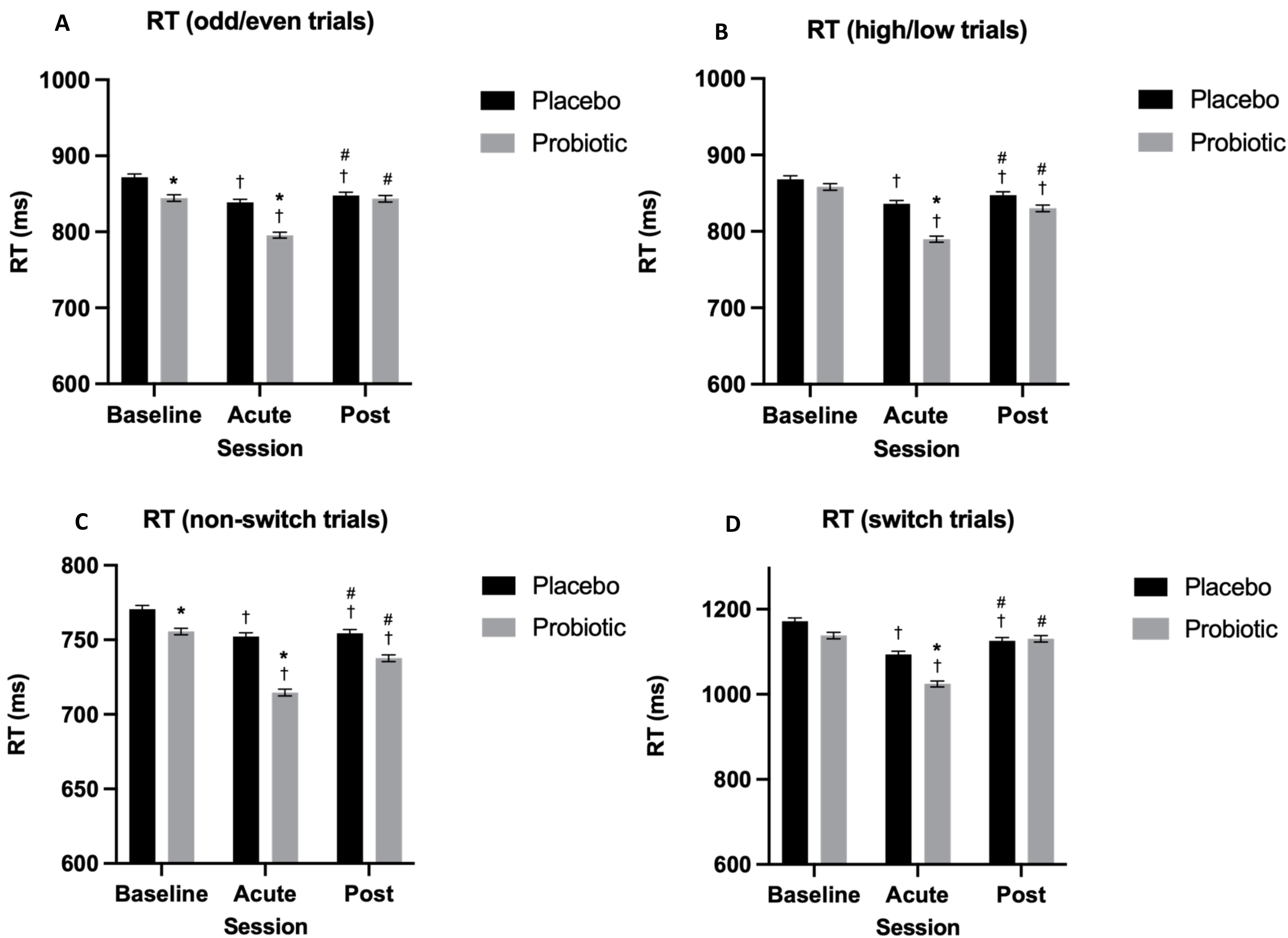


Figure 5.4 – TST RTs on odd/even (A), high/low (B), non-switch (C), and switch trials (D). Values are represented as M ± SE (between-subject). * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment, p <0.05.

Table 5.4 – TST data per outcome measure of interest as mean (M) and standard deviation (SD)

Variable	Treatment	Test session					
		Baseline		Acute		Post	
		M	SD	M	SD	M	SD
Accuracy (%)	Placebo	97.76	3.17	97.88	2.70	97.98	2.63
	Probiotic	97.38	3.27	97.72	2.86	97.47	2.90
Accuracy on non-switch trials (%)	Placebo	99.08	1.05	98.79	1.25	98.78	1.16
	Probiotic	98.85	1.22	98.59	1.41	98.71	1.27
Accuracy on switch trials (%)	Placebo	96.49	3.94	96.95	3.39	97.17	3.36
	Probiotic	95.94	3.96	96.86	3.61	96.28	3.48
Accuracy on odd/even trials (%)	Placebo	98.40	2.12	98.27	2.65	98.46	2.03
	Probiotic	97.94	2.61	97.59	3.14	97.30	3.05
Accuracy on high/low trails (%)	Placebo	97.12	3.87	97.48	2.72	97.47	3.09
	Probiotic	96.82	3.77	97.85	2.57	97.66	2.75
RT (ms)	Placebo	870.01	318.73	837.55†	306.32	847.65$^{\dagger\#}$	322.04
	Probiotic	851.40	306.32	792.62†*	282.39	836.87$^{\dagger\#}$	308.51
RT on non-switch trials (ms)	Placebo	770.61	216.40	752.32†	213.49	754.39$^{\dagger\#}$	223.96
	Probiotic	755.57*	196.26	714.65†*	198.22	737.70$^{\dagger\#}$	198.35
RT on switch trials (ms)	Placebo	1171.96	216.4	1093.94†	376.85	1125.89$^{\dagger\#}$	400.18
	Probiotic	1138.32	386.85	1024.15†*	358.10	1130.63$^{\#}$	381.05
RT on odd/even trials (ms)	Placebo	871.75	316.52	838.91†	296.07	847.83$^{\dagger\#}$	309.39
	Probiotic	844.49*	302.05	795.52†*	274.22	843.64$^{\#}$	307.51
RT on high/low trials (ms)	Placebo	868.23	320.99	836.18†	308.80	847.47$^{\dagger\#}$	322.25
	Probiotic	858.30	310.39	789.73†*	290.29	830.14$^{\dagger\#}$	309.39

Significant pairwise comparisons are represented, * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment

Go/No-Go task

Mean and standard deviation for all Go/No-Go outcome measures can be found in Table 5.5.

Commission errors fell from baseline in the acute session and increased again at post-intervention. Session was found to be a significant predictor of commission errors [$F(2,83.59) = 5.27$, $p = 0.007$] (Model 4A), where significantly fewer commission errors were made in the acute session compared to post-intervention [$p = 0.005$]. A similar pattern of results was revealed for omission errors, which were lowest in the acute session and highest in the post-intervention session. Again, session significantly predicted omission errors [$F(2, 107.79) = 4.92$, $p = 0.01$] (Model 4B) where errors were significantly lower in the acute session than post-intervention [$p = 0.008$]. Treatment was not a significant predictor of either error type, nor was there a session x treatment interaction.

Session [$F(2,18524.6) = 8.11$, $p < 0.001$] was a significant predictor of RT on correct go trials, along with a significant session x treatment interaction [$F(2,18547.7) = 7.06$, $p < 0.001$] (Model 4C). Pairwise comparisons indicate that RT was significantly quicker post-intervention than at baseline [$p = 0.003$] and the acute session [$p < 0.001$], but this was true only of the placebo condition [$p < 0.001$].

Table 5.5 – Go/No-Go data per outcome measure of interest as mean (M) and standard deviation (SD)

Variable	Treatment	Test session					
		Baseline		Acute		Post	
		M	SD	M	SD	M	SD
Commission errors (as a % of 'no-go' trials)	Placebo	9.17	7.17	7.04	3.43	11.15	7.99
	Probiotic	10.55	8.95	7.74	4.11	10.49	8.43
Omission errors (as a % of 'go' trials)	Placebo	8.89	8.17	6.98	4.70	8.64	8.02
	Probiotic	9.03	8.32	4.64	4.13	11.44	9.19
RT (ms)	Placebo	374.63	61.54	372.94	61.76	$366.46^{\dagger \#}$	63.11
	Probiotic	371.55	61.21	370.84	59.06	372.17	63.34

Significant pairwise comparisons are represented, * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment

5.3.4 Mood measures

Mean and standard deviation for all mood measures can be found in Table 5.6.

PANAS

None of the included factors significantly predicted positive affect, although treatment was a trending factor [F(1,28.43) = 2.91, p = 0.099]. Pairwise comparisons indicate that general positive affect was trending lower in the placebo condition than the probiotic [p = 0.099]. Session significantly predicted negative affect scores [F(2,93.74) = 7.97, p < 0.001] (Model 5A), where negative affect significantly reduced between baseline and acute [p<0.001] and baseline and post-intervention [p=0.001] sessions. Further analysis indicates that the decrease in negative affect between baseline and acute [p = 0.018] and baseline and post-intervention sessions [p=0.011] was significant in the probiotic group, but also significant between baseline and acute sessions [p = 0.023] in the placebo condition. In addition, fall in negative affect between baseline and post-intervention was trending in the placebo condition [p=0.083]. No significant differences in negative affect were evident between treatments.

Looking at the additional subscales, none of the included factors predicted sadness, hostility, joviality, serenity, or attentiveness (Models 5C, D, F, I & G, respectively). Treatment significantly predicted fear [F(1,26.05) = 6.42, p = 0.018], while the treatment x session interaction was trending [F(2,102.66) = 2.46, p = 0.091] (Model 5E). Fear scores fell significantly between baseline and post-intervention in the probiotic condition [p=0.016], although fear was significantly higher in the probiotic condition compared to placebo at baseline [p = 0.006] and in the acute session [p = 0.017]. The covariate sex significantly predicted fatigue [F(1,9.12) = 10.24, p = 0.011] (Model 5H), whereby female participants reported significantly higher fatigue than male participants [p=0.017]. Finally, treatment significantly predicted self-assurance [F(1,124.62) =3.87, p = 0.051], where self-assurance in the placebo condition trended significantly lower than in the probiotic condition[p=0.059], and this was only true post-intervention [p = 0.099] (Model 5J). In addition, the difference in self-assurance score between treatments was trending post-intervention [p=0.010].

PSS

Average PSS score at baseline in both conditions was 12, which corresponds to low levels of stress. None of the included factors significantly predicted PSS score (Model 6A).

STAI

Average state anxiety scores at baseline were 27, which corresponds to low or no anxiety. Treatment was trending towards being a significant predictor [$F(1,125.48) = 3.69$, $p = 0.057$] (Model 7A). STAI scores were trending lower in the placebo condition [$p = 0.07$], which appear to be driven by significantly lower STAI scores in the acute session [$p=0.04$].

CESD

Average CESD score at baseline was 8, which is below the cut-off for identifying individuals at risk of depression, as was to be expected when recruiting a healthy cohort with no diagnosed mental health conditions. Neither treatment nor session were found to be significant predictors of CESD score (Model 8A).

LEIDS-r

LEIDS-r total score was not significantly predicted by any of the included factors (Model 9A). However, session significantly predicted scores for the LEIDS-r sub-scales hopelessness (HOP) [$F(2, 110.30) = 5.05$, $p = 0.008$], aggression (AGG) [$F(2,111.53) = 3.23$, $p = 0.037$], and rumination (RUM) [$F(2,110.90) = 3.39$, $p = 0.043$] (Models 9B, D & G, respectively). Session x treatment interaction was also significant for AGG [$F(2,111.50) = 4.11$, $p = 0.019$] and trending for HOP [$F(2,110.26) = 2.65$, $p = 0.075$]. Pairwise comparisons reveal a significant reduction in HOP [$p = 0.006$], AGG [$p = 0.001$] and RUM [$p = 0.010$] from baseline following chronic probiotic intervention, but not placebo (Figure 5.5). Additionally, the fall in AGG score from acute to post-intervention sessions was trending towards significant [$p = 0.060$]. It should be noted that AGG scores differed significantly between the two treatments at the baseline [$p = 0.013$] and acute [$p= 0.043$] sessions, such that scores were significantly lower in the placebo group. This, however, was not the case post-intervention, where scores were non-significantly higher in the placebo than in the probiotic group. Additionally, RUM scores were higher at baseline in the probiotic condition, and this difference was trending [$p = 0.057$].

Treatment was a trending factor for the prediction of control (CON) [$F(1,26.86) = 3.84$, $p = 0.060$] (Model 9E), which post-hoc investigation revealed was due to lower CON scores in the placebo group at

baseline [p=0.041] and post-intervention [p=0.048]. None of the included factors were found to be significant predictors of acceptance (ACC) or risk avoidance (RAV) (Models 9C & F, respectively).

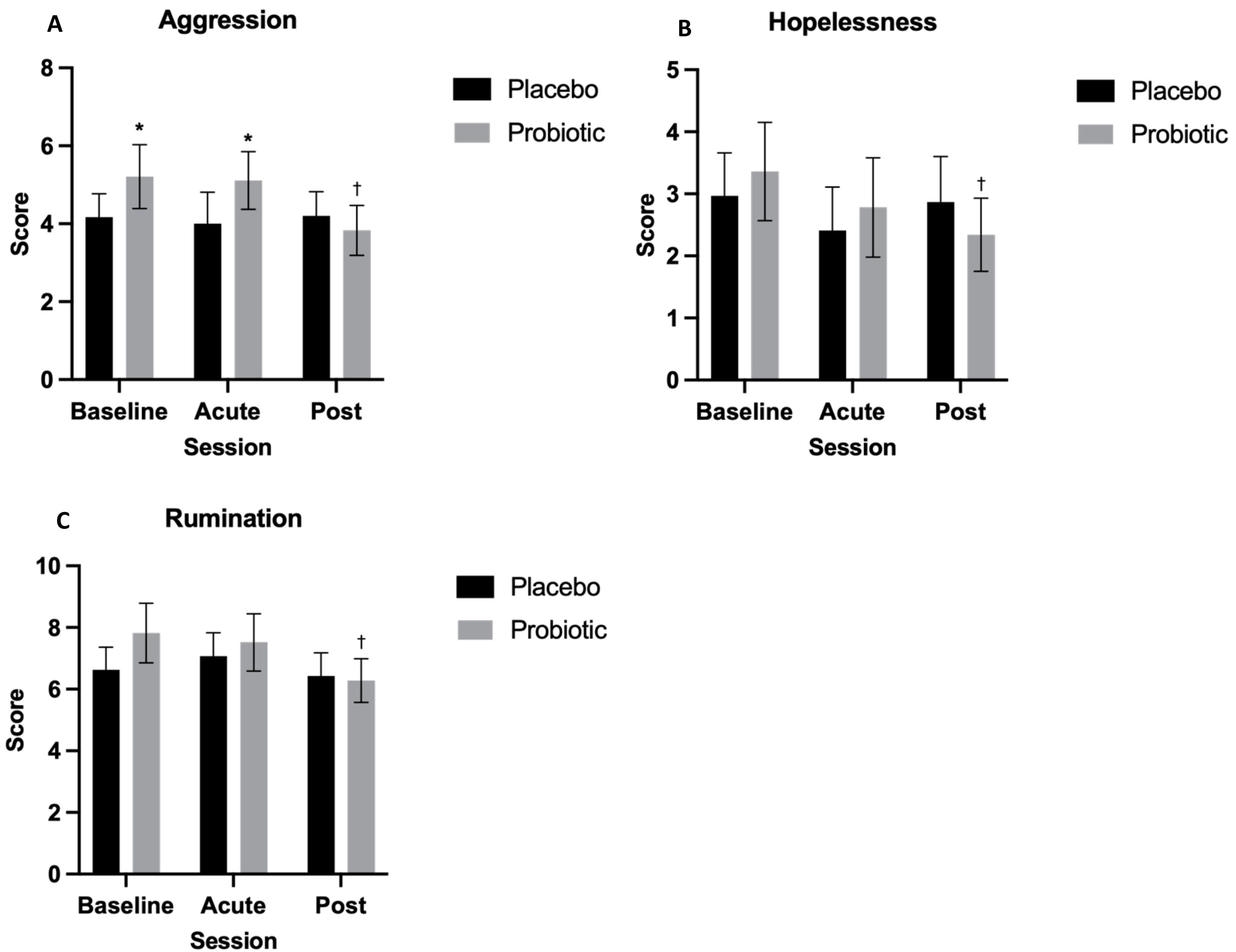


Figure 5.5 – LEIDS-r subscale scores for aggression (A), hopelessness (B) and rumination (C). Values are represented as M ± SE (between subject). * indicates significant difference between treatment groups within session, † indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment, p <0.05.

Table 5.6 –Mood data per outcome measure and sub-scales of interest as mean (M) and standard deviation (SD)

Variable	Treatment	Test session					
		Baseline		Acute		Post	
		M	SD	M	SD	M	SD
PANAS							
positive affect	Placebo	33.57	7.25	33.69	9.04	33.40	8.58
	Probiotic	35.39	7.79	34.85	8.68	35.11	7.40
negative affect	Placebo	10.93	1.26	10.38†	0.57	10.32	0.56
	Probiotic	11.04	1.23	10.43†	0.66	10.38†	0.58
sadness	Placebo	5.48	0.82	5.85	1.52	5.38	0.77
	Probiotic	5.85	1.35	5.83	1.64	6.27	1.87
hostility	Placebo	7.79	0.94	7.83	1.10	7.90	1.24
	Probiotic	7.89	1.12	7.78	0.43	8.03	1.09
fear	Placebo	6.32	0.61	6.22	0.42	6.32	0.55
	Probiotic	6.86*	1.18	6.69*	1.01	6.35†	0.63
joviality	Placebo	25.63	7.28	25.83	8.45	25.80	7.98
	Probiotic	28.09	4.69	27.28	7.20	27.56	6.65
attentiveness	Placebo	15.03	2.85	15.07	2.91	14.40	3.40
	Probiotic	15.61	2.85	15.48	3.09	15.39	2.64
fatigue	Placebo	6.10	1.99	6.79	0.43	6.32	1.79
	Probiotic	6.11	2.21	5.92	2.04	6.25	2.68
Serenity	Placebo	11.47	2.30	11.86	2.13	11.40	2.50
	Probiotic	12.04	2.20	12.19	2.13	12.25	2.08
self-assurance	Placebo	18.27	4.13	18.31	4.77	18.20	4.44
	Probiotic	19.59	3.75	18.74	4.61	19.34	4.61
PSS	Placebo	12.30	5.82	10.88	4.26	11.17	4.91
	Probiotic	12.00	8.11	11.63	6.68	11.52	6.98
STAI	Placebo	27.60	6.17	26.25	5.49	25.30	4.17
	Probiotic	27.03	5.36	28.04	6.41	27.04	5.40
CESD	Placebo	7.79	5.72	NA	NA	7.00	5.61
	Probiotic	8.43	7.89	NA	NA	8.07	7.94
LEIDS-r							
Total score	Placebo	25.87	15.95	25.21	16.08	25.10	16.86
	Probiotic	29.25	19.63	28.67	20.26	26.38	16.90

HOP	Placebo	2.97	3.79	2.41	3.75	2.87	4.01
	Probiotic	3.36	4.19	2.78	4.14	2.34ᵗ	3.21
ACC	Placebo	2.80	2.35	2.83	2.41	2.27	2.50
	Probiotics	2.96	2.46	3.11	3.30	3.07	2.48
AGG	Placebo	4.17	3.26	4.00	4.35	4.20	3.41
	Probiotic	5.21*	4.35	5.11*	3.87	3.83ᵗ	3.44
CON	Placebo	5.53	3.41	5.76	3.70	5.20	3.45
	Probiotic	6.82*	4.46	5.89	3.72	6.41*	3.31
RAV	Placebo	7.50	4.30	6.97	4.40	7.47	4.42
	Probiotic	7.79	4.77	8.07	4.75	7.48	4.10
RUM	Placebo	6.63	4.00	7.07	4.08	6.43	4.08
	Probiotic	7.82	5.16	7.52	4.84	6.28ᵗ	3.82

Significant pairwise comparisons are represented, * indicates significant difference between treatment groups within session, ᵗ indicates significant difference from baseline within treatment, and # indicates significant difference from the acute session within treatment

5.3.5 Additional measures

Food Frequency Questionnaire data

Average estimated daily consumption of the 56 nutrients and food items assessed by the Epic-Norfolk FFQ are presented at the end of this chapter in Table 5.8. Average energy intake (kcal) was in line with estimated average energy requirements for adults assuming a low level of physical activity (Department of Health., 1991). Fruit and vegetable intake was higher than the recommended daily intake of 5 portions at 7 portions, and higher than the average intake reported in the 2020 National Diet and Nutrition Survey (NDNS) for adults ages 75+. Average daily protein intake was also higher at 81.62g/day than the current reference nutritional intake (RNI) of 53.3g/day for adults aged 50+, contributing a higher proportion of total energy intake, as it commonly reported in older adults. However, it has been suggested that healthy older adults should be consuming 1-1.2 g protein/kg per day, rather than the 0.75 g protein/kg currently recommended in order to maintain muscle mass and mitigate frailty (Deutz et al., 2014). Total fat intake was marginally higher than is recommended, particularly saturated fatty

acids, while non starch polysaccharide intake fell within the recommended range for the general adult population. Generally, intake of vitamins and minerals met recommendations with the exception of vitamin D, which averaged 2.93 mcg/day compared to the 10 mcg/day recommendation. Although below the RDI, this data is in line with research indicating a large proportion of UK residents are vitamin D deficient (Lin et al., 2021). Overall, the dietary patterns evidenced in this cohort are in line with that previously described in older adult populations (Zaragoza-Martí et al., 2020).

16S rRNA sequencing

Diversity metrices

Average Shannon and Simpson diversity across this cohort were 4.15 and 0.03, respectively. No significant effect of session, treatment or session x treatment interaction was found on any of the assessed diversity indices, including Shannon alpha diversity, Simpson diversity, richness, or Beta diversity.

Taxonomic composition

Relative abundance of bacteria at the level of order is illustrated per sampling timepoint in Figure 5.6. No significant change in taxonomy between sampling timepoints was observed at the level of order or family. With regards to specific genera of interest, relative abundance of *Bifidobacterium*, *Lactobacillus, Bacteroides, Akkermansia, Blautia, Roseburia,* and *Clostridium* was not affected by treatment. However, there was a significant effect of treatment on *Lactococcus* [$F(1,109) = 5.93$, $p = 0.017$]. Further analysis indicates that abundance of *Lactococcus* did not differ at baseline but was significantly higher post-intervention following probiotic intervention as compared to placebo [$p = 0.007$] (Figure 5.7).

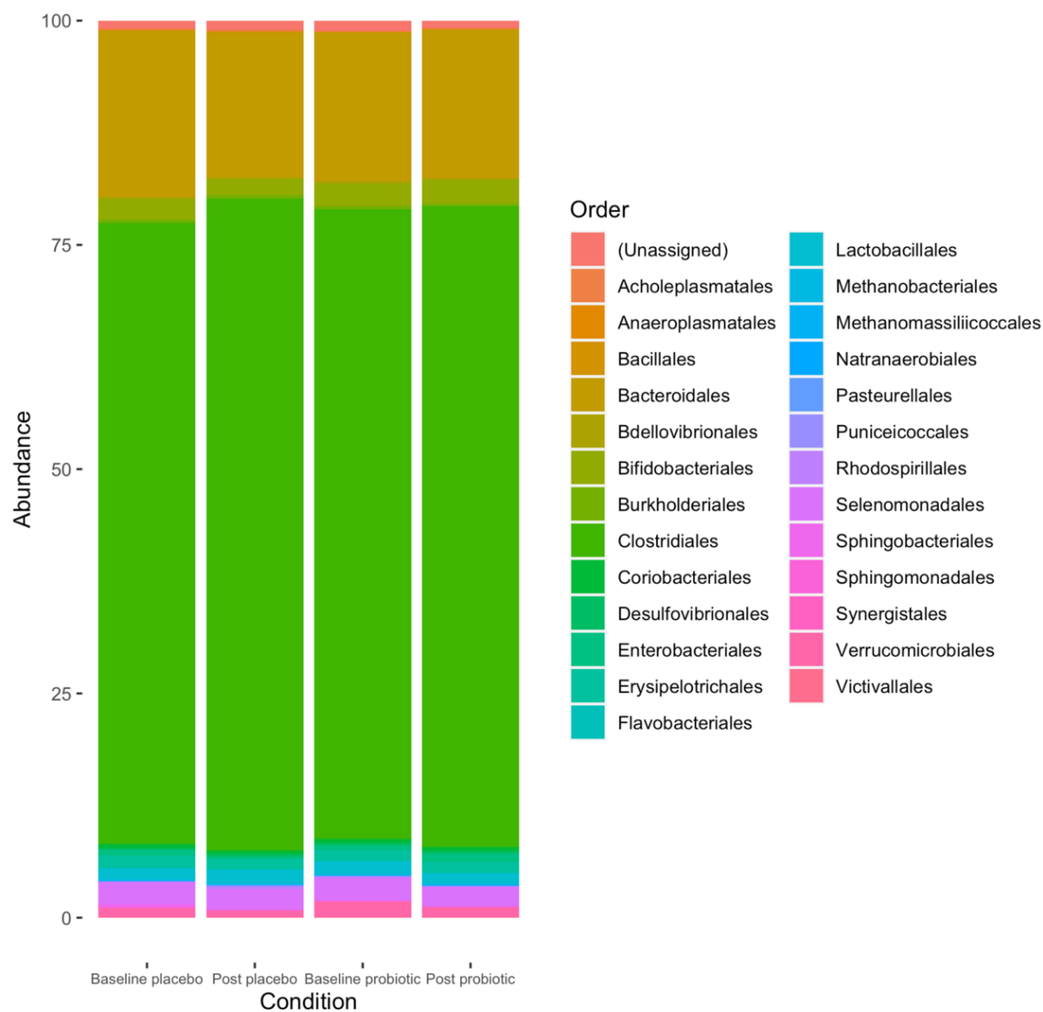
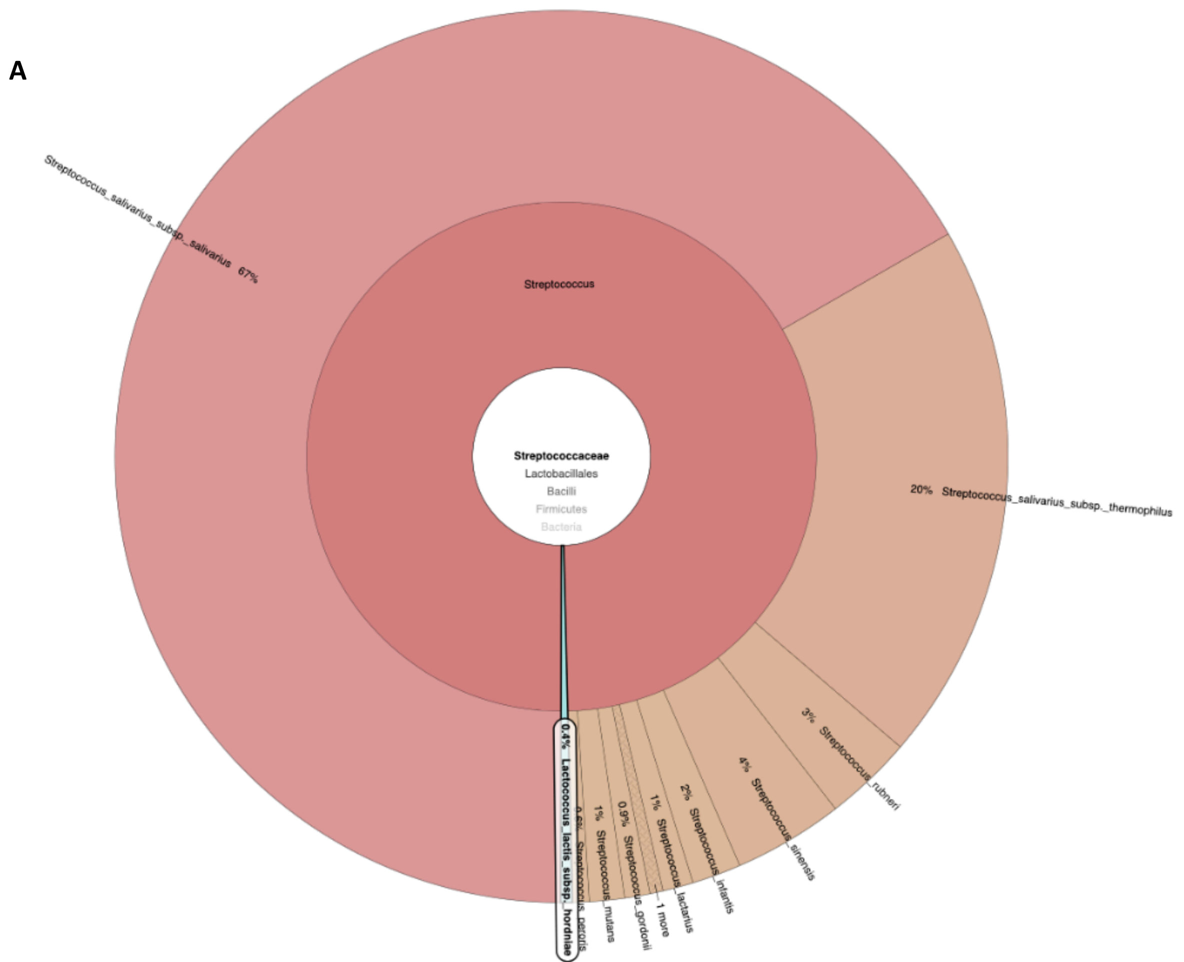


Figure 5.6 – Relative abundance of bacteria at the level of Order at each sampling timepoint.

Regarding correlation between relative abundance of genera present in the supplement and cognitive outcomes significantly affected by probiotic treatment, a significant weak, positive correlation between relative abundance of *Bifidobacterium* and accuracy on high/low TST trials was found [$r_s = 0.20$, $p = 0.003$]. In addition, relative abundance of *Bifidobacterium* was significantly negatively correlated with RT on switch trials in the TST, although again this correlation was weak [rs = 0.21, $p < 0.001$].



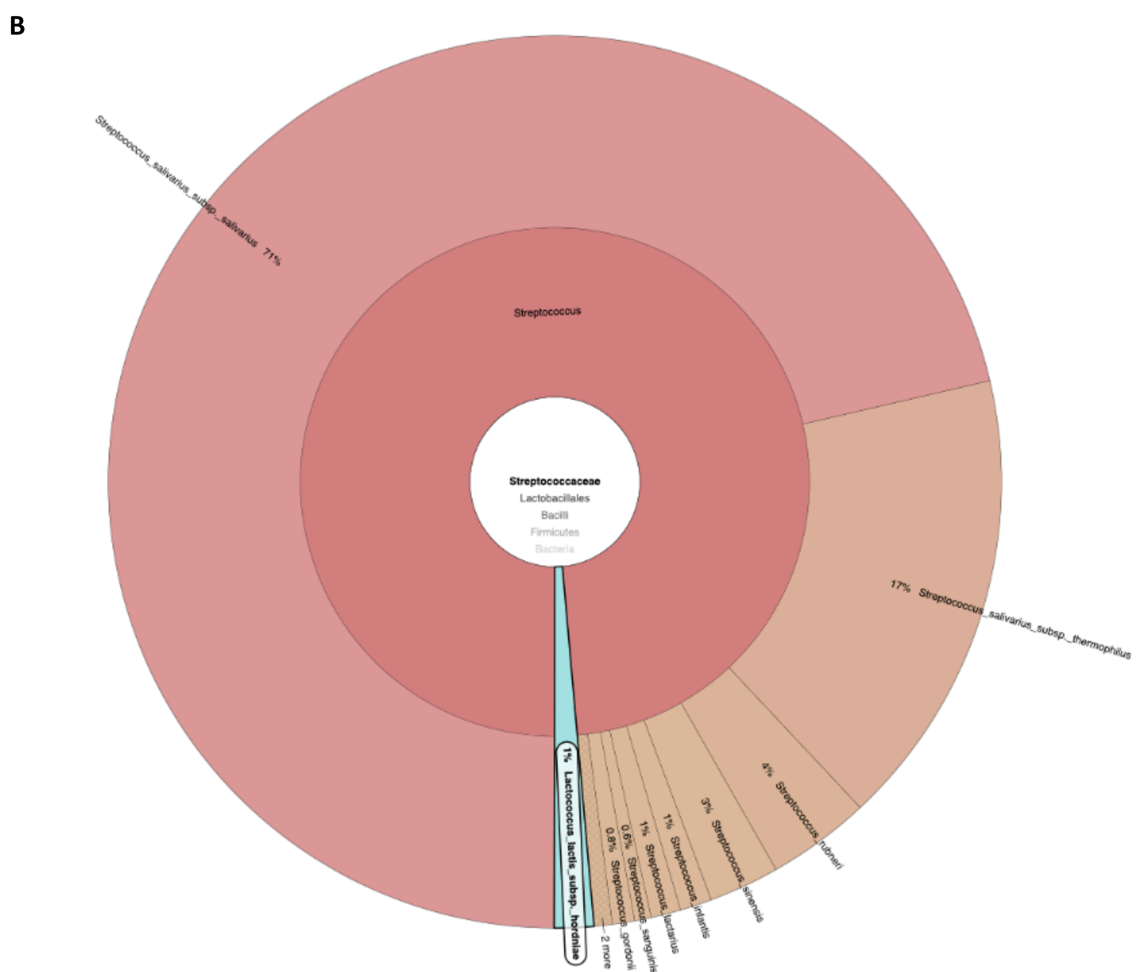


Figure 5.7 – Metagenomic Krona charts illustrating increase in *Lactococcus* between baseline (A) and post-intervention (B) following probiotic intervention.

5.3.6 Summary of results

Given the complexity of the findings, significant effects of treatment on behavioural outcomes are summarised below in Table 5.7, which provides an overview of the outcome measures affected, the treatment conditions in which these effects were found, and highlights cases where the effect is likely attributable to significant difference between treatments at baseline, as opposed to a genuine effect of treatment.

Table 5.7 – Summary of statistically significant results

Outcome variable	Session	Direction of Change in performance	Associated treatment	Effect possibly due to difference at baseline
Total acquisition (RAVLT)	Acute	Declined	Placebo	×
Retroactive interference (RAVLT)	Acute	Declined	Probiotic	✓
Recognition of list B	Post-intervention	Improved	Probiotic	✓
Correct sequence accuracy (CBTT) – 4 block trials	Acute & post-intervention	Improved	Placebo	×
Correct sequence accuracy (CBTT) – 5 block trials	Acute	Improved	Placebo	×
Correct sequence accuracy (CBTT) – 8 block trials	Post-intervention	Declined	Placebo	✓

Correct block accuracy (CBTT) – 4 block trials	Acute & post-intervention	Improved	Placebo	×
Correct block accuracy (CBTT) – 8 block trials	Post-intervention	Improved	Probiotic	✓
Accuracy high/low trials (TST)	Acute and post-intervention	Improved	Probiotic	×
General RT (TST)	Acute	Improved	Probiotic	×
RT switch trials (TST)	Acute	Improved	Probiotic	×
RT non-switch trials (TST)	Acute	Improved	Probiotic	✓
RT on odd/even trials (TST)	Acute	Improved	Probiotic	✓
RT on high/low trials (TST)	Acute	Improved	Probiotic	×
RT (Go/No-Go)	Acute & Post-intervention	Improved	Placebo	×
Negative affect (PANAS)	Acute	Improved	Probiotic & placebo	×
Negative affect (PANAS)	Post-intervention	Improved	Probiotic	×
State anxiety (STAI)	Acute	Improved	Placebo	×

Hopelessness (LEIDS-R)	Post-intervention	Improved	Probiotic	×
Rumination (LEIDS-R)	Post-intervention	Improved	Probiotic	×
Aggression (LEIDS-R)	Post-intervention	Improved	Probiotic	✓

5.4 Discussion

The aim of this work was to explore whether a chronic 8-week multi-strain probiotic intervention could improve cognitive function and mood in a healthy older adult population, with cognitive outcome measures including verbal learning and memory, spatial working memory and executive functioning, and mood measures including general positive and negative affect, stress, anxiety, depression, and cognitive reactivity to sad mood. Additionally, the current study included a novel investigation into the acute (23-hour) effect of supplementation on these outcome measures.

The primary outcome measure of delayed recall saw no improvement following either acute or chronic probiotic supplementation, contrary to predictions. In fact, contrary to the hypotheses, treatment did not significantly predict performance on any of the outcome variables assessed in the RAVLT. Acutely, performance across several variables got worse, and this was true across both treatment conditions. This is likely due to interference from carrying out the same task with different word lists the previous day. As for the effect of chronic supplementation, no significant changes to verbal learning performance were demonstrated. Although a number of previous chronic supplementation studies report significantly improved performance on a verbal learning task following probiotic intervention, particularly in delayed and total recall (Ceccarelli et al., 2017a; Ceccarelli et al., 2017b; Rudzki et al., 2019), these improvements have been reported in middle-aged clinical populations with conditions affecting cognitive function. Additionally, in older adults with MCI, change in composite z score for verbal learning, comprising of immediate recall, delayed recall, and recognition, showed greater improvement following *Lactobacillus plantarum* compared to a placebo (Hwang et al., 2019), although this failed to reach statistical significance and the improvement in the placebo group suggests the presence of practice effects. The current findings are in line with two prior studies in young/middle-aged adults which report no improvement on verbal learning tasks (Czajeczny et al., 2021; Wallis et al., 2018),

perhaps suggesting the RAVLT is not sensitive to change in cognitive function following probiotic intervention in healthy populations, or that the scope to improve verbal learning and memory is limited in such populations.

The current data provides no evidence for an enhancement in the second primary outcome of spatial working memory, as measured by the CBTT, following probiotic intervention. In fact, this task elicited unexpected results in that accuracy in selecting the correct sequence actually improved in the placebo condition on 4 and 5-block trials to be significantly greater than that seen in the probiotic condition. Accuracy on 8-block trials was significantly better post-intervention in the probiotic condition, but this was a reflection of a fall in performance in the placebo condition, rather than an improvement following probiotic intervention. A similar pattern of results was elicited in participant ability to select the correct blocks, but in the incorrect order. Type 1 error due to a high number of comparisons is a possibility, but since the comparisons were Bonferroni corrected and the same pattern of results was evident across both correct sequence and correct block outcome measures, this is perhaps unlikely. As such, the results remain challenging to interpret, but provide no evidence for a beneficial effect of the probiotics used in spatial working memory.

Despite no evidence for an effect on either primary outcome measure, there does appear to be an interaction between the probiotic treatment and performance in the TST. Unexpectedly, accuracy on trials requiring participants to decide whether the number was higher or lower than five was significantly poorer than trials requiring a decision on whether the number was odd or even, suggesting high/low trials provided higher cognitive load. One possible explanation for high/low trials being more demanding is that they required a quick decision to be made relative to a target number (number five), whereas numbers are learned as odd or even, meaning no additional mental process was required on odd/even trials. This effect of cognitive load was attenuated following the probiotic intervention, and performance improved, albeit non-significantly, between baseline and post-intervention, meaning accuracy was higher on average in high/low trials than odd/even trials by the end of the trial. However, this attenuation in the effect of cognitive load appears to come at the expense of odd/even trials, where accuracy post-intervention was significantly lower in the probiotic condition than in the placebo. With that said, performance on this task was towards ceiling, leaving little scope for an improvement in performance. As such, a relative improvement in executive function on higher cognitive load trials and concurrent fall in accuracy on lower cognitive load trials, although interesting, is arguably negligible in terms of benefit to the individual. This also highlights that the TST was likely not demanding enough to explore the full extent of a probiotic effect on task switching.

In terms of RT on the TST, general RT, as well as RT on higher cognitive load trials (switch trials and high/low trials), was significantly faster in the probiotic condition compared to the placebo condition at the acute timepoint. Improved RT occurred across treatments, which is likely a practice effect from completing the task the day before, reflecting improved confidence in response keys and task requirements. While practice effects make the results more challenging to interpret, the reduction in RT was significantly greater in the probiotic condition compared to placebo, suggesting a potential benefit to RT of acute probiotic supplement.

In addition to cognitive function, the present study explored the potential to improve mood and mental wellbeing via probiotic supplementation. Probiotic intervention was associated with a significant reduction in hopelessness and rumination following 8 weeks of probiotic supplementation, but not placebo, as measured by the LEIDS-R. The same pattern of results was also evident in the aggression subscale, but for aggression baseline scores in the probiotic arm were significantly higher, so this reduction should be interpreted with caution. These results indicate that, when in a sad mood, participants were less consumed with aggressive, hopeless, and ruminative thoughts when taking the probiotic supplement compared to the placebo. Interestingly, similar findings were reported in two prior studies utilising Ecologic Barrier as a chronic probiotic intervention. The first assessed cognitive reactivity to sad mood using the LEIDS-R in healthy young adults with no evidence of depression or anxiety (Steenbergen et al., 2015). Here, participants randomised to receive the probiotic showed marked reductions in total LEIDS-R score and rumination and aggression subscales. It is unclear whether post-intervention scores in these outcome variables were significantly different to that of those who were randomised to the placebo condition, but those taking the placebo showed comparable scores pre- and post-intervention. The second study enrolled middle-aged volunteers with self-reported depressive symptoms, as indicated by a Beck Depression Index score of 12 or higher, and found a significant reduction in LEIDS-R total cognitive reactivity score in those with mild to moderate depression scores compared to a placebo (Chahwan et al., 2019). Average total LEIDS-R scores were higher at baseline in both previous studies (approximately 44 and 65, respectively) compared to the current study, where average total score at baseline was 28. Replication of these results within a healthy older adult cohort provides further support for an effect of Ecologic Barrier in reducing reactivity to sad mood and therefore susceptibility to depression, which is particularly important within the ageing population. The fact that cognitive reactivity scores were lower at baseline in this population than in the previous studies aligns with the generally low scores demonstrated across the other mood measures included here, and may explain why treatment did not significantly predict LEIDS-R total score. The present results therefore highlight that this multispecies probiotic may be beneficial in reducing cognitive biases that increase risk of depression, even in those who experience lower levels of cognitive reactivity to sad mood. These results also highlight that the LEIDS-R was sensitive to change in reactivity

to sad mood in a population who generally reported low levels of depression and evidenced no significant effect of probiotic intervention on depressive symptomology. As such, it may be a useful tool for assessing reactivity to sad mood, and therefore risk of depression, in sub-clinical populations who may be more vulnerable to depression, which is important for the prevention of mental health disorders.

16s sequencing highlighted a significant increase in *Lactococcus* following chronic probiotic intervention which was not evident following the placebo. These results compliment the *in vitro* work in chapter four, where daily addition of the same multi-species supplement to continuous culture models resulted in increased relative abundance of *Lactococcus*. The current data provides evidence that this probiotic supplement is successfully reaching the gut, and the increase in relative abundance of *Lactococcus* may reflect that present in the supplement, which is perhaps easier to detect than change in *Lactobacillus* or *Bifidobacterium* due to lower starting levels, or may suggest that probiotic bacteria interact with the enteric microbiota to support the growth of *Lactococcus* species. It is unclear whether this shift would be behaviourally relevant and result in measurable change in health parameters and neural function in healthy older adults. However, *Lactococcus* is typically low in relative abundance and yet is proposed to play a significant role in host immunity through enhanced response to pathogenic bacteria, inhibition of inflammatory cytokines and stimulation of gut mucosal immunity (Santibañez et al., 2021; Han et al., 2015; Yu et al., 2021). As such, supporting the abundance of *Lactococcus* may in turn have neuroprotective effect in older adults.

Exploratory correlation analysis highlighted significant but weak correlations between the relative abundance of *Bifidobacterium* and performance on the more cognitively demanding elements of the TST, including accuracy on high/low trials RT on switch trials. Limited studies perform 16s after probiotic supplementation, so although cognitive performance is reported to improve following *Bifidobacterium* supplements, it is unclear whether this is reflected in changes relative abundance, and whether this correlates with change in cog function. Given that the correlation is weak, this finding requires replication before exploring further.

One of the core considerations of this work was to address frequent limitations in this field of research that were highlighted in the literature review in chapter two, such as poor randomisation, practice effects, and lack of regard for potential confounds such as diet and time-of-day effects. The relative strengths of this work therefore include proper randomisation in a well-controlled, double-blind trial, mitigation of practice effects through practice sessions and alternative task versions, and control of diet in the acute period prior to testing. However, this work is not without limitations. The combination of attrition and missing data mean this work is likely underpowered to see an effect of probiotic

intervention, which should be taken into consideration when interpreting the results. Although efforts were made in line with previous recommendations to attenuate learning effects, some evidence of practice remained at the acute timepoint, particularly in the executive function tasks. Although research suggests that practice effects cannot be removed entirely (Bell et al., 2018), this makes interpretation of results more difficult and remain a challenge in this field of work. Additionally, it should be noted that beneficial effects of the probiotic were evident among a number of placebo effects, particularly in the CBTT. Spurious results can arise across treatments when utilising assessment batteries with multiple cognitive and mood measures as, although attenuated through randomisation procedures and robust statistical modelling, this increases the likelihood of type 1 error. Sufficiently powered trials and replication of results is therefore important in interpreting the effects of probiotic intervention. Use of a cognitive task battery that generates multiple metrics per task may also pose an issue in that the matrices are often highly correlated. In such cases, Bonferroni correction may not be appropriate, and may not address the problem of type 1 error due to potential correlation across different tasks.

To conclude, chronic multi-species probiotic supplementation with Ecologic® Barrier may improve executive function under higher levels of cognitive demand in healthy older adults, in addition to reducing cognitive biases associated with cognitive reactivity to sad mood and therefore vulnerability to depression. Novel acute supplementation may be associated with quicker reaction times during executive function, although practice effects make this challenging to interpret in the current data. 16s sequencing indicates that the probiotic bacteria are successfully able to reach the colon when administered as a dietary supplement, and consistent evidence across the *in vitro* and *in vivo* experiments suggests this particular supplement may support the growth *Lactococcus* species.

Table 5.8 – Baseline FFQ data

Item	M	SD
Nutrient		
Alcohol (g)	10.47	8.31
Alpha carotene (mcg)	606.06	540.06
Beta carotene (mcg)	407.29	2674.16
Calcium (mg)	967.58	374.19
Carbohydrate – fructose (g)	23.68	8.99
Carbohydrate – galactose (g)	0.82	0.58
Carbohydrate – glucose (g)	21.14	8.17
Carbohydrate – lactose (g)	16.79	7.71
Carbohydrate – starch (g)	104.27	44.64
Carbohydrate – sucrose (g)	42.35	19.84
Carbohydrate (g)	217.16	76.99
Carbohydrate sugars (total) (g)	110.66	38.15
Carotene (total) (mcg)	4630.39	2964.28
Chloride (mg)	3981.18	1365.67
Cholesterol (mg)	278.23	94.24
Copper (mg)	1.32	0.49
Englyst fibre – non starch polysaccharides (g)	20.71	7.81
Energy (kcal)	1937.91	666.89
Energy (kj)	8145.24	2792.43
Fat (g)	81.22	35.83
Folate (mcg)	349.83	126.93
Iron (mg)	12.61	4.30
Magnesium (mg)	363.73	119.40
Manganese (mg)	4.21	1.78
Monounsaturated fatty acids (g)	30.27	15.76
Niacin (mg)	24.00	7.50
Nitrogen (g)	13.21	4.31
Phosphorus (mg)	1474.04	474.94
Potassium (mg)	3922.52	1024.89
Protein (g)	81.62	26.32
Polyunsaturatd fatty acids (g)	13.04	6.31
Selenium (mcg)	61.72	22.72
Saturated fatty acids (g)	31.00	13.93
Sodium (mg)	2619.84	905.38
Vitamin A retinol (mcg)	604.16	483.04
Vitamin A retinal equivalents (mcg)	1381.51	699.50
Vitamin B1 (mg)	1.58	0.50
Vitamin B12 (mcg)	6.33	3.13
Vitamin B6 (mg)	2.41	0.79
Vitamin C (mg)	137.97	57.23
Vitamin D (mcg)	2.93	1.54
Vitamin E (mg)	12.78	5.32
Zinc (mg)	9.58	3.23

Food categories (g)		
Alcoholic beverage	138.38	124.20
Cereals	225.33	110.11
Eggs	21.07	11.10
Fats (oils)	22.74	14.85
Fish	42.44	29.24
Fruit	270.63	134.13
Meat	83.32	62.77
Milk	354.43	166.22
Nuts/seeds	24.09	39.88
Potatoes	84.15	47.18
Soups/sauces	65.26	39.30
Added sugars	34.78	25.23
Vegetables	341.13	218.76

Chapter 6 - Final discussion

The aims of this thesis were: 1) to systematically review the existing literature exploring how probiotic supplementation may influence cognitive function 2) to assess whether microbes produce neurotransmitters under physiologically relevant conditions *in vitro*, 3) explore how probiotic bacteria might influence the production of these neuroactive metabolites, alongside short-chain fatty acids and the composition of the microbial community, and 4) determine whether a multispecies probiotic supplement beneficially affects cognitive function and mood in healthy older adults.

6.1 Assessing microbial production of neurotransmitters and exploring how probiotic bacteria might influence the production of neuroactive metabolites and microbial community

Batch culture fermentation models were initially used to assess production of a range of neurotransmitters, precursors and SCFAs under physiologically relevant conditions, and explore how the addition of 6 select probiotic strains (*Lactobacillus rhamnosus* W198, *Lactobacillus reuteri* W192, *Bacillus coagulans* W64, *Propionibacterium freudenreichii* W200, *Lactococcus lactis* W58 & *Bacillus subtilis* W201) influenced bacterial composition and metabolite production compared to the natural bacterial community. Enumeration of bacteria, short-chain fatty acids and neuroactive metabolites were analysed using Flow-FISH, GC, and LCMS/MS, respectively, at baseline and following 8 and 24 hours of fermentation. As expected, microbial production of GABA was evident, alongside lower concentrations of serotonin, dopamine, and tryptophan. No significant effect of the probiotic strains was observed on these metabolites, although trending increases in GABA production in the vessels with *Lactobacillus reuteri, Lacctococcus lactis, Lactobacillus rhamnosus*, and *Bacillus coagulans* suggest these strains may help to enhance GABA production in the faecal bacterial community. Regarding SCFAs, the addition of *Lc. lactis* and *L. rhamnosus* resulted in significantly increased concentrations of lactate after 8 hours of fermentation. No significant shifts in microbial composition were evidenced in the probiotic vessels compared with the control vessel, although visual trends in the current data suggest these probiotics may support the growth of *Bacteroides-Prevotella* spp. and *Clostridium* cluster IX.

Following successful detection of several neuroactive metabolites in the batch culture fermentation models, change in bacterial community and metabolite production following the addition of multi-strain probiotic treatment (*Bifidobacterium lactis* W51*, Bifidobacterium lactis* W52*, Lactobacillus acidophilus*

W37*, Lactobacillus salivarius* W24*, Lactobacillus casei* W56*, Bifidobacterium bifidum* W23*, Lactobacillus brevis* W63*, Lactococcus lactis* W19*, Lactococcus lactis* W58) was assessed using a more sophisticated three-stage continuous fermentation model. Here, using a more complex nutritional input, observations at the first equilibrium phase could be compared to the second, post-treatment equilibrium across vessels mimicking the proximal, transverse, and distal regions of the colon. In these experiments, GABA, dopamine, kynurenic acid, tryptophan, and norepinephrine were detected pre- and post-treatment. Daily feeding of the probiotic supplement did not result in any statistically significant changes to neuroactive metabolite synthesis, but a trending significant increase in valerate production was observed in the distal vessel. In terms of bacterial community, increases in the *Roseburia* and *Lactococcus,* specifically in the subspecies *Lactoccocus lactis* ssp *hordinae,* were observed in the continuous culture experiments.

Together, the data from these *in vitro* experiments provide evidence for the bacterial derivation of several neurotransmitters under physiologically relevant conditions, in the absence of colonic cells, with standard nutrient input. As hypothesised, GABA was measured at relatively high concentrations compared to the other metabolites in both fermentation models, particularly under conditions mimicking the proximal vessel (pH 5.5). This provides further support for *in silico* work proposing that microbes found enterically encode for the glutamic acid decarboxylase gene (Valles-Colomer et al., 2019), and supports previous *in vitro* work evidencing the production of GABA in pure cultures (Das & Goyal., 2015; Otaru et al., 2021). Since the present work was conducted, a more recent study assessed the production of GABA by several lactic acid producing strains in pure culture, showed that *Levilacobacillus brevis* LB01 and *Lactiplantibacillus plantarum* 299v were the most efficient producers of GABA in a culture medium enriched with monosodium glutamate (MSG) (Monteagudo-Mera et al., 2022). Subsequently, the authors utilised a batch culture fermentation system similar to that described in chapter 3 (but using MSG enriched media) to assess conversion of glutamate to GABA in the faecal microbiota of four healthy middle-aged male donors following the addition of each probiotic strain. Substantially higher concentrations of GABA were measured in the vessel with *L. brevis* compared with *L. plantarum* and the negative control. For comparison, quantity of GABA produced by *L. brevis* was approximately 10x higher than that found in the probiotic vessels in the current batch culture fermentations, although that is to be expected given the use of MSG enriched media. As with the current data, GABA synthesis was highest at a pH of 5.4 – 5.6, which is in line with previous work indicating that the glutamic acid decarboxylase (GAD) pathway is activated as a glutamate-dependent acid resistance mechanism under low pH (Dhakal et al., 2012). Such conditions are found in the proximal region of the human colon, which is innervated by vagal afferent fibres (Wang & Powley., 2007) expressing $GABA_A$ and $GABA_B$ receptors, providing a potential mechanism for gut-derived GABA to influence the central nervous system. Although further work is required to elucidate the precise

mechanisms via which microbially derived GABA may influence the brain, the current data provides further support for the production of GABA under conditions relevant to the large intestine, without the enrichment of precursors or prebiotic fibre, and suggests that GABA synthesised by bacteria in the gut may represent a significant gut-brain axis pathway.

Several other neurotransmitters and neuroactive metabolites were detected across the two *in vitro* experiments, but at substantially lower concentrations than GABA. The majority of *in vitro* work to date has assessed microbial production of these metabolites under conditions engineered to optimise production, typically adding vast quantities of precursors to media and working under physiologically irrelevant temperature and pH (Villegas et al., 2016; Li & Cao., 2010; Özoğul et al., 2012). However, the detection of these metabolites in the present work designed to mimic physiologically relevant conditions is novel and suggests bacterial derivation of these compounds could occur *in vivo*. With that said, the low concentrations detected may indicate that bacterial derivation does not provide a primary production pathway, and instead interactions between microbial metabolites and host cells are required. For example, while microbes may directly produce serotonin, microbially derived SCFAs are thought to influence the transcription of tryptophan hydroxylase 1 enzyme in enterochromaffin cells, which is necessary for the conversion of dietary and microbially derived tryptophan to serotonin in much greater quantities (Reigstad et al., 2015). Additionally, metabolites produced in the gut may exert beneficial effects on neural function and behaviour indirectly through microbe-host interactions. For example, microbially derived kynurenic acid may exert mucosal protective and immunoregulatory effects via G protein coupled receptors expressed in epithelial cells (Gao et al., 2018), which in turn may be neuroprotective (Gwak & Chang., 2021).

The current work did not provide evidence for a significant effect of probiotic bacteria on the production of neuroactive metabolites assessed, both when administered as individual strains or as a combined multi-species supplement. Trends within the batch culture data suggest a few of the selected strains may have potential to enhance GABA production in the microbial community under conditions relevant to the proximal colon, and, although concentration of GABA did increase in the proximal vessel in the gut model experiments, this once again failed to reach significance. Additionally, probiotic administration did not impact the production of any of the other detected metabolites *in vitro*. Given that effects on cognitive outcomes are reported following at least 4 weeks of probiotic supplementation, it may be argued that 16 days of probiotic administration between the model equilibrium states is perhaps not sufficient time to evidence metabolite changes that may be implicated in these improvements in cognitive function. However, it may also be argued that the model has reached steady state at this point, and therefore wouldn't alter after this state is reached. It should also

be acknowledged that, although these models have their strengths, without the inclusion of colonic cells we do not get the full picture.

Based on findings in previous supplementation studies (Wang et al., 2014; Kim et al., 2015) it was anticipated that the addition of probiotic bacteria to the faecal microbiota community may increase the synthesis of SCFAs, particularly acetate, propionate, and butyrate, but this was not supported by the current data. SCFAs are produced as a product of non-digestible fibre fermentation, suggesting that the introduction of a relatively small number of microbes to an existing community may not influence SCFAs without concurrently changing the availability of non-digestible fibres. Having said this, addition of *Lc. lactis* and *L. rhamnosus* led to a significant increase in lactate after 8 hours of fermentation, which was not evident in the control vessel. Lactate was not detected in the gut models in any of the modelled regions, likely due to utilisation for SCFAs, so this finding cannot be compared across the *in vitro* experiments. However, an increase in valerate was evident in the distal vessel at the second equilibrium following daily probiotic feeding, which was trending towards significance. Valerate is produced in smaller quantities compared with other SCFAs and is readily absorbed from the gut lumen, meaning it is difficult to measure *in vivo*. This highlights the benefit of using *in vitro* fermentation, which allows the production of less dominant metabolites to be better mapped. Valerate may play an important role in gut barrier integrity (Gao et al., 2022) and elicit a neuroprotective effect against inflammatory cytokines (Dulla et al., 2022), suggesting that enhancing microbial production of valerate may be beneficial for neural function via various pathways.

Fluorescence in situ hybridisation with flow cytometry (Flow-FISH) was selected as an appropriate method of bacterial enumeration in the *in vitro* experiments (chapters 3 & 4), as it allows for quantification of functional microbial groups of interest. Flow-FISH enables a quantitative assessment of how the addition of probiotic bacteria impacts these functional microbial groups. In an *in vitro* setting, this provides sufficient evidence to determine if the treatment is likely to positively or negatively influence the microbial community. In contrast, and to provide further information in the randomised, placebo controlled human trial, 16s rRNA sequencing was selected (chapter 5). The reason for this is because sequencing provides more information about the changes occurring, detailing relative abundance of the full microbiome down to the level of species, and in some cases strain, giving a broader overview of the microbial community and providing a more complete picture than Flow-FISH data. It was decided that this level of detail was appropriate for a human study to determine exactly where changes were occurring. Each technique presents different limitations. For example, Flow-FISH is a quantitative approach that actually determines the number of cells present in a sample, but it can be limited by the probes being used not providing complete coverage of the microbial community. In contrast, 16s sequencing provides a fuller picture, but may miss bacteria that are very low in relative

abundance. Multiple copies of DNA also means abundance data does not quantitatively relate to bacterial numbers. However, for an intervention study, to provide a fuller picture of which bacteria were altered due to the intervention, the sequencing approach was selected. Given that measurable shifts in gut microbiota community are not always reported alongside behavioural change and the interaction between probiotic bacteria and commensal microbes is likely of greater importance (Sandler et al., 2018), changes in the bacterial community following the addition of probiotic bacteria were not necessarily expected, particularly in the faecal microbiota of healthy young adults such as that utilised in the batch culture experiments. However, the addition of the multispecies probiotic supplement did result in a shift in *Roseburia* and significant increase in *Lactococcus* in faecal microbiota of healthy older adults. Maintenance of *Roseburia* species has been associated with healthier ageing (Claesson et al., 2012) and promotion of *Roseburia*, along with other butyrate producing bacteria, through a whole-diet approach has been associated with reduced incidence of frailty and reduced cognitive decline (Ghosh et al., 2020). Additionally, *Lactococcus* species may be particularly important for stimulation of ileal mucosal immunity (Yu et al., 2021) and therefore play into immune gut-brain pathways.

6.2 Exploring the effect of a multispecies probiotic supplement on cognition and mood in healthy older adults

To address the final aim of the thesis, a randomised, placebo-controlled cross-over trial in 30 healthy older adults was employed to explore both the acute (1 day) and chronic (8 weeks) effects of a multispecies probiotic supplement (Ecologic® Barrier) on primary outcome measures of verbal memory and learning (Ray Auditory Verbal Learning Task) and spatial working memory (Corsi Block Tapping Task), and secondary cognitive and mood outcomes, including executive functions, perceived stress, anxiety, depression, and cognitive reactivity to sad mood. 16s rRNA sequencing of stool samples was also performed pre- and post-intervention to assess potential effects on the gut microbiota community. Chronic probiotic supplementation was associated with the attenuation of poorer executive function during higher cognitive demand, and improvement in cognitive biases such as hopelessness, rumination and aggression that contribute to reactivity to sad mood and therefore vulnerability to depression. Novel acute probiotic supplementation was associated with significantly faster reaction times on tasks targeting executive function, particularly on higher cognitive load trials. In addition, significantly higher relative abundance of the *Lactococcus* genus was found following chronic probiotic supplementation compared to the placebo.

Contrary to expectations, no beneficial effect on either primary outcome measure was observed. The primary outcome measures were selected based on previous literature, where improvements in verbal learning and memory were observed in older adults with mild cognitive impairment and Alzheimer's disease (Ton et al., 2020; Xiao et al., 2020; Kobayashi et al., 2019b), and spatial working memory was consistently improved when measured (Ceccarelli et al., 2017a; Ceccarelli et al., 2017b; Ton et al., 2020; Xiao et al., 2020). Decline in these domains is also common in ageing (Harada et al., 2013). However, since baseline cognition scores were within the healthy ageing range for all participants, there was perhaps less scope for an improvement in memory domains in the current population, unlike in individuals with age-related disorders. Despite no effect on the primary outcome measures, some limited evidence for an effect on executive function, specifically on cognitive flexibility, was observed. In the Switching task, subjects demonstrated poorer accuracy on high/low trials compared to odd/even trials, suggesting the high/low trials were found to be more cognitively demanding. While this differentiation between trial type persisted at the acute and chronic timepoints when consuming the placebo, subjects demonstrated a shift in performance while taking the probiotic supplement, such that trial type no longer significantly predicted cognitive performance and participants performed equally across trial types. While this improvement in accuracy following probiotic intervention on the more cognitively demanding trials did not reach statistical significance, the current data illustrates a clear interaction between probiotic treatment and performance which suggests that the probiotic supplement may improve executive function under high cognitive load. A comparison may be drawn between the current data and previous work exploring the effect of probiotic supplementation on cognition under conditions of acute stress, where tasks place higher cognitive demand on the individual than they otherwise would due to cognitive resources being dampened by stress (Shields, Sazma & Yonelinas., 2016). For example, following 4 weeks of supplementation with Ecologic® Barrier, Papalini and colleagues (2019) found a buffering effect of the probiotic against stress-induced decline in working memory in young adults, where working memory performance after an acute stressor was maintained following probiotic intervention but declined following the placebo. Importantly, this beneficial effect of supplementation was only found under conditions of stress, as probiotics did not improve working memory under normal conditions. Interestingly, similar findings have consistently been reported following other dietary interventions such as polyphenol-rich foods, where significant beneficial effects of treatment were only found under conditions of cognitive fatigue or more challenging executive function tasks that elicit higher cognitive demand (Scholey et al. 2010; Miller at al., 2018; Whyte, Schafter & Williams., 2017). As such, these behavioural results may highlight the need for more cognitively demanding tasks in order to be sensitive to a potential effect of probiotic function in healthy older adults, and cognitive demand should therefore be carefully considered in future research with this population.

An effect on reaction times (RTs) in the Switching task was also evident following acute probiotic supplementation, where general RTs across all trials were significant quicker following probiotic supplementation compared to the placebo. Additionally, in the more cognitively demanding switch and high/low trials, performance at baseline did not differ between treatments, but at the acute timepoint RTs were significantly quicker after a single dose of the probiotic intervention relative to the placebo. However, RTs significantly improved between baseline and acute sessions in both the probiotic and placebo conditions, so although the improvement was significantly greater following probiotic, this indicates an element of practice. The same pattern of results was evident in the less cognitively demanding non-switch and odd/even trails, but here RT in the probiotic condition was also significantly faster at baseline. As such, these results are challenging to interpret, and the potential beneficial effect of acute probiotic supplementation should be interpreted with caution. Although extensive efforts were made to reduce practice effects in line with recommendations in the literature, it is not uncommon for these effects to persist over an acute timeframe, particularly in more complex tasks of executive function (Bell et al., 2018). As such, learning effects remain a challenge to be considered in future work.

In addition to consistent evidence for an effect of probiotics under conditions of heightened cognitive demand, the beneficial effects of probiotic supplementation on cognitive reactivity to sad mood demonstrated in the current study are aligned with that previously described in younger, non-clinical populations. Three previous studies utilising Ecologic® Barrier as a probiotic intervention have included the LEIDS-R as a measure of cognitive reactivity. Improvement in rumination, hopelessness, and aggression sub-scales of the LEIDS-R, as well as total score, was reported in two of these studies when supplementing young healthy adults (Steenbergen et al., 2015) and middle-aged adults with self-reported low mood (Chahwan et al., 2019). In both studies, participants were supplemented with double the quantity of Ecologic® Barrier provided in the present study ($1x10^{10}$ CFU/day compared to $5x10^{9}$ CFU/day) for four and eight weeks, respectively. In the third, however, no effect of probiotic supplementation was found on reactivity to sad mood in healthy young adults (Papalini et al., 2019). Bagga and colleagues (2018) also utilised the LEIDS-R to assess cognitive reactivity in healthy young-middle/aged adults following 4 weeks of a similar multispecies supplement, containing strains from the *Bifidobacterium*, *Lactobacillus* and *Lactococcus* genera, and found a significant benefit to the hopelessness and risk aversion subscales of the LEIDS-R but no effect on total score. As such, the current data replicates previous findings of reduced cognitive biases for rumination, hopelessness, and aggression in a healthy older adult population, and adds to a growing evidence base for a beneficial effect of multispecies probiotic supplementation on cognitive reactivity to sad mood. These findings therefore provide further support that probiotic supplementation may influence cognitive mechanisms associated with vulnerability to depression.

Four other RCTs have explored the effect of probiotic supplements in healthy older adults to date, although a number of methodological issues are evident, such as combined probiotic intervention with resistance training (Inoue et al., 2018) and utilisation of dementia screening tools as cognitive assessments despite recruiting healthy adult participants (Kim et al., 2021). In the most robust study to date, Chung and colleagues (2014) report no effect of probiotic intervention on cognitive tasks administered individually, including a digit span task, story recall test and verbal learning task, but did find an improvement in response accuracy during rapid visual information processing (sustained attention/working memory) and a classic Stroop task (executive function) when these tasks were administered 4 times consecutively as part of a cognitive fatigue battery specifically designed to be cognitively demanding and induce mental fatigue. Once again, these findings provide further evidence that probiotic supplementation may be beneficial in healthy adults, including older adults, under cognitively demanding conditions, and highlight that executive function rather than memory may be more sensitive to probiotic intervention in healthy older adults.

A number of potential mechanisms may underpin the behavioural effects of probiotoc supplementation demonstrated in the present work. The present RCT incorporated 16s rRNA sequencing to capture any shifts in taxonomic community which may allude to potential mechanisms of action behind behavioural effects. Relative abundance of *Lactococcus* was significantly higher following probiotic intervention relative to the placebo, which is consistent with findings from the continuous fermentation models following the addition of the same multispecies supplement. The *Lactococcus* genus represents a relatively small proportion of the human GM, but is proposed to play a significant role in host immunity through enhanced response to pathogenic bacteria, inhibition of inflammatory cytokines and stimulation of gut mucosal immunity (Santibañez et al., 2021; Han et al., 2015; Yu et al., 2021) which may in turn have neuroprotective effect on the host. To date there is limited research as to the possible role of *Lactococcus* in cognitive function. In a *Caenorhabditis elegans* (nematode) model of ageing, recent research reported increased host defence to *Salmonella* or *Staphylococcus* infection, improved epithelial barrier function, and an amelioration of cognitive decline naturally occurring with age following *Lactococcus lactis* spp. *cremoris* (Komura et al., 2021). These effects were not demonstrated in mutated animals with defects to the transcription factor SKN-1, which is equivalent to the Nrf-2 transcription factor in humans and associated with protection against oxidative damage in cells (Ma., 2013). Although based on limited evidence in an animal model, these findings suggest that *Lactococcus* species may benefit the host by reducing incidence of oxidative stress, which has been implicated in neurodegenerative diseases such as MCI, AD and Parkinson’s (Kim et al., 2015) and decline in executive function in healthy ageing (Hajjar et al., 2018).

Changes to brain derived neurotropic factor (BDNF) may provide another plausible mechanism behind improvement in executive function. BDNF is neuroprotective and essential for synaptic plasticity (Miranda et al., 2019). It is therefore integral to cognitive functions including learning and memory and executive, and, unsurprisingly, decreased levels of BDNF are associated with multiple neurodegenerative diseases (Bathina & Das., 2015). A role for the GM in mediating BDNF is supported by studies in germ-free mice, who evidence reduced expression of BDNF in the brain, particularly in the hippocampus (Sudo et al., 2004; Heijtz et al., 2011), although precise mechanisms remain unclear. In support of this theory, Kim et al (2021) report a significant increase in serum BDNF following probiotic supplementation in healthy older adults in tandem with improved scores on the cognitive flexibility subtest on the CERAD-K. However, an improvement in cognitive function is not always associated with increased BDNF following probiotic supplementation (Chung et al., 2014), highlighting that mediation of BDNF may provide just one possible mechanism of action.

Cognitive reactivity to sad mood has been associated with serotonin, such that individuals with a genetic polymorphism affecting serotonin transportation (5-HTTLPR) evidence increased cognitive reactivity to sad mood (Wells, Beevers & McGeary., 2010). Interestingly, this was compounded in individuals who also presented a common polymorphism in the BDNF gene (Val66Met). Further support for an association between cognitive reactivity with serotonin is provided by Booji and Van der Does (2007), who found that individuals with higher cognitive reactivity scores were more sensitive to the effects of a 24-hour tryptophan depletion protocol, which reduces serum tryptophan concentrations by approximately 90%, and thus serotonin availability, as indicated by higher depression scores. As such, it is plausible that microbial pathways affecting serotonin availability may offer a biological mechanism, such as the promotion of transcription for tryptophan hydroxylase (TPH1) in enterochromaffin cells, which in turn influences circulating serotonin (Reigstad et al., 2015; Yano et al., 2015). Of course, several other possibilities including altered GABA synthesis, improved gut and blood brain barrier integrity and vagal interactions remain viable, as outlined in chapter one.

Although the beneficial effect of probiotic supplementation was limited to two outcome measures within a larger battery and did not affect the primary outcome measures, the effects seen on executive function and cognitive reactivity in the present study are in line with those previously reported in the literature. Consideration was taken when designing the trial to ensure that it addressed many of the limitations in existing research, such as mitigating practice effects via practice sessions and alternative task versions, counterbalancing of alternate task versions, proper randomisation procedure, and controlling for time-of-day and acute dietary effects on cognition. As such, this RCT adds to the limited pool of research investigating the potential for improved cognition in healthy older adults following probiotic intervention, and provides evidence that supplementation with a multispecies probiotic may

improve reaction times, cognitive reactivity to sad mood, and executive function under cognitively demanding conditions.

6.3 Limitations

6.3.1 Limitations of *in vitro* models

While *in vitro* models such as the batch culture fermentation and continuous culture models utilised in the present work provide valuable data regarding the microbial production of metabolites, lack of human tissue absorption or interaction is a clear limitation as it is not possible to explore how metabolites subsequently interact with human tissues to influence potential GBA pathways. However, given that the aim of this work was to assess microbial production of neuroactive compounds under controlled conditions, the selected fermentation models were appropriate.

It is also important to acknowledge that, while faecal microbiota currently provide a standard proxy for the GM, the microbial content of faeces may not accurately represent the GM, as faecal samples do not capture the microbial community within the gut mucosa which are important to consider, particularly in immune pathways (Tang et al., 2020). Additionally, microbiota is not equally distributed within a faecal samples (Swidsinski et al., 2008), which could lead to inaccurate representation depending on sampling methods. Efforts were made to homogenise samples prior to aliquoting and storage in line with recommendations (Hsieh et al., 2016), although there is debate over whether this is best practise (Swidsinski et al., 2008). Finally, while efforts were made preserve the faecal microbiota for the human trial by using anaerobic sachets and storing at -80°C at the earliest possibility, immediate storage following collection was not possible, and therefore degradation of bacteria and possible overgrowth due to aerobic conditions are a possibility.

6.3.2 Limitations of the behavioural trial

6.3.2.1 Cognitive tasks

It should be noted that although cognitive effects relating to reaction time and executive function were evident, these significant outcomes relate only to performance on those specific tasks. It is therefore not valid to generalise these effects to other related tasks or domains. It also remains unclear at

present how improvement within these tasks could have clinical application and manifest in day-to-day life for participants.

6.3.2.2 Ceiling effects

Participants performed near ceiling on both the Task Switching Task (TST) and Go/No-Go task. This is problematic in nutritional cognition research as high scores leaves little scope for improvement, and suggests the tasks are not sufficiently demanding to be sensitive to a potential effect of intervention. Although this version of the TST has been used in this population previously without evidence of ceiling effects (Miller et al., 2018) and the Go/No-Go task was piloted prior to recruitment in 3 healthy older adults to ensure individuals were not performing at ceiling, the final cohort of participants performed well. Ceiling effects may be avoided in future work by refining recruitment to include only those at the lower end of healthy cognition, as defined by a measure such as the Montreal Cognitive Assessment, or by including cognitive tasks where the cognitive load can be systematically increased, in order to create sufficient cognitive demand.

6.3.3 Practice effects

Although the design of the study was tailored to minimise practice effects in the cognitive task battery based on previous recommendation (Bell et al., 2018; Goldberg et al., 2015), evidence of practice was still apparent in reaction times on the TST and accuracy in the Go/No-Go task, where performance was improved across treatments in the acute session compared to baseline. Such practice effects hinder interpretation of results and may increase variance within each treatment condition, influencing the statistical power to detect small changes in cognition following the active intervention, particularly when using conservative models such as LMMs. Practice presents a particular issue in older adult populations as it may occlude subtle declines in cognition, which reduces the ability to detect a protective effect of intervention on cognition against a placebo. However, research suggests that it is not possible to completely remove practice effects, particularly in serial testing over short periods (Bell et al., 2018). As such, it remains a challenge in this field of work, and replication od results is important to reduce inaccurate interpretation of practice as improvement (type 1 error), or masking efficacy of an intervention (type 2 error).

6.3.4 Attrition and missing data

Three participants were lost after the initial screening session, and a fourth declined to participate after completing the first arm. Although the number of participants recruited accounted for a 10% attrition

rate to meet the aim of 30 participants, as indicated was necessary for sufficient statistical power, attrition of the fourth participant meant that a full dataset was acquired for only 29 subjects. In addition, technical difficulties resulted in missing data across of a number of the outcome measures. As such, it is likely that the statistical analysis was underpowered to detect an effect of probiotic intervention.

6.4 Future work

6.4.1 *in vitro* considerations

A synbiotic approach using *in vitro* and *in vivo* methodologies is essential to furthering our understanding of how the GM influences human cognition and behaviour. Since the present work indicated that direct production of the neurotransmitters other than GABA may be limited under physiological conditions, it would be beneficial for future work to assess the effect of probiotic bacteria on a broader range of metabolites. For example, the GM may mediate the availability of several precursors such as tyrosine, tryptophan, and phenylalanine (Chen et al., 2021) as opposed to the neurotransmitters themselves. In addition, modulation of the GM via probiotics may instead have a greater influence over how tryptophan is metabolised across the kynurenic, serotonin and indole pathways, so assessment of a range of metabolites across these pathways may prove more insightful (O'Mahony et al., 2015; Clarke et a., 2013). The indole pathway in particular may be of interest as this is a microbial pathway that does not require interaction with host cells, suggesting more scope for an effect of probiotic intervention which could be assessed using *in vitro* fermentation models (Gao et al., 2018). Future work assessing the activity of microbial communities from older adults may also wish to consider manipulating the nutrient contents of the media to increase protein and reduce fibre, as is commonly reported in this population (Zaragoza-Martí et al., 2020). Similar manipulation of media has been conducted recently to successfully model conditions under anorexia nervosa (Liu et al., 2022). Finally, complimentary use of *in vitro* models that include absorption, mucus, and cell lines, such as the simulator of the human intestinal microbial ecosystem (M-SHIME – Van den Abbeele et al., 2012), host-microbiota interaction (HMI – Mazorati et al., 2014) and gut-on-a-chip models (Kim et al., 2012), may provide further insight into how microbial activity identified in batch and continuous culture fermentation models may interact with host cells and thus influence potential GBA pathways.

6.4.2 Considerations for future RCTs

The observed effects on executive function in the current work, alongside results from previous studies, suggest that cognitive demand of tasks is important in assessing potential efficacy of probiotic supplements. Future work should therefore consider systematically manipulating the cognitive demand placed on participants during cognitive assessment, in order to further explore this relationship between cognitive load and performance and improve the sensitivity of tasks for detecting beneficial effect of probiotic treatment. The current data also provides evidence that a larger, well-powered replication study is warranted. In addition, the inclusion of a follow-up assessment would be useful to explore whether microbiota and behavioural changes are sustained after ceasing supplementation.

One of the core challenges in gut-brain axis research is exploring underlying mechanisms of action *in vivo* in a feasible, non-invasive manner. While the current study incorporated 16s rRNA sequencing, future work should also consider the collection of urine and blood samples. Recent work has begun to identify metabolites present in urine that are associated with cognitive status and may be used as biomarkers to distinguish individuals with AD from those with MCI and healthy controls (Yilmaz et al., 2020; Wang et al. 2022). Urinary metabolites have been shown to correlate well with peripheral metabolites and provide a useful non-invasive tool for diagnosis of various medical conditions (Kim et al., 2009; Echeverry et al., 2010; Liu et al., 2018). Metabolomics techniques such as Nuclear Magnetic Resonance (NMR) and Ultra High Performance Liquid Chromatography (UHPLC) may therefore allow changes in urinary metabolites to be assessed following probiotic intervention. Further, concentrations of relevant compounds such as BDNF, kynurenine and tryptophan can be measured in serum, providing insight into changes in circulating neuroactive metabolites following probiotic intervention. Finally, promising magnetic resonance imaging (MRI) techniques such as magnetic resonance spectroscopy (MRS), as well as Positron emission Tomography (PET), could provide powerful tools for exploring changes in the brain at a metabolite level following probiotic intervention. Mescher-Garwood point-resolved (MEGA-PRESS) and ^{1}H spectroscopy allow for estimation of neurotransmitter concentration in various regions of the brain, such as GABA (Song et al., 2020) and tryptophan (Nanga et al., 2022). Additionally, PET imaging has been used to assess neurotransmission through neuroreceptor targets across a range of neurotransmitter systems including dopamine, norepinephrine, acetylcholine, and GABA (Sandler & Hesse., 2017). Where previous work has employed functional MRI to explore changes in neural activation and structure following supplementation (Tillisch et al., 2013), incorporating these techniques into future human trials may begin to provide an insight into how the effect of probiotic bacteria on the GM may manifest at the level of neurotransmission in the human brain.

6.5 Final conclusions

The *in vitro* work carried out in this thesis provides novel evidence for the production of several neurotransmitters in faecal microbiota under conditions relevant to the human colon, but provided limited evidence for an effect of additional probiotic bacteria on microbially derived metabolites. *In vivo*, primary cognitive outcomes were not significantly affected, but chronic supplementation in healthy older adults elicited beneficial effects on cognitive reactivity to sad mood, providing further support to previous work and suggests a potential role for probiotics in the prevention of depression. Additionally, chronic supplementation was associated with potential improvement in executive function under high cognitive demand, which once again aligned with previous research and highlights that probiotics may be beneficial in attenuating decline in executive function in healthy older adults. Novel acute supplementation was associated with improved reaction times during a task of executive function. Consistent changes in the microbial community following Ecologic® barrier, both *in vitro* and *in vivo*, indicates that this combination of probiotic strains may support the growth of *Lactoccocus* species in the GM of healthy older adults, which may have implications for cognitive function via immune pathways. The work outlined in this thesis contributes to the growing body of research exploring how probiotic bacteria may provide a therapeutic tool for the attenuation of cognitive decline in healthy ageing and expands our understanding of microbial production of neurotransmitters.

References

Aarts, E., Ederveen, T. H., Naaijen, J., Zwiers, M. P., Boekhorst, J., Timmerman, H. M., ... & Arias Vasquez, A. (2017). Gut microbiome in ADHD and its relation to neural reward anticipation. *PloS one*, *12*(9), e0183509.

Abildgaard, A., Elfving, B., Hokland, M., Wegener, G., & Lund, S. (2017). Probiotic treatment reduces depressive-like behaviour in rats independently of diet. *Psychoneuroendocrinology*, *79*, 40-48.

Academy of Nutrition and Dietetics. (2016). Quality Criteria Checklist: Primary Research. *Evidence Analysis Manual: Steps in the Academy Evidence Analysis Process*.

Adikari, A. M. G. C. P., Appukutty, M., & Kuan, G. (2020). Effects of daily probiotics supplementation on anxiety induced physiological parameters among competitive football players. *Nutrients*, *12*(7), 1920.

Agahi, A., Hamidi, G. A., Daneshvar, R., Hamdieh, M., Soheili, M., Alinaghipour, A., ... & Salami, M. (2018). Does severity of Alzheimer's disease contribute to its responsiveness to modifying gut microbiota? A double blind clinical trial. *Frontiers in Neurology*, *9*, 662.

Agostoni, E., Chinnock, J. E., Daly, M. D. B., & Murray, J. (1957). Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. *The Journal of physiology*, *135*(1), 182.

Agus, A., Planchais, J., & Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell host & microbe*, *23*(6), 716-724.

Ahmed, W., & Rashid, S. (2019). Functional and therapeutic potential of inulin: A comprehensive review. *Critical reviews in food science and nutrition*, *59*(1), 1-13.

Aiello, E. N., Gramegna, C., Esposito, A., Gazzaniga, V., Zago, S., Difonzo, T., ... & Bolognini, N. (2022). The Montreal Cognitive Assessment (MoCA): updated norms and psychometric insights into adaptive testing from healthy individuals in Northern Italy. *Aging clinical and experimental research*, 1-8.

Ait-Belgnaoui, A., Colom, A., Braniste, V., Ramalho, L., Marrot, A., Cartier, C., ... & Tompkins, T. (2014). Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterology & Motility*, *26*(4), 510-520.

Akar, M., Eras, Z., Oncel, M. Y., Arayici, S., Guzoglu, N., Canpolat, F. E., ... & Oguz, S. S. (2017). Impact of oral probiotics on neurodevelopmental outcomes in preterm infants. *The Journal of Maternal-Fetal & Neonatal Medicine*, *30*(4), 411-415.

Akbari, E., Asemi, Z., Daneshvar Kakhaki, R., Bahmani, F., Kouchaki, E., Tamtaji, O. R., ... & Salami, M. (2016). Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: a randomized, double-blind and controlled trial. *Frontiers in aging neuroscience*, *8*, 256.

Akhtar, M., Naqvi, S. U. A. S., Liu, Q., Pan, H., Ma, Z., Kong, N., ... & Liu, H. (2022). Short Chain Fatty Acids (SCFAs) Are the Potential Immunomodulatory Metabolites in Controlling Staphylococcus aureus-Mediated Mastitis. *Nutrients*, *14*(18), 3687.

Akkasheh, G., Kashani-Poor, Z., Tajabadi-Ebrahimi, M., Jafari, P., Akbari, H., Taghizadeh, M., ... & Esmaillzadeh, A. (2016). Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomized, double-blind, placebo-controlled trial. *Nutrition*, *32*(3), 315-320.

Allegretti, J. R., Mullish, B. H., Kelly, C., & Fischer, M. (2019). The evolution of the use of faecal microbiota transplantation and emerging therapeutic indications. *The Lancet*, *394*(10196), 420-431.

Allen, A. P., Hutch, W., Borre, Y. E., Kennedy, P. J., Temko, A., Boylan, G., ... & Clarke, G. (2016). Bifidobacterium longum 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. *Translational psychiatry*, *6*(11), e939-e939.

Anderton, B. H. (2002). Ageing of the brain. *Mechanisms of ageing and development*, *123*(7), 811-817.

Appleton, J. (2018). The gut-brain axis: Influence of microbiota on mood and mental health. *Integrative Medicine: A Clinician's Journal*, *17*(4), 28.

Arentsen, T., Raith, H., Qian, Y., Forssberg, H., & Heijtz, R. D. (2015). Host microbiota modulates development of social preference in mice. *Microbial ecology in health and disease*, *26*(1), 29719.

Asaoka, D., Xiao, J., Takeda, T., Yanagisawa, N., Yamazaki, T., Matsubara, Y., ... & Sato, N. (2022). Effect of probiotic Bifidobacterium breve in improving cognitive function and preventing brain atrophy in older patients with suspected mild cognitive impairment: results of a 24-week randomized, double-blind, placebo-controlled trial. *Journal of Alzheimer's Disease*, (Preprint), 1-21.

Bagga, D., Reichert, J. L., Koschutnig, K., Aigner, C. S., Holzer, P., Koskinen, K., ... & Schöpf, V. (2018). Probiotics drive gut microbiome triggering emotional brain signatures. *Gut microbes*, *9*(6), 486-496.

Bajaj, J. S., Heuman, D. M., Hylemon, P. B., Sanyal, A. J., Puri, P., Sterling, R. K., ... & Gillevet, P. M. (2014). Randomised clinical trial: Lactobacillus GG modulates gut microbiome, metabolome and endotoxemia in patients with cirrhosis. *Alimentary pharmacology & therapeutics*, *39*(10), 1113-1125.

Barbato, G., Barini, E. M., Genta, G., & Levi, R. (2011). Features and performance of some outlier detection methods. *Journal of Applied Statistics*, *38*(10), 2133-2149.

Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F., & Stanton, C. (2012). γ-Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of applied microbiology*, *113*(2), 411-417.

Bates, D. M. B. S., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. J Stat Softw.

Bathina, S., & Das, U. N. (2015). Brain-derived neurotrophic factor and its clinical implications. *Archives of Medical Science*, *11*(6), 1164-1178.

Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C., & Schmidt, T. M. (2019). Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. *MBio*, *10*(1), e02566-18.

Bell, L., Lamport, D. J., Field, D. T., Butler, L. T., & Williams, C. M. (2018). Practice effects in nutrition intervention studies with repeated cognitive testing. *Nutrition and healthy aging*, *4*(4), 309-322.

Ben-Menachem, E., Hamberger, A., Hedner, T., Hammond, E. J., Uthman, B. M., Slater, J., ... & Wilder, B. J. (1995). Effects of vagus nerve stimulation on amino acids and other metabolites in the CSF of patients with partial seizures. Epilepsy research, 20(3), 221-227.

Benton, D., Williams, C., & Brown, A. (2007). Impact of consuming a milk drink containing a probiotic on mood and cognition. *European journal of clinical nutrition*, *61*(3), 355-361.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., ... & Collins, S. M. (2011). The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology, 141(2), 599-609.

Bercik, P., & Collins, S. M. (2014). The effects of inflammation, infection and antibiotics on the microbiota-gut-brain axis. *microbial endocrinology: the microbiota-gut-brain axis in health and disease*, 279-289.

Bergua, V., Meillon, C., Potvin, O., Ritchie, K., Tzourio, C., Bouisson, J., ... & Amieva, H. (2016). Short STAI-Y anxiety scales: validation and normative data for elderly subjects. *Aging & mental health*, *20*(9),
987-995.

Biagi, E., Rampelli, S., Turroni, S., Quercia, S., Candela, M., & Brigidi, P. (2017). The gut microbiota of centenarians: signatures of longevity in the gut microbiota profile. *Mechanisms of ageing and development*, *165*, 180-184.

Bingham, S. A., Gill, C., Welch, A., Cassidy, A., Runswick, S. A., Oakes, S., ... & Day, N. E. (1997). Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers. *International journal of epidemiology*, *26*(suppl_1), S137.

Bloemendaal, M., Szopinska-Tokov, J., Belzer, C., Boverhoff, D., Papalini, S., Michels, F., ... & Aarts, E. (2021). Probiotics-induced changes in gut microbial composition and its effects on cognitive performance after stress: exploratory analyses. *Translational psychiatry*, *11*(1), 300.

Booij, L., & Van der Does, A. J. (2007). Cognitive and serotonergic vulnerability to depression: convergent findings. *Journal of Abnormal psychology*, *116*(1), 86.

Boonstra, E., De Kleijn, R., Colzato, L. S., Alkemade, A., Forstmann, B. U., & Nieuwenhuis, S. (2015). Neurotransmitters as food supplements: the effects of GABA on brain and behavior. *Frontiers in psychology*, 1520.

Bouhnik, Y., Raskine, L., Simoneau, G., Vicaut, E., Neut, C., Flourié, B., ... & Bornet, F. R. (2004). The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. *The American journal of clinical nutrition*, *80*(6), 1658-1664.

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., ... & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences*, *108*(38), 16050-16055.

Browning, K. N. (2015). Role of central vagal 5-HT3 receptors in gastrointestinal physiology and pathophysiology. *Frontiers in Neuroscience*, *9*, 413.

Burke, S. N., & Barnes, C. A. (2006). Neural plasticity in the ageing brain. *Nature reviews neuroscience*, *7*(1), 30-40.

Canipe III, L. G., Sioda, M., & Cheatham, C. L. (2021). Diversity of the gut-microbiome related to cognitive behavioral outcomes in healthy older adults. *Archives of Gerontology and Geriatrics*, *96*, 104464.

Cao, G., Tao, F., Hu, Y., Li, Z., Zhang, Y., & Deng, B. (2019). Positive effects of a Clostridium butyricum-based compound probiotic on growth performance, immune responses, intestinal morphology, hypothalamic neurotransmitters, and colonic microbiota in weaned piglets. *Food & function*, *10*(5), 2926-2934.

Caspani, G., & Swann, J. (2019). Small talk: microbial metabolites involved in the signaling from microbiota to brain. *Current opinion in pharmacology*, *48*, 99-106.

Castelli, V., d'Angelo, M., Lombardi, F., Alfonsetti, M., Antonosante, A., Catanesi, M., ... & Cimini, A. (2020). Effects of the probiotic formulation SLAB51 in in vitro and in vivo Parkinson's disease models. *Aging (albany NY)*, *12*(5), 4641.

Cattaneo, A., Cattane, N., Galluzzi, S., Provasi, S., Lopizzo, N., Festari, C., ... & Group, I. F. (2017). Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiology of aging*, *49*, 60-68.

Ceccarelli, G., Brenchley, J. M., Cavallari, E. N., Corano Scheri, G., Fratino, M., Pinacchio, C., ... & d'Ettorre, G. (2017b). Impact of high-dose multi-strain probiotic supplementation on neurocognitive performance and central nervous system immune activation of HIV-1 infected individuals. *Nutrients*, *9*(11), 1269.

Ceccarelli, G., Fratino, M., Selvaggi, C., Giustini, N., Serafino, S., Schietroma, I., ... & d'Ettorre, G. (2017a). A pilot study on the effects of probiotic supplementation on neuropsychological performance and micro RNA-29a-c levels in antiretroviral-treated HIV-1-infected patients. *Brain and behavior*, *7*(8), e00756.

Cenit, M. C., Nuevo, I. C., Codoñer-Franch, P., Dinan, T. G., & Sanz, Y. (2017). Gut microbiota and attention deficit hyperactivity disorder: new perspectives for a challenging condition. *European child & adolescent psychiatry*, *26*, 1081-1092.

Chahwan, B., Kwan, S., Isik, A., van Hemert, S., Burke, C., & Roberts, L. (2019). Gut feelings: A randomised, triple-blind, placebo-controlled trial of probiotics for depressive symptoms. *Journal of affective disorders*, *253*, 317-326.

Chakrabarti, A., Geurts, L., Hoyles, L., Iozzo, P., Kraneveld, A. D., La Fata, G., ... & Vauzour, D. (2022). The microbiota–gut–brain axis: pathways to better brain health. Perspectives on what we know, what we need to investigate and how to put knowledge into practice. *Cellular and Molecular Life Sciences*, *79*(2), 80.

Chambers, J. M., Freeny, A and Heiberger, R. M. (1992) *Analysis of variance; designed experiments.* Chapter 5 of *Statistical Models in S* eds J. M. Chambers and T. J. Hastie, Wadsworth & Brooks/Cole.

Chapman, C. M. C., Gibson, G. R., & Rowland, I. (2012). In vitro evaluation of single-and multi-strain probiotics: Inter-species inhibition between probiotic strains, and inhibition of pathogens. *Anaerobe*, *18*(4), 405-413.

Chen, L. M., Bao, C. H., Wu, Y., Liang, S. H., Wang, D., Wu, L. Y., ... & Wu, H. G. (2021). Tryptophan-kynurenine metabolism: a link between the gut and brain for depression in inflammatory bowel disease. *Journal of neuroinflammation*, *18*(1), 1-13.

Chen, Y., Xu, J., & Chen, Y. (2021). Regulation of Neurotransmitters by the Gut Microbiota and Effects on Cognition in Neurological Disorders. *Nutrients*, *13*(6), 2099. https://doi.org/10.3390/nu13062099

Cheng, Y., Liu, J., & Ling, Z. (2022). Short-chain fatty acids-producing probiotics: A novel source of psychobiotics. *Critical reviews in food science and nutrition*, *62*(28), 7929-7959.

Chou, I. C., Kuo, H. T., Chang, J. S., Wu, S. F., Chiu, H. Y., Su, B. H., & Lin, H. C. (2010). Lack of effects of oral probiotics on growth and neurodevelopmental outcomes in preterm very low birth weight infants. *The Journal of pediatrics*, *156*(3), 393-396.

Christ, S. E., White, D. A., Mandernach, T., & Keys, B. A. (2001). Inhibitory control across the life span. *Developmental neuropsychology*, *20*(3), 653-669.

Chung, Y. C., Jin, H. M., Cui, Y., Jung, J. M., Park, J. I., Jung, E. S., ... & Chae, S. W. (2014). Fermented milk of Lactobacillus helveticus IDCC3801 improves cognitive functioning during cognitive fatigue tests in healthy older adults. *Journal of functional foods*, *10*, 465-474.

Cimler, R., Maresova, P., Kuhnova, J., & Kuca, K. (2019). Predictions of Alzheimer’s disease treatment and care costs in European countries. *PLoS One*, *14*(1), e0210958.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O’connor, E. M., Cusack, S., ... & O’toole, P. W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature*, *488*(7410), 178-184.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R. D., Shanahan, F., ... & Cryan, J. (2013). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular psychiatry*, *18*(6), 666-673.

Clarke, G., O'mahony, S. M., Dinan, T. G., & Cryan, J. F. (2014). Priming for health: gut microbiota acquired in early life regulates physiology, brain and behaviour. *Acta Paediatrica*, *103*(8), 812-819.

Cohen, J. (1992). Statistical power analysis. *Current directions in psychological science*, *1*(3), 98-101.

Cohen, S., Kamarck, T., & Mermelstein, R. (1983). A global measure of perceived stress. *Journal of health and social behavior*, 385-396.

Connell, E., Le Gall, G., Pontifex, M. G., Sami, S., Cryan, J. F., Clarke, G., ... & Vauzour, D. (2022). Microbial-derived metabolites as a risk factor of age-related cognitive decline and dementia. *Molecular Neurodegeneration*, *17*(1), 43.

Cook, T. M., Gavini, C. K., Jesse, J., Aubert, G., Gornick, E., Bonomo, R., ... & Mansuy-Aubert, V. (2021). Vagal neuron expression of the microbiota-derived metabolite receptor, free fatty acid receptor (FFAR3), is necessary for normal feeding behavior. *Molecular Metabolism*, *54*, 101350.

Craig, A. (1986). Acute effects of meals on perceptual and cognitive efficiency. *Nutrition reviews (USA)*.

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature reviews neuroscience*, *13*(10), 701-712.

Cunha, C., Brambilla, R., & Thomas, K. L. (2010). A simple role for BDNF in learning and memory?. *Frontiers in molecular neuroscience*, 1.

Cunningham, J. L., Bramstång, L., Singh, A., Jayarathna, S., Rasmusson, A. J., Moazzami, A., & Müller, B. (2020). Impact of time and temperature on gut microbiota and SCFA composition in stool samples. *PLoS One*, *15*(8), e0236944.

Curby, K. M., Johnson, K. J., & Tyson, A. (2012). Face to face with emotion: Holistic face processing is modulated by emotional state. *Cognition & emotion*, *26*(1), 93-102.

Czajeczny, D., Kabzińska, K., & Wójciak, R. W. (2021). Effects of Bifidobacterium Lactis BS01 and Lactobacillus Acidophilus LA02 on cognitive functioning in healthy women. *Applied Neuropsychology: Adult*, 1-9.

Daims, H., Brühl, A., Amann, R., Schleifer, K. H., & Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Systematic and applied microbiology*, *22*(3), 434-444.

Dalile, B., Van Oudenhove, L., Vervliet, B., & Verbeke, K. (2019). The role of short-chain fatty acids in microbiota–gut–brain communication. *Nature reviews Gastroenterology & hepatology*, *16*(8), 461-478.

Dao, V. H., Hoang, L. B., Trinh, T. O., Tran, T. T. T., & Dao, V. L. (2021). Psychobiotics for patients with chronic gastrointestinal disorders having anxiety or depression symptoms. *Journal of Multidisciplinary Healthcare*, 1395-1402.

Das, D., & Goyal, A. (2015). Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic Lactobacillus plantarum DM5 isolated from Marcha of Sikkim. *LWT-food Science and Technology*, *61*(1), 263-268.

Davari, S., Talaei, S. A., & Alaei, H. (2013). Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome–gut–brain axis. *Neuroscience*, *240*, 287-296.

De Vries, M. C., Vaughan, E. E., Kleerebezem, M., & de Vos, W. M. (2006). Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal*, *16*(9), 1018-1028.

Deary, I. J., Corley, J., Gow, A. J., Harris, S. E., Houlihan, L. M., Marioni, R. E., ... & Starr, J. M. (2009). Age-associated cognitive decline. *British medical bulletin*, *92*(1), 135-152.

Den Besten, G., Van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research*, *54*(9), 2325-2340.

Den, H., Dong, X., Chen, M., & Zou, Z. (2020). Efficacy of probiotics on cognition, and biomarkers of inflammation and oxidative stress in adults with Alzheimer's disease or mild cognitive impairment—A meta-analysis of randomized controlled trials. *Aging (Albany NY)*, *12*(4), 4010.

Deoni, S., Dean III, D., Joelson, S., O'Regan, J., & Schneider, N. (2018). Early nutrition influences developmental myelination and cognition in infants and young children. *Neuroimage*, *178*, 649-659.

Department of Health.(1991).Dietary Reference Values for Food and Energy and Nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G., & Cryan, J. (2014). Microbiota is essential for social development in the mouse. *Molecular psychiatry*, *19*(2), 146-148.

Deutz, N. E., Bauer, J. M., Barazzoni, R., Biolo, G., Boirie, Y., Bosy-Westphal, A., ... & Calder, P. C. (2014). Protein intake and exercise for optimal muscle function with aging: recommendations from the ESPEN Expert Group. *Clinical nutrition*, *33*(6), 929-936.

Devereux, R., Kane, M. D., Winfrey, J., & Stahl, D. A. (1992). Genus-and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *Systematic and applied microbiology*, *15*(4), 601-609.

Dhakal, R., Bajpai, V. K., & Baek, K. H. (2012). Production of GABA (γ-aminobutyric acid) by microorganisms: a review. *Brazilian Journal of Microbiology*, *43*, 1230-1241.

Diez-Gutierrez, L., San Vicente, L., Barron, L. J. R., del Carmen Villaran, M., & Chavarri, M. (2020). Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market. *Journal of Functional Foods*, *64*, 103669.

Dinan, T. G., Quigley, E. M., Ahmed, S. M., Scully, P., O'Brien, S., O'Mahony, L., ... & Keeling, P. N. (2006). Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker?. *Gastroenterology*, *130*(2), 304-311.

Donato, K. A., Gareau, M. G., Wang, Y. J. J., & Sherman, P. M. (2010). Lactobacillus rhamnosus GG attenuates interferon-γ and tumour necrosis factor-α-induced barrier dysfunction and pro-inflammatory signalling. *Microbiology*, *156*(11), 3288-3297.

Dorrestein, P. C., Mazmanian, S. K., & Knight, R. (2014). Finding the missing links among metabolites, microbes, and the host. *Immunity*, *40*(6), 824-832.

Dulla, B. S., & Bindhu, S. (2022). A Study on the Effect of Valeric Acid in Alzheimer's Induced Rats by the Estimation of Aβ 1-42 Biomarker. *Journal of Health and Allied Sciences NU*, *12*(02), 134-138.

Duncan, S. H., Hold, G. L., Barcenilla, A., Stewart, C. S., & Flint, H. J. (2002). Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *International journal of systematic and evolutionary microbiology*, *52*(5), 1615-1620.

Duncan, S. H., Louis, P., & Flint, H. J. (2004). Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied and environmental microbiology*, *70*(10), 5810-5817.

Echeverry, G., Hortin, G. L., & Rai, A. J. (2010). Introduction to urinalysis: historical perspectives and clinical application. *The urinary proteome: methods and protocols*, 1-12.

Elfil, M., Kamel, S., Kandil, M., Koo, B. B., & Schaefer, S. M. (2020). Implications of the gut microbiome in Parkinson's disease. *Movement Disorders*, *35*(6), 921-933.

Erny, D., Hrabě de Angelis, A. L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., ... & Prinz, M. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nature neuroscience*, *18*(7), 965-977.

Ezzati, A., Jiang, J., Katz, M. J., Sliwinski, M. J., Zimmerman, M. E., & Lipton, R. B. (2014). Validation of the Perceived Stress Scale in a community sample of older adults. *International journal of geriatric psychiatry*, *29*(6), 645-652.

Fadgyas-Stanculete, M., Buga, A. M., Popa-Wagner, A., & Dumitrascu, D. L. (2014). The relationship between irritable bowel syndrome and psychiatric disorders: from molecular changes to clinical manifestations. *Journal of molecular psychiatry*, *2*(1), 1-7.

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., ... & Raes, J. (2016). Population-level analysis of gut microbiome variation. *Science*, *352*(6285), 560-564.

Fan, Y., Støving, R. K., Berreira Ibraim, S., Hyötyläinen, T., Thirion, F., Arora, T., ... & Pedersen, O. (2023). The gut microbiota contributes to the pathogenesis of anorexia nervosa in humans and mice. Nature Microbiology, 1-16.

Farhadi, A., Fields, J. Z., & Keshavarzian, A. (2007). Mucosal mast cells are pivotal elements in inflammatory bowel disease that connect the dots: stress, intestinal hyperpermeability and inflammation. World Journal of Gastroenterology, 13(22), 3027.

Farrall, A. J., & Wardlaw, J. M. (2009). Blood–brain barrier: ageing and microvascular disease–systematic review and meta-analysis. *Neurobiology of aging*, *30*(3), 337-352.

Field, A. (2009). Discovering Statistics using SPSS (3rd Editio). SAGE Publications Ltd.

Finegold, S. M. (2011). Desulfovibrio species are potentially important in regressive autism. *Medical hypotheses*, *77*(2), 270-274.

Firmansyah, A., Dwipoerwantoro, P. G., Kadim, M., Alatas, S., Conus, N., Lestarina, L., ... & Steenhout, P. (2011). Improved growth of toddlers fed a milk containing synbiotics. *Asia Pacific journal of clinical nutrition*, *20*(1), 69-76.

Fiske, A., Wetherell, J. L., & Gatz, M. (2009). Depression in older adults. *Annual review of clinical psychology*, *5*, 363-389.

Flanagan, E., Lamport, D., Brennan, L., Burnet, P., Calabrese, V., Cunnane, S. C., ... & Vauzour, D. (2020). Nutrition and the ageing brain: Moving towards clinical applications. *Ageing Research Reviews*, *62*, 101079.

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society*, *74*(1), 13-22.

Fond, G. B., Lagier, J. C., Honore, S., Lancon, C., Korchia, T., Verville, P. L. S. D., ... & Boyer, L. (2020). Microbiota-orientated treatments for major depression and schizophrenia. *Nutrients*, *12*(4), 1024.

Forgas, J. P. (2017). Mood effects on cognition: Affective influences on the content and process of information processing and behavior. *Emotions and affect in human factors and human-computer interaction*, 89-122.

Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F., & Welling, G. W. (1998). Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Applied and environmental microbiology*, *64*(9), 3336-3345.

Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., ... & Bell, J. D. (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nature communications*, *5*(1), 3611.

Fujimori, S., Gudis, K., Mitsui, K., Seo, T., Yonezawa, M., Tanaka, S., ... & Sakamoto, C. (2009). A randomized controlled trial on the efficacy of synbiotic versus probiotic or prebiotic treatment to improve the quality of life in patients with ulcerative colitis. *Nutrition*, *25*(5), 520-525.

Galkin, F., Mamoshina, P., Aliper, A., Putin, E., Moskalev, V., Gladyshev, V. N., & Zhavoronkov, A. (2020). Human gut microbiome aging clock based on taxonomic profiling and deep learning. *Iscience*, *23*(6), 101199.

Gao, G., Zhou, J., Wang, H., Ding, Y., Zhou, J., Chong, P. H., ... & Zhang, L. (2022). Effects of valerate on intestinal barrier function in cultured Caco-2 epithelial cell monolayers. *Molecular Biology Reports*, 1-9.

Gao, J., Xu, K., Liu, H., Liu, G., Bai, M., Peng, C., ... & Yin, Y. (2018). Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Frontiers in cellular and infection microbiology*, *8*, 13.

Gareau, M. G. (2014). Microbiota-gut-brain axis and cognitive function. *Microbial endocrinology: The microbiota-gut-brain axis in health and disease*, 357-371.

Gareau, M. G., Wine, E., Rodrigues, D. M., Cho, J. H., Whary, M. T., Philpott, D. J., ... & Sherman, P. M. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut*, *60*(3), 307-317.

Ghosh, S. S., Wang, J., Yannie, P. J., & Ghosh, S. (2020). Intestinal barrier dysfunction, LPS translocation, and disease development. *Journal of the Endocrine Society*, *4*(2), bvz039.

Ghosh, T. S., Rampelli, S., Jeffery, I. B., Santoro, A., Neto, M., Capri, M., ... & O'Toole, P. W. (2020). Mediterranean diet intervention alters the gut microbiome in older people reducing frailty and improving health status: the NU-AGE 1-year dietary intervention across five European countries. *Gut*, *69*(7), 1218-1228.

Gibbons, S. M., Gurry, T., Lampe, J. W., Chakrabarti, A., Dam, V., Everard, A., ... & Miani, M. (2022). Perspective: Leveraging the gut microbiota to predict personalized responses to dietary, prebiotic, and probiotic interventions. *Advances in Nutrition*, *13*(5), 1450-1461.

Gibbs, R. A. (2020). The human genome project changed everything. *Nature Reviews Genetics*, *21*(10), 575-576.

Gibson, G. R., Cummings, J. H., & Macfarlane, G. T. (1988). Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. *Applied and environmental microbiology*, *54*(11), 2750-2755.

Gilbert, J. A., Blaser, M. J., Caporaso, J. G., Jansson, J. K., Lynch, S. V., & Knight, R. (2018). Current understanding of the human microbiome. *Nature medicine*, *24*(4), 392-400.

Gilboa-Schechtman, E., Erhard-Weiss, D., & Jeczemien, P. (2002). Interpersonal deficits meet cognitive biases: memory for facial expressions in depressed and anxious men and women. *Psychiatry research*, *113*(3), 279-293.

Girvin, G. T., & Stevenson, J. W. (1954). Cell free" choline acetylase" from Lactobacillus plantarum. *Canadian journal of biochemistry and physiology*, *32*(2), 131-146.

Goldberg, T. E., Harvey, P. D., Wesnes, K. A., Snyder, P. J., & Schneider, L. S. (2015). Practice effects due to serial cognitive assessment: implications for preclinical Alzheimer's disease randomized controlled trials. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, *1*(1), 103-111.

Grimaldi, R., Cela, D., Swann, J. R., Vulevic, J., Gibson, G. R., Tzortzis, G., & Costabile, A. (2017). In vitro fermentation of B-GOS: impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. *FEMS microbiology ecology*, *93*(2), fiw233.

Guarner, F., Khan, A. G., Garisch, J., Eliakim, R., Gangl, A., Thomson, A., ... & Kim, N. (2012). World gastroenterology organisation global guidelines: probiotics and prebiotics october 2011. *Journal of clinical gastroenterology*, *46*(6), 468-481.

Gubert, C., Choo, J. M., Love, C. J., Kodikara, S., Masson, B. A., Liew, J. J., ... & Hannan, A. J. (2022). Faecal microbiota transplant ameliorates gut dysbiosis and cognitive deficits in Huntington's disease mice. *Brain Communications*, *4*(4), fcac205.

Gwak, M. G., & Chang, S. Y. (2021). Gut-brain connection: microbiome, gut barrier, and environmental sensors. *Immune Network*, *21*(3).

Hajjar, I., Hayek, S. S., Goldstein, F. C., Martin, G., Jones, D. P., & Quyyumi, A. (2018). Oxidative stress predicts cognitive decline with aging in healthy adults: an observational study. *Journal of neuroinflammation*, *15*(1), 1-7.

Han, K. J., Lee, N. K., Park, H., & Paik, H. D. (2015). Anticancer and anti-inflammatory activity of probiotic Lactococcus lactis NK34. *Journal of microbiology and biotechnology*, *25*(10), 1697-1701.

Harada, C. N., Love, M. C. N., & Triebel, K. L. (2013). Normal cognitive aging. *Clinics in geriatric medicine*, *29*(4), 737-752.

Harmsen, H. J., Wildeboer-Veloo, A. C., Grijpstra, J., Knol, J., Degener, J. E., & Welling, G. W. (2000). Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Applied and Environmental Microbiology*, *66*(10), 4523-4527.

Hart, A., & Kamm, M. A. (2002). Mechanisms of initiation and perpetuation of gut inflammation by stress. Alimentary pharmacology & therapeutics, 16(12), 2017-2028.

Hata, T., Asano, Y., Yoshihara, K., Kimura-Todani, T., Miyata, N., Zhang, X. T., ... & Sudo, N. (2017). Regulation of gut luminal serotonin by commensal microbiota in mice. *PloS one*, *12*(7), e0180745.

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., ... & Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences*, *108*(7), 3047-3052.

Herselman, M. F., Bailey, S., & Bobrovskaya, L. (2022). The Effects of Stress and Diet on the "Brain–Gut" and "Gut–Brain" Pathways in Animal Models of Stress and Depression. International Journal of Molecular Sciences, 23(4), 2013.

Hoffman, L., & Rovine, M. J. (2007). Multilevel models for the experimental psychologist: Foundations and illustrative examples. *Faculty Publications, Department of Psychology*, 421.

Holzer, P., & Farzi, A. (2014). Neuropeptides and the microbiota-gut-brain axis. *Microbial endocrinology: the microbiota-gut-brain axis in health and disease*, 195-219.

Howell, D. C. (2010). Statistical methods for psychology (8th Ed). Belmont, CA: Cengage Wadsworth.

Hoyles, L., Snelling, T., Umlai, U. K., Nicholson, J. K., Carding, S. R., Glen, R. C., & McArthur, S. (2018). Microbiome–host systems interactions: protective effects of propionate upon the blood–brain barrier. *Microbiome*, *6*(1), 1-13.

Hsieh, S., Wu, M., & Tang, C. H. (2016). Adaptive strategies for the elderly in inhibiting irrelevant and conflict no-go trials while performing the go/no-go task. *Frontiers in Aging Neuroscience*, *7*, 243.

Hsieh, Y. H., Peterson, C. M., Raggio, A., Keenan, M. J., Martin, R. J., Ravussin, E., & Marco, M. L. (2016). Impact of different fecal processing methods on assessments of bacterial diversity in the human intestine. *Frontiers in microbiology*, *7*, 1643.

Huck, S. W. (2015). Statistical Misconceptions (Classic Ed). Routledge.

Hwang, Y. H., Park, S., Paik, J. W., Chae, S. W., Kim, D. H., Jeong, D. G., ... & Chung, Y. C. (2019). Efficacy and safety of Lactobacillus plantarum C29-fermented soybean (DW2009) in individuals with mild cognitive impairment: a 12-week, multi-center, randomized, double-blind, placebo-controlled clinical trial. *Nutrients*, *11*(2), 305.

Hyland, N. P., Cavanaugh, C. R., & Hornby, P. J. (2022). Emerging effects of tryptophan pathway metabolites and intestinal microbiota on metabolism and intestinal function. *Amino Acids*, *54*(1), 57-70.

Inazu, M. (2019). Functional Expression of Choline Transporters in the Blood–Brain Barrier. *Nutrients*, *11*(10), 2265.

Inoue, T., Kobayashi, Y., Mori, N., Sakagawa, M., Xiao, J. Z., Moritani, T., ... & Nagai, N. (2018). Effect of combined bifidobacteria supplementation and resistance training on cognitive function, body composition and bowel habits of healthy elderly subjects. *Beneficial microbes*, *9*(6), 843-853.

Jackson, P. P. J., Wijeyesekera, A., van Harsselaar, J., Theis, S., & Rastall, R. A. (2022). Oligofructose, 2'fucosyllatose and β-glucan in combination induce specific changes in microbial composition and short-chain fatty acid production compared to sole supplementation. *Journal of Applied Microbiology*.

Jacobs, S. E., Hickey, L., Donath, S., Opie, G. F., Anderson, P. J., Garland, S. M., ... & Groups, P. (2017). Probiotics, prematurity and neurodevelopment: follow-up of a randomised trial. *BMJ Paediatrics Open*, *1*(1).

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N. (2015). Role of the normal gut microbiota. *World journal of gastroenterology: WJG*, *21*(29), 8787.

Janik, R., Thomason, L. A., Stanisz, A. M., Forsythe, P., Bienenstock, J., & Stanisz, G. J. (2016). Magnetic resonance spectroscopy reveals oral Lactobacillus promotion of increases in brain GABA, N-acetyl aspartate and glutamate. *Neuroimage*, *125*, 988-995.

Jayaraj, R. L., Beiram, R., Azimullah, S., Mf, N. M., Ojha, S. K., Adem, A., & Jalal, F. Y. (2020). Valeric acid protects dopaminergic neurons by suppressing oxidative stress, neuroinflammation and modulating autophagy pathways. *International journal of molecular sciences*, *21*(20), 7670.

Jeong, J. J., Woo, J. Y., Kim, K. A., Han, M. J., & Kim, D. H. (2015). Lactobacillus pentosus var. plantarum C29 ameliorates age-dependent memory impairment in Fischer 344 rats. *Letters in applied microbiology*, *60*(4), 307-314.

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., ... & Ruan, B. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain, behavior, and immunity*, *48*, 186-194.

Kang, D. W., Adams, J. B., Coleman, D. M., Pollard, E. L., Maldonado, J., McDonough-Means, S., ... & Krajmalnik-Brown, R. (2019). Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Scientific reports*, *9*(1), 5821.

Kang, D. W., Adams, J. B., Gregory, A. C., Borody, T., Chittick, L., Fasano, A., ... & Krajmalnik-Brown, R. (2017). Microbiota transfer therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome*, *5*(1), 1-16.

Kang, D. W., Park, J. G., Ilhan, Z. E., Wallstrom, G., LaBaer, J., Adams, J. B., & Krajmalnik-Brown, R. (2013). Reduced incidence of Prevotella and other fermenters in intestinal microflora of autistic children. *PloS one*, *8*(7), e68322.

Kaur, H., Bose, C., & Mande, S. S. (2019). Tryptophan metabolism by gut microbiome and gut-brain-axis: an in silico analysis. *Frontiers in neuroscience*, *13*, 1365.

Kazemi, A., Noorbala, A. A., Azam, K., Eskandari, M. H., & Djafarian, K. (2019). Effect of probiotic and prebiotic vs placebo on psychological outcomes in patients with major depressive disorder: A randomized clinical trial. *Clinical Nutrition*, *38*(2), 522-528.

Kekkonen, R. A., Lummela, N., Karjalainen, H., Latvala, S., Tynkkynen, S., Järvenpää, S., ... & Korpela, R. (2008). Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. *World journal of gastroenterology: WJG*, *14*(13), 2029.

Keller, J., Gomez, R., Williams, G., Lembke, A., Lazzeroni, L., Murphy, G. M., & Schatzberg, A. F. (2017). HPA axis in major depression: cortisol, clinical symptomatology and genetic variation predict cognition. *Molecular psychiatry*, *22*(4), 527-536.

Kelly, J. R., Allen, A. P., Temko, A., Hutch, W., Kennedy, P. J., Farid, N., ... & Dinan, T. G. (2017). Lost in translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects. *Brain, behavior, and immunity*, *61*, 50-59.

Kelly, J. R., Borre, Y., O'Brien, C., Patterson, E., El Aidy, S., Deane, J., ... & Dinan, T. G. (2016). Transferring the blues: depression-associated gut microbiota induces neurobehavioural changes in the rat. *Journal of psychiatric research*, *82*, 109-118.

Kelly, J. R., Minuto, C., Cryan, J. F., Clarke, G., & Dinan, T. G. (2017). Cross talk: the microbiota and neurodevelopmental disorders. *Frontiers in neuroscience*, *11*, 490.

Kennedy, P. J., Cryan, J. F., Dinan, T. G., & Clarke, G. (2017). Kynurenine pathway metabolism and the microbiota-gut-brain axis. Neuropharmacology, 112, 399-412.

Kenny, R. A., Coen, R. F., Frewen, J., Donoghue, O. A., Cronin, H., & Savva, G. M. (2013). Normative values of cognitive and physical function in older adults: findings from the Irish Longitudinal Study on Ageing. *Journal of the American Geriatrics Society*, *61*, S279-S290.

Kim, C. S., Cha, L., Sim, M., Jung, S., Chun, W. Y., Baik, H. W., & Shin, D. M. (2021). Probiotic supplementation improves cognitive function and mood with changes in gut microbiota in community-dwelling older adults: a randomized, double-blind, placebo-controlled, multicenter trial. *The Journals of Gerontology: Series A*, *76*(1), 32-40.

Kim, G. H., Kim, J. E., Rhie, S. J., & Yoon, S. (2015). The role of oxidative stress in neurodegenerative diseases. *Experimental neurobiology*, *24*(4), 325.

Kim, H. J., Huh, D., Hamilton, G., & Ingber, D. E. (2012). Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab on a Chip*, *12*(12), 2165-2174.

Kim, H. K., Rutten, N. B. M. M., Besseling-van Der Vaart, I., Niers, L. E. M., Choi, Y. H., Rijkers, G. T., & van Hemert, S. (2015). Probiotic supplementation influences faecal short chain fatty acids in infants at high risk for eczema. *Beneficial microbes*, *6*(6), 783-790.

Kim, K., Aronov, P., Zakharkin, S. O., Anderson, D., Perroud, B., Thompson, I. M., & Weiss, R. H. (2009). Urine metabolomics analysis for kidney cancer detection and biomarker discovery. *Molecular & cellular proteomics*, *8*(3), 558-570.

Kim, M., & Benayoun, B. A. (2020). The microbiome: an emerging key player in aging and longevity. *Translational medicine of aging*, *4*, 103-116.

Knief, U., & Forstmeier, W. (2021). Violating the normality assumption may be the lesser of two evils. *Behavior Research Methods*, *53*(6), 2576-2590.

Knudsen, G. M., Poulsen, H. E., & Paulson, O. B. (1988). Blood-brain barrier permeability in galactosamine-induced hepatic encephalopathy: no evidence for increased GABA-transport. *Journal of hepatology*, *6*(2), 187-192.

Kobayashi, Y., Kinoshita, T., Matsumoto, A., Yoshino, K., Saito, I., & Xiao, J. Z. (2019a). Bifidobacterium breve A1 supplementation improved cognitive decline in older adults with mild cognitive impairment: an open-label, single-arm study. *The Journal of Prevention of Alzheimer's Disease*, *6*, 70-75.

Kobayashi, Y., Kuhara, T., Oki, M., & Xiao, J. Z. (2019b). Effects of Bifidobacterium breve A1 on the cognitive function of older adults with memory complaints: a randomised, double-blind, placebo-controlled trial. *Beneficial microbes*, *10*(5), 511-520.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & Bäckhed, F. (2016). From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell*, *165*(6), 1332-1345.

Komura, T., Takemoto, A., Kosaka, H., Suzuki, T., & Nishikawa, Y. (2021). Prolonged Lifespan, Ameliorated Cognition, and Improved Host Defense of Caenorhabditis elegans by Lactococcus lactis subsp. cremoris. *bioRxiv*, 2021-04.

Kristensen, N. B., Bryrup, T., Allin, K. H., Nielsen, T., Hansen, T. H., & Pedersen, O. (2016). Alterations in fecal microbiota composition by probiotic supplementation in healthy adults: a systematic review of randomized controlled trials. *Genome medicine*, *8*(1), 1-11.

Kruijt, A. W., Antypa, N., Booij, L., de Jong, P. J., Glashouwer, K., Penninx, B. W., & Van der Does, W. (2013). Cognitive reactivity, implicit associations, and the incidence of depression: a two-year prospective study. *PLoS One*, *8*(7), e70245.

Kuc, D., Zgrajka, W., Parada-Turska, J., Urbanik-Sypniewska, T., & Turski, W. A. (2008). Micromolar concentration of kynurenic acid in rat small intestine. *Amino Acids*, *35*, 503-505.

Kwon, S., Yoo, I. K., Lee, W. G., Chang, H. N., & Chang, Y. K. (2001). High-rate continuous production of lactic acid by Lactobacillus rhamnosus in a two-stage membrane cell-recycle bioreactor. *Biotechnology and bioengineering*, *73*(1), 25-34.

La-Ongkham, O., Nakphaichit, M., Nakayama, J., Keawsompong, S., & Nitisinprasert, S. (2020). Age-related changes in the gut microbiota and the core gut microbiome of healthy Thai humans. *3 Biotech*, *10*, 1-14.

Labanski, A., Langhorst, J., Engler, H., & Elsenbruch, S. (2020). Stress and the brain-gut axis in functional and chronic-inflammatory gastrointestinal diseases: A transdisciplinary challenge. *Psychoneuroendocrinology*, *111*, 104501.

Labus, J. S., Hollister, E. B., Jacobs, J., Kirbach, K., Oezguen, N., Gupta, A., ... & Mayer, E. A. (2017). Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome. *Microbiome*, *5*, 1-17.

Landete, J. M., Ferrer, S., & Pardo, I. (2007). Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control*, *18*(12), 1569-1574.

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H., & Welling, G. W. (1995). Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Applied and environmental microbiology*, *61*(8), 3069-3075.

Larsen, N., Vogensen, F. K., Gøbel, R. J., Michaelsen, K. F., Forssten, S. D., Lahtinen, S. J., & Jakobsen, M. (2013). Effect of Lactobacillus salivarius Ls-33 on fecal microbiota in obese adolescents. *Clinical nutrition*, *32*(6), 935-940.

Leblhuber, F., Steiner, K., Schuetz, B., Fuchs, D., & Gostner, J. M. (2018). Probiotic supplementation in patients with Alzheimer's dementia-an explorative intervention study. *Current Alzheimer Research*, *15*(12), 1106-1113.

Leppänen, J. M., Milders, M., Bell, J. S., Terriere, E., & Hietanen, J. K. (2004). Depression biases the recognition of emotionally neutral faces. *Psychiatry research*, *128*(2), 123-133.

Lew, L. C., Hor, Y. Y., Yusoff, N. A. A., Choi, S. B., Yusoff, M. S., Roslan, N. S., ... & Liong, M. T. (2019). Probiotic Lactobacillus plantarum P8 alleviated stress and anxiety while enhancing memory and cognition in stressed adults: A randomised, double-blind, placebo-controlled study. *Clinical Nutrition*, *38*(5), 2053-2064.

Lewinsohn, P. M., Seeley, J. R., Roberts, R. E., & Allen, N. B. (1997). Center for Epidemiologic Studies Depression Scale (CES-D) as a screening instrument for depression among community-residing older adults. *Psychology and aging*, *12*(2), 277.

Lezak, M. D., Howieson, D. B., Bigler, E. D., & Tranel, D. (2012). Neuropsychological Assessment (5th edition). Oxford University Press.

Li, H., & Cao, Y. (2010). Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino acids*, *39*, 1107-1116.

Li, M., Van Esch, B. C., Henricks, P. A., Folkerts, G., & Garssen, J. (2018). The anti-inflammatory effects of short chain fatty acids on lipopolysaccharide-or tumor necrosis factor α-stimulated endothelial cells via activation of GPR41/43 and inhibition of HDACs. *Frontiers in pharmacology*, *9*, 533.

Lichtenstein, A. H., Petersen, K., Barger, K., Hansen, K. E., Anderson, C. A., Baer, D. J., ... & Matthan, N. R. (2021). Perspective: design and conduct of human nutrition randomized controlled trials. *Advances in Nutrition*, *12*(1), 4-20.

Likotrafiti, E., Tuohy, K. M., Gibson, G. R., & Rastall, R. A. (2014). An in vitro study of the effect of probiotics, prebiotics and synbiotics on the elderly faecal microbiota. *Anaerobe*, *27*, 50-55.

Lin, L. Y., Smeeth, L., Langan, S., & Warren-Gash, C. (2021). Distribution of vitamin D status in the UK: a cross-sectional analysis of UK Biobank. *BMJ open*, *11*(1), e038503.

Liu, H., Ding, L., Zhang, H., Mellor, D., Wu, H., Zhao, D., ... & Peng, D. (2018). The metabolic factor kynurenic acid of kynurenine pathway predicts major depressive disorder. *Frontiers in psychiatry*, *9*, 552.

Liu, L., Poveda, C., Jenkins, P. E., & Walton, G. E. (2021). An in vitro approach to studying the microbial community and impact of pre and probiotics under anorexia nervosa related dietary restrictions. *Nutrients*, *13*(12), 4447.

Liu, S., Guo, R., Liu, F., Yuan, Q., Yu, Y., & Ren, F. (2020). Gut microbiota regulates depression-like behavior in rats through the neuroendocrine-immune-mitochondrial pathway. *Neuropsychiatric Disease and Treatment*, 859-869.

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., ... & Chen, W. (2021). Blautia—a new functional genus with potential probiotic properties?. *Gut microbes*, *13*(1), 1875796.

Liu, Y., Gibson, G. R., & Walton, G. E. (2016). An in vitro approach to study effects of prebiotics and probiotics on the faecal microbiota and selected immune parameters relevant to the elderly. *PLoS One*, *11*(9), e0162604.

Liu, Y., Wei, M., Yue, K., Hu, M., Li, S., Men, L., ... & Liu, Z. (2018). Study on urine metabolic profile of Aβ25–35-induced Alzheimer's disease using UHPLC-Q-TOF-MS. *Neuroscience*, *394*, 30-43.

Logsdon, A. F., Erickson, M. A., Rhea, E. M., Salameh, T. S., & Banks, W. A. (2018). Gut reactions: How the blood–brain barrier connects the microbiome and the brain. *Experimental Biology and Medicine*, *243*(2), 159-165.

Long-Smith, C., O'Riordan, K. J., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2020). Microbiota-gut-brain axis: new therapeutic opportunities. *Annual review of pharmacology and toxicology*, *60*, 477-502.

Louis, P., & Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environmental microbiology*, *19*(1), 29-41.

Louis, P., Duncan, S. H., Sheridan, P. O., Walker, A. W., & Flint, H. J. (2022). Microbial lactate utilisation and the stability of the gut microbiome. *Gut Microbiome*, *3*, e3.

Luczynski, P., McVey Neufeld, K. A., Oriach, C. S., Clarke, G., Dinan, T. G., & Cryan, J. F. (2016). Growing up in a bubble: using germ-free animals to assess the influence of the gut microbiota on brain and behavior. *International Journal of Neuropsychopharmacology*, *19*(8).

Lunia, M. K., Sharma, B. C., Sharma, P., Sachdeva, S., & Srivastava, S. (2014). Probiotics prevent hepatic encephalopathy in patients with cirrhosis: a randomized controlled trial. *Clinical Gastroenterology and Hepatology*, *12*(6), 1003-1008.

Lv, T., Ye, M., Luo, F., Hu, B., Wang, A., Chen, J., ... & Ding, Z. (2021). Probiotics treatment improves cognitive impairment in patients and animals: A systematic review and meta-analysis. *Neuroscience & Biobehavioral Reviews*, *120*, 159-172.

Lyons, A., Alba, B., Heywood, W., Fileborn, B., Minichiello, V., Barrett, C., ... & Dow, B. (2018). Experiences of ageism and the mental health of older adults. *Aging & mental health*, *22*(11), 1456-1464.

Lyte, M., Vulchanova, L., & Brown, D. R. (2011). Stress at the intestinal surface: catecholamines and mucosa–bacteria interactions. Cell and tissue research, 343, 23-32.

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annual review of pharmacology and toxicology*, *53*, 401-426.

Ma, T., Jin, H., Kwok, L. Y., Sun, Z., Liong, M. T., & Zhang, H. (2021). Probiotic consumption relieved human stress and anxiety symptoms possibly via modulating the neuroactive potential of the gut microbiota. *Neurobiology of Stress*, *14*, 100294.

Macfarlane, G. T., Macfarlane, S., & Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial ecology*, *35*, 180-187.

Mackos, A. R., Galley, J. D., Eubank, T. D., Easterling, R. S., Parry, N. M., Fox, J. G., ... & Bailey, M. T. (2016). Social stress-enhanced severity of Citrobacter rodentium-induced colitis is CCL2-dependent and attenuated by probiotic Lactobacillus reuteri. *Mucosal immunology*, *9*(2), 515-526.

Maia, L. P., Levi, Y. L. D. A. S., do Prado, R. L., dos Santos Santinoni, C., & Marsicano, J. A. (2019). Effects of probiotic therapy on serum inflammatory markers: A systematic review and meta-analysis. *Journal of functional foods*, *54*, 466-478.

Malaguarnera, M., Gargante, M. P., Malaguarnera, G., Salmeri, M., Mastrojeni, S., Rampello, L., ... & Galvano, F. (2010). Bifidobacterium combined with fructo-oligosaccharide versus lactulose in the treatment of patients with hepatic encephalopathy. *European journal of gastroenterology & hepatology*, *22*(2), 199-206.

Malan-Muller, S., Valles-Colomer, M., Raes, J., Lowry, C. A., Seedat, S., & Hemmings, S. M. (2018). The gut microbiome and mental health: implications for anxiety-and trauma-related disorders. *Omics: a journal of integrative biology*, *22*(2), 90-107.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., & Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology*, *142*(5), 1097-1106.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., ... & Hutkins, R. (2017). Health benefits of fermented foods: microbiota and beyond. *Current opinion in biotechnology*, *44*, 94-102.

Margolis, K. G., Cryan, J. F., & Mayer, E. A. (2021). The microbiota-gut-brain axis: from motility to mood. *Gastroenterology*, *160*(5), 1486-1501.

Markowiak-Kopeć, P., & Śliżewska, K. (2020). The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients*, *12*(4), 1107.

Marrosu, F., Serra, A., Maleci, A., Puligheddu, M., Biggio, G., & Piga, M. (2003). Correlation between GABAA receptor density and vagus nerve stimulation in individuals with drug-resistant partial epilepsy. Epilepsy research, 55(1-2), 59-70.

Marx, W., Scholey, A., Firth, J., D'Cunha, N. M., Lane, M., Hockey, M., ... & Jacka, F. (2020). Prebiotics, probiotics, fermented foods and cognitive outcomes: A meta-analysis of randomized controlled trials. *Neuroscience & Biobehavioral Reviews*, *118*, 472-484.

Marzorati, M., Vanhoecke, B., De Ryck, T., Sadaghian Sadabad, M., Pinheiro, I., Possemiers, S., ... & Van de Wiele, T. (2014). The HMI™ module: a new tool to study the Host-Microbiota Interaction in the human gastrointestinal tract in vitro. *BMC microbiology*, *14*, 1-14.

Mattson, M. P., & Arumugam, T. V. (2018). Hallmarks of brain aging: adaptive and pathological modification by metabolic states. Cell metabolism, 27(6), 1176-1199.

Mayer, E. A., & Tillisch, K. (2011). The brain-gut axis in abdominal pain syndromes. Annual review of medicine, 62, 381-396.

McCue, J. D., & Zandt, J. R. (1991). Acute psychoses associated with the use of ciprofloxacin and trimethoprim-sulfamethoxazole. *The American journal of medicine*, *90*(1), 528-529.

McFarland, L. V. (2021). Efficacy of single-strain probiotics versus multi-strain mixtures: systematic review of strain and disease specificity. *Digestive diseases and sciences*, *66*, 694-704.

Mennigen, R., & Bruewer, M. (2009). Effect of probiotics on intestinal barrier function. *Annals of the New York Academy of Sciences*, *1165*(1), 183-189.

Meyer, D., & Stasse-Wolthuis, M. (2009). The bifidogenic effect of inulin and oligofructose and its consequences for gut health. *European journal of clinical nutrition*, *63*(11), 1277-1289.

Miller, M. G., Hamilton, D. A., Joseph, J. A., & Shukitt-Hale, B. (2018). Dietary blueberry improves cognition among older adults in a randomized, double-blind, placebo-controlled trial. *European journal of nutrition*, *57*, 1169-1180.

Miranda, M., Morici, J. F., Zanoni, M. B., & Bekinschtein, P. (2019). Brain-derived neurotrophic factor: a key molecule for memory in the healthy and the pathological brain. *Frontiers in cellular neuroscience*, 363.

Mobini, R., Tremaroli, V., Ståhlman, M., Karlsson, F., Levin, M., Ljungberg, M., ... & Jansson, P. A. (2017). Metabolic effects of L actobacillus reuteri DSM 17938 in people with type 2 diabetes: A randomized controlled trial. *Diabetes, Obesity and Metabolism*, *19*(4), 579-589.

Monteagudo-Mera, A., Fanti, V., Rodriguez-Sobstel, C., Gibson, G., Wijeyesekera, A., Karatzas, K. A., & Chakrabarti, B. (2022). Gamma aminobutyric acid production by commercially available probiotic strains. *Journal of Applied Microbiology*.

Montejo, P., Montenegro, M., Fernández, M. A., & Maestú, F. (2011). Subjective memory complaints in the elderly: prevalence and influence of temporal orientation, depression and quality of life in a population-based study in the city of Madrid. *Aging & mental health*, *15*(1), 85-96.

Morgan, D. G., May, P. C., & Finch, C. E. (1987). Dopamine and serotonin systems in human and rodent brain: effects of age and neurodegenerative disease. *Journal of the American Geriatrics Society*, *35*(4), 334-345.

Mosca, A., Leclerc, M., & Hugot, J. P. (2016). Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem?. *Frontiers in microbiology*, *7*, 455.

Moulds, M. L., Kandris, E., Williams, A. D., Lang, T., Yap, C., & Hoffmeister, K. (2008). An investigation of the relationship between cognitive reactivity and rumination. *Behavior Therapy*, *39*(1), 65-71.

Mulhall, J. P., & Bergmann, L. S. (1995). Ciprofloxacin-induced acute psychosis. *Urology*, *46*(1), 102-103.

Mulligan, A. A., Luben, R. N., Bhaniani, A., Parry-Smith, D. J., O'Connor, L., Khawaja, A. P., ... & Khaw, K. T. (2014). A new tool for converting food frequency questionnaire data into nutrient and food group values: FETA research methods and availability. *BMJ open*, *4*(3), e004503.

Murray, C. D., Flynn, J., Ratcliffe, L., Jacyna, M. R., Kamm, M. A., & Emmanuel, A. V. (2004). Effect of acute physical and psychological stress on gut autonomic innervation in irritable bowel syndrome. *Gastroenterology*, *127*(6), 1695-1703.

Musazadeh, V., Zarezadeh, M., Faghfouri, A. H., Keramati, M., Jamilian, P., Jamilian, P., ... & Farnam, A. (2022). Probiotics as an effective therapeutic approach in alleviating depression symptoms: an umbrella meta-analysis. *Critical Reviews in Food Science and Nutrition*, 1-9.

Nagpal, R., Wang, S., Ahmadi, S., Hayes, J., Gagliano, J., Subashchandrabose, S., ... & Yadav, H. (2018). Human-origin probiotic cocktail increases short-chain fatty acid production via modulation of mice and human gut microbiome. *Scientific reports*, *8*(1), 1-15.

Nanga, R. P. R., Elliott, M. A., Swain, A., Wilson, N., Swago, S., Soni, N. D., ... & Reddy, R. (2022). Identification of l-Tryptophan by down-field 1H MRS: A precursor for brain NAD+ and serotonin syntheses. *Magnetic Resonance in Medicine*, *88*(6), 2371-2377.

Nankova, B. B., Agarwal, R., MacFabe, D. F., & La Gamma, E. F. (2014). Enteric bacterial metabolites propionic and butyric acid modulate gene expression, including CREB-dependent catecholaminergic neurotransmission, in PC12 cells-possible relevance to autism spectrum disorders. *PLoS One*, *9*(8), e103740.

Naomi, R., Embong, H., Othman, F., Ghazi, H. F., Maruthey, N., & Bahari, H. (2022). Probiotics for Alzheimer’s disease: A systematic review. *Nutrients*, *14*(1), 20.

Nasreddine, Z. S., Phillips, N. A., Bédirian, V., Charbonneau, S., Whitehead, V., Collin, I., ... & Chertkow, H. (2005). The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. *Journal of the American Geriatrics Society*, *53*(4), 695-699.

Neufeld, K. M., Kang, N., Bienenstock, J., & Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, *23*(3), 255-e119.

Ng, Q. X., Soh, A. Y. S., Venkatanarayanan, N., Ho, C. Y. X., Lim, D. Y., & Yeo, W. S. (2019). A systematic review of the effect of probiotic supplementation on schizophrenia symptoms. *Neuropsychobiology*, *78*(1), 1-6.

Nie, K., Ma, K., Luo, W., Shen, Z., Yang, Z., Xiao, M., ... & Wang, X. (2021). Roseburia intestinalis: A beneficial gut organism from the discoveries in genus and species. *Frontiers in Cellular and Infection Microbiology*, 1147.

Nixon, A. F., Cunningham, S. J., Cohen, H. W., & Crain, E. F. (2012). The effect of lactobacillus GG (LGG) on acute diarrheal Illness in the pediatric emergency department (PED). *Pediatric emergency care*, *28*(10), 1048.

Nobile, V., Giardina, S., & Puoci, F. (2022). The effect of a probiotic complex on the gut-brain axis: a translational study. *Neuropsychobiology*, *81*(2), 116-126.

Noorwali, E. A., Beaumont, J. D., Corfe, B. M., & Owen, L. (2017). The effects of probiotic supplementation on emotional memory and pain response. *Proceedings of the Nutrition Society*, *76*(OCE1), E6.

O'Toole, P. W., & Claesson, M. J. (2010). Gut microbiota: changes throughout the lifespan from infancy to elderly. *International Dairy Journal*, *20*(4), 281-291.

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., & Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behavioural brain research*, *277*, 32-48.

O'Toole, P. W., & Jeffery, I. B. (2015). Gut microbiota and aging. *Science*, *350*(6265), 1214-1215.

O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017). Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nature microbiology*, *2*(5), 1-6.

Ohsawa, K., Nakamura, F., Uchida, N., Mizuno, S., & Yokogoshi, H. (2018). Lactobacillus helveticus-fermented milk containing lactononadecapeptide (NIPPLTQTPVVVPPFLQPE) improves cognitive function in healthy middle-aged adults: A randomised, double-blind, placebo-controlled trial. *International journal of food sciences and nutrition*, *69*(3), 369-376.

Oleskin, A. V., & Shenderov, B. A. (2019). Probiotics and psychobiotics: the role of microbial neurochemicals. *Probiotics and antimicrobial proteins*, *11*, 1071-1085.

Oliphant, K., & Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome*, *7*(1), 1-15.

Otaru, N., Ye, K., Mujezinovic, D., Berchtold, L., Constancias, F., Cornejo, F. A., ... & Pugin, B. (2021). GABA production by human intestinal Bacteroides spp.: Prevalence, regulation, and role in acid stress tolerance. *Frontiers in microbiology*, *12*, 656895.

Otaru, N., Ye, K., Mujezinovic, D., Berchtold, L., Constancias, F., Cornejo, F. A., ... & Pugin, B. (2021). GABA production by human intestinal Bacteroides spp.: Prevalence, regulation, and role in acid stress tolerance. *Frontiers in microbiology*, *12*, 656895.

Owen, L., Reinders, M., Narramore, R., Marsh, A. R., Lui, F. G., Baron, R., ... & Corfe, B. M. (2014). A double blind, placebo controlled, randomised pilot trial examining the effects of probiotic administration on mood and cognitive function. *Proceedings of the Nutrition Society*, *73*(OCE1), E29.

Özoğul, F., Kuley, E., ÖZOĞUL, Y., & ÖZOĞUL, İ. (2012). The function of lactic acid bacteria on biogenic amines production by food-borne pathogens in arginine decarboxylase broth. *Food Science and Technology Research*, *18*(6), 795-804.

Papalini, S., Michels, F., Kohn, N., Wegman, J., van Hemert, S., Roelofs, K., ... & Aarts, E. (2019). Stress matters: randomized controlled trial on the effect of probiotics on neurocognition. *Neurobiology of stress*, *10*, 100141.

Pappolla, M. A., Perry, G., Fang, X., Zagorski, M., Sambamurti, K., & Poeggeler, B. (2021). Indoles as essential mediators in the gut-brain axis. Their role in Alzheimer's disease. *Neurobiology of disease*, *156*, 105403.

Park, S. H., & Lee, H. (2021). Is the center for epidemiologic studies depression scale as useful as the geriatric depression scale in screening for late-life depression? A systematic review. *Journal of Affective Disorders*, *292*, 454-463.

Park, S. H., Kim, K. A., Ahn, Y. T., Jeong, J. J., Huh, C. S., & Kim, D. H. (2015). Comparative analysis of gut microbiota in elderly people of urbanized towns and longevity villages. *BMC microbiology*, *15*(1), 1-9.

Patterson, E., Ryan, P. M., Wiley, N., Carafa, I., Sherwin, E., Moloney, G., ... & Stanton, C. (2019). Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome. *Scientific reports*, *9*(1), 16323.

Pellanda, P., Ghosh, T. S., & O'Toole, P. W. (2021). Understanding the impact of age-related changes in the gut microbiome on chronic diseases and the prospect of elderly-specific dietary interventions. *Current Opinion in Biotechnology*, *70*, 48-55.

Peng, L., Li, Z. R., Green, R. S., Holzman, I. R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *The Journal of nutrition*, *139*(9), 1619-1625.

Perez Chaia, A., & Oliver, G. (2003). Intestinal microflora and metabolic activity. *Gut flora, nutrition, immunity and health*, 77-98.

Pessione, E. (2012). Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Frontiers in cellular and infection microbiology*, *2*, 86.

Petrof, E. O., Claud, E. C., Gloor, G. B., & Allen-Vercoe, E. (2013). Microbial ecosystems therapeutics: a new paradigm in medicine?. *Beneficial microbes*, *4*(1), 53-65.

Pinzone, M. R., Celesia, B. M., Di Rosa, M., Cacopardo, B., & Nunnari, G. (2012). Microbial translocation in chronic liver diseases. *International journal of microbiology*, *2012*.

Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., ... & Versalovic, J. (2017). GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. *Neurogastroenterology & Motility*, *29*(1), e12904.

Potvin, O., Bergua, V., Meillon, C., Le Goff, M., Bouisson, J., Dartigues, J. F., & Amieva, H. (2011). Norms and associated factors of the STAI-Y State anxiety inventory in older adults: results from the PAQUID study. *International psychogeriatrics*, *23*(6), 869-879.

Poveda, C., Pereira, D. I., Lewis, M. C., & Walton, G. E. (2020). The impact of low-level iron supplements on the faecal microbiota of irritable bowel syndrome and healthy donors using in vitro batch cultures. *Nutrients*, *12*(12), 3819.

Purton, T., Staskova, L., Lane, M. M., Dawson, S. L., West, M., Firth, J., ... & Marx, W. (2021). Prebiotic and probiotic supplementation and the tryptophan-kynurenine pathway: A systematic review and meta analysis. *Neuroscience & Biobehavioral Reviews*, *123*, 1-13.

R Core Team. (2022). R: A Language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Radloff, L. S. (1977). The CES-D scale: A self-report depression scale for research in the general population. *Applied psychological measurement*, *1*(3), 385-401.

Rainey-Smith, S. R., Brown, B. M., Sohrabi, H. R., Shah, T., Goozee, K. G., Gupta, V. B., & Martins, R. N. (2016). Curcumin and cognition: a randomised, placebo-controlled, double-blind study of community-dwelling older adults. *British Journal of Nutrition*, *115*(12), 2106-2113.

Rajkumar, H., Kumar, M., Das, N., Kumar, S. N., Challa, H. R., & Nagpal, R. (2015). Effect of probiotic Lactobacillus salivarius UBL S22 and prebiotic fructo-oligosaccharide on serum lipids, inflammatory markers, insulin sensitivity, and gut bacteria in healthy young volunteers: a randomized controlled single-blind pilot study. *Journal of cardiovascular pharmacology and therapeutics*, *20*(3), 289-298.

Ratto, D., Roda, E., Romeo, M., Venuti, M. T., Desiderio, A., Lupo, G., ... & Rossi, P. (2022). The Many Ages of Microbiome–Gut–Brain Axis. *Nutrients*, *14*(14), 2937.

Raymond, C., Marin, M. F., Majeur, D., & Lupien, S. (2018). Early child adversity and psychopathology in adulthood: HPA axis and cognitive dysregulations as potential mechanisms. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *85*, 152-160.

Redruello, B., Saidi, Y., Sampedro, L., Ladero, V., Del Rio, B., & Alvarez, M. A. (2021). GABA-producing Lactococcus lactis strains isolated from camel's milk as starters for the production of GABA-enriched cheese. *Foods*, *10*(3), 633.

Reigstad, C. S., Salmonson, C. E., Rainey III, J. F., Szurszewski, J. H., Linden, D. R., Sonnenburg, J. L., ... & Kashyap, P. C. (2015). Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *The FASEB Journal*, *29*(4), 1395.

Reis, D. J., Ilardi, S. S., & Punt, S. E. (2018). The anxiolytic effect of probiotics: A systematic review and meta-analysis of the clinical and preclinical literature. *PloS one*, *13*(6), e0199041.

Rey, A. (1964). L'e xamen en clinique psychologique.

Rianda, D., Agustina, R., Setiawan, E. A., & Manikam, N. R. M. (2019). Effect of probiotic supplementation on cognitive function in children and adolescents: A systematic review of randomised trials. *Beneficial microbes*, *10*(8), 873-882.

Richardson, A. J., Calder, A. G., Stewart, C. S., & Smith, A. (1989). Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. *Letters in applied microbiology*, *9*(1), 5-8.

Rieger, A., Bengesser, S., Platzer, M., Fellendorf, F., Queissner, R., Birner, A., ... & Reininghaus, E. (2019). SA46THE effects of probiotics on gene expression and cognitive function in depressive patients. *European Neuropsychopharmacology*, *29*, S1213.

Rogers, P. J., & Lloyd, H. M. (1994). Nutrition and mental performance. *Proceedings of the Nutrition Society*, *53*(2), 443-456.

Román, E., Nieto, J. C., Gely, C., Vidal, S., Pozuelo, M., Poca, M., ... & Soriano, G. (2019). Effect of a multistrain probiotic on cognitive function and risk of falls in patients with cirrhosis: a randomized trial. *Hepatology communications*, *3*(5), 632-645.

Roman, P., Estévez, A. F., Miras, A., Sánchez-Labraca, N., Cañadas, F., Vivas, A. B., & Cardona, D. (2018). A pilot randomized controlled trial to explore cognitive and emotional effects of probiotics in fibromyalgia. *Scientific reports*, *8*(1), 1-9.

Romijn, A. R., Rucklidge, J. J., Kuijer, R. G., & Frampton, C. (2017). A double-blind, randomized, placebo-controlled trial of Lactobacillus helveticus and Bifidobacterium longum for the symptoms of depression. *Australian & New Zealand Journal of Psychiatry*, *51*(8), 810-821.

Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. *Nature reviews immunology*, *16*(6), 341-352.

Rudzki, L., Ostrowska, L., Pawlak, D., Małus, A., Pawlak, K., Waszkiewicz, N., & Szulc, A. (2019). Probiotic Lactobacillus Plantarum 299v decreases kynurenine concentration and improves cognitive

functions in patients with major depression: A double-blind, randomized, placebo controlled study. *Psychoneuroendocrinology*, *100*, 213-222.

Ruiz-Gonzalez, C., Cardona, D., Rodriguez-Arrastia, M., Ropero-Padilla, C., Rueda-Ruzafa, L., Carvajal, F., ... & Roman, P. (2022). Effects of probiotics on cognitive and emotional functions in healthy older adults: Protocol for a double-blind randomized placebo-controlled crossover trial. *Research in Nursing & Health*, *45*(3), 274-286.

Rutledge, G. A., Sandhu, A. K., Miller, M. G., Edirisinghe, I., Burton-Freeman, B. B., & Shukitt-Hale, B. (2021). Blueberry phenolics are associated with cognitive enhancement in supplemented healthy older adults. *Food & function*, *12*(1), 107-118.

Sachs, B. C., Chelune, G. J., Rapp, S. R., Couto, A. M., Willard, J. J., Williamson, J. D., ... & Pajewski, N. M. (2022). Robust demographically-adjusted normative data for the Montreal Cognitive Assessment (MoCA): Results from the systolic blood pressure intervention trial. *The Clinical Neuropsychologist*, *36*(8), 2237-2259.

Sakurai, K., Shen, C., Shiraishi, I., Inamura, N., & Hisatsune, T. (2021). Consumption of oleic acid on the preservation of cognitive functions in Japanese elderly individuals. *Nutrients*, *13*(2), 284.

Salazar, N., Arboleya, S., Fernández-Navarro, T., de Los Reyes-Gavilán, C. G., Gonzalez, S., & Gueimonde, M. (2019). Age-associated changes in gut microbiota and dietary components related with the immune system in adulthood and old age: a cross-sectional study. *Nutrients*, *11*(8), 1765.

Samardzic, J., Jadzic, D., Hencic, B., Jancic, J., & Strac, D. S. (2018). Introductory chapter: GABA/Glutamate balance: A key for normal brain functioning. *GABA And Glutamate-New Developments In Neurotransmission Research*, 1-9.

Sanborn, V., Azcarate-Peril, M. A., Updegraff, J., Manderino, L., & Gunstad, J. (2020). Randomized clinical trial examining the impact of Lactobacillus rhamnosus GG probiotic supplementation on cognitive functioning in middle-aged and older adults. *Neuropsychiatric disease and treatment*, 2765-2777.

Sander, C. Y., & Hesse, S. (2017). News and views on in vivo imaging of neurotransmission using PET and MRI. *The quarterly journal of nuclear medicine and molecular imaging: official publication of the*

Italian Association of Nuclear Medicine (AIMN)[and] the International Association of Radiopharmacology (IAR),[and] Section of the Society of..., *61*(4), 414.

Sanders, M. E., Merenstein, D., Merrifield, C. A., & Hutkins, R. (2018). Probiotics for human use. *Nutrition bulletin*, *43*(3), 212-225.

Sandler, R. H., Finegold, S. M., Bolte, E. R., Buchanan, C. P., Maxwell, A. P., Väisänen, M. L., ... & Wexler, H. M. (2000). Short-term benefit from oral vancomycin treatment of regressive-onset autism. *Journal of child neurology*, *15*(7), 429-435.

Santibañez, A., Paine, D., Parra, M., Muñoz, C., Valdes, N., Zapata, C., ... & Tello, M. (2021). Oral administration of Lactococcus lactis producing interferon type ii, enhances the immune response against bacterial pathogens in rainbow trout. *Frontiers in Immunology*, *12*, 696803.

Savignac, H. M., Kiely, B., Dinan, T. G., & Cryan, J. F. (2014). B ifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterology & Motility*, *26*(11), 1615-1627.

Savignac, H. M., Tramullas, M., Kiely, B., Dinan, T. G., & Cryan, J. F. (2015). Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behavioural brain research*, *287*, 59-72.

Schielzeth, H., Dingemanse, N. J., Nakagawa, S., Westneat, D. F., Allegue, H., Teplitsky, C., ... & Araya-Ajoy, Y. G. (2020). Robustness of linear mixed-effects models to violations of distributional assumptions. *Methods in ecology and evolution*, *11*(9), 1141-1152.

Schmidt, C., Collette, F., Cajochen, C., & Peigneux, P. (2007). A time to think: circadian rhythms in human cognition. *Cognitive neuropsychology*, *24*(7), 755-789.

Schmidt-Wilcke, T., Fuchs, E., Funke, K., Vlachos, A., Müller-Dahlhaus, F., Puts, N. A. J., ... & Edden, R. A. (2018). GABA—from inhibition to cognition: emerging concepts. The Neuroscientist, 24(5), 501-515.

Scholey, A. B., French, S. J., Morris, P. J., Kennedy, D. O., Milne, A. L., & Haskell, C. F. (2010). Consumption of cocoa flavanols results in acute improvements in mood and cognitive performance during sustained mental effort. *Journal of Psychopharmacology*, *24*(10), 1505-1514.

Schütte, K., Schulz, C., Vilchez-Vargas, R., Vasapolli, R., Palm, F., Simon, B., ... & Malfertheiner, P. (2021). Impact of healthy aging on active bacterial assemblages throughout the gastrointestinal tract. *Gut Microbes*, *13*(1), 1966261.

Schwarcz, R., Bruno, J. P., Muchowski, P. J., & Wu, H. Q. (2012). Kynurenines in the mammalian brain: when physiology meets pathology. *Nature Reviews Neuroscience*, *13*(7), 465-477.

Senan, S., Prajapati, J. B., Joshi, C. G., Sreeja, V., Gohel, M. K., Trivedi, S., ... & Patel, H. A. (2015). Geriatric respondents and non-respondents to probiotic intervention can be differentiated by inherent gut microbiome composition. *Frontiers in microbiology*, *6*, 944.

Settanni, C. R., Ianiro, G., Bibbò, S., Cammarota, G., & Gasbarrini, A. (2021). Gut microbiota alteration and modulation in psychiatric disorders: Current evidence on fecal microbiota transplantation. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *109*, 110258.

Sgritta, M., Dooling, S. W., Buffington, S. A., Momin, E. N., Francis, M. B., Britton, R. A., & Costa-Mattioli, M. (2019). Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder. *Neuron*, *101*(2), 246-259.

Shajib, M. S., & Khan, W. I. (2015). The role of serotonin and its receptors in activation of immune responses and inflammation. *Acta physiologica*, *213*(3), 561-574.

Shields, G. S., Sazma, M. A., & Yonelinas, A. P. (2016). The effects of acute stress on core executive functions: A meta-analysis and comparison with cortisol. *Neuroscience & Biobehavioral Reviews*, *68*, 651-668.

Silva, Y. P., Bernardi, A., & Frozza, R. L. (2020). The role of short-chain fatty acids from gut microbiota in gut-brain communication. *Frontiers in endocrinology*, *11*, 25.

Simensky, J. D., & Abeles, N. (2002). Decline in verbal memory performance with advancing age: the role of frontal lobe functioning. *Aging & Mental Health*, *6*(3), 293-303.

Singmann, H., & Kellen, D. (2019). An introduction to mixed models for experimental psychology. In *New methods in cognitive psychology* (pp. 4-31). Routledge.

Sivieri, K., Morales, M. L. V., Adorno, M. A. T., Sakamoto, I. K., Saad, S. M. I., & Rossi, E. A. (2013). Lactobacillus acidophilus CRL 1014 improved "gut health" in the SHIME® reactor. *BMC gastroenterology*, *13*, 1-9.

Slykerman, R. F., Kang, J., Van Zyl, N., Barthow, C., Wickens, K., Stanley, T., ... & Mitchell, E. A. (2018). Effect of early probiotic supplementation on childhood cognition, behaviour and mood a randomised, placebo-controlled trial. *Acta Paediatrica*, *107*(12), 2172-2178.

Sochocka, M., Donskow-Łysoniewska, K., Diniz, B. S., Kurpas, D., Brzozowska, E., & Leszek, J. (2019). The gut microbiome alterations and inflammation-driven pathogenesis of Alzheimer's disease—a critical review. *Molecular neurobiology*, *56*, 1841-1851.

Song, A. A. L., In, L. L., Lim, S. H. E., & Rahim, R. A. (2017). A review on Lactococcus lactis: from food to factory. *Microbial cell factories*, *16*(1), 1-15.

Song, Y., Gong, T., Edden, R. A., & Wang, G. (2020). Feasibility of measuring GABA levels in the upper brainstem in healthy volunteers using edited MRS. *Frontiers in Psychiatry*, *11*, 813.

Spielberger, C. D. (1983). State-trait anxiety inventory for adults.

Staal, M. A., & Bolton, A. E. (2008). Cognitive Performance and Resilience to Stress RITA A. YAROUSH, AND LYLE E. BOURNE Jr. In *Biobehavioral resilience to stress* (pp. 281-322). Routledge.

Stackebrandt, E. (2014). 23 The Emended Family Peptococcaceae and Description of the Families Desulfitobacteriaceae, Desulfotomaculaceae, and Thermincolaceae.

Steenbergen, L., Sellaro, R., van Hemert, S., Bosch, J. A., & Colzato, L. S. (2015). A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, behavior, and immunity*, *48*, 258-264.

Steinberg, S. I., Negash, S., Sammel, M. D., Bogner, H., Harel, B. T., Livney, M. G., ... & Arnold, S. E. (2013). Subjective memory complaints, cognitive performance, and psychological factors in healthy older adults. *American Journal of Alzheimer's Disease & Other Dementias®*, *28*(8), 776-783.

Stoidis, C. N., Misiakos, E. P., Patapis, P., Fotiadis, C. I., & Spyropoulos, B. G. (2011). Potential benefits of pro-and prebiotics on intestinal mucosal immunity and intestinal barrier in short bowel syndrome. *Nutrition research reviews*, *24*(1), 21-30.

Strandwitz, P. (2018). Neurotransmitter modulation by the gut microbiota. *Brain research*, *1693*, 128-133.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X. N., ... & Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *The Journal of physiology*, *558*(1), 263-275.

Sun, J., Xu, J., Ling, Y., Wang, F., Gong, T., Yang, C., ... & Liu, J. (2019). Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Translational psychiatry*, *9*(1), 189.

Sweeney, J. A., Rosano, C., Berman, R. A., & Luna, B. (2001). Inhibitory control of attention declines more than working memory during normal aging. *Neurobiology of aging*, *22*(1), 39-47.

Swidsinski, A., Loening–Baucke, V., Verstraelen, H., Osowska, S., & Doerffel, Y. (2008). Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. *Gastroenterology*, *135*(2), 568-579.

Szalardy, L., Zadori, D., Toldi, J., Fulop, F., Klivenyi, P., & Vecsei, L. (2012). Manipulating kynurenic acid levels in the brain–on the edge between neuroprotection and cognitive dysfunction. Current topics in medicinal chemistry, 12(16), 1797-1806.

Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S. B., ... & Jolivet-Gougeon, A. (2017). Roseburia spp.: a marker of health?. *Future microbiology*, *12*(2), 157-170.

Tamtaji, O. R., Heidari-Soureshjani, R., Mirhosseini, N., Kouchaki, E., Bahmani, F., Aghadavod, E., ... & Asemi, Z. (2019). Probiotic and selenium co-supplementation, and the effects on clinical, metabolic and genetic status in Alzheimer's disease: a randomized, double-blind, controlled trial. *Clinical Nutrition*, *38*(6), 2569-2575.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Advances in immunology*, *121*, 91-119.

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., Wang, B., & Cao, H. (2020). Current sampling methods for gut microbiota: a call for more precise devices. *Frontiers in cellular and infection microbiology*, 151.

Tengeler, A. C., Dam, S. A., Wiesmann, M., Naaijen, J., Van Bodegom, M., Belzer, C., ... & Kiliaan, A. J. (2020). Gut microbiota from persons with attention-deficit/hyperactivity disorder affects the brain in mice. *Microbiome*, *8*, 1-14.

Tette, F. M., Kwofie, S. K., & Wilson, M. D. (2022). Therapeutic anti-depressant potential of microbial GABA produced by Lactobacillus rhamnosus strains for GABAergic signaling restoration and inhibition of addiction-induced HPA axis hyperactivity. *Current Issues in Molecular Biology*, *44*(4), 1434-1451.

Tillisch, K., Labus, J., Kilpatrick, L., Jiang, Z., Stains, J., Ebrat, B., ... & Mayer, E. A. (2013). Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology*, *144*(7), 1394-1401.

Tillisch, K., Mayer, E., Gupta, A., Gill, Z., Brazeilles, R., Le Nevé, B., ... & Labus, J. (2017). Brain structure and response to emotional stimuli as related to gut microbial profiles in healthy women. *Psychosomatic medicine*, *79*(8), 905.

Ton, A. M. M., Campagnaro, B. P., Alves, G. A., Aires, R., Côco, L. Z., Arpini, C. M., ... & Vasquez, E. C. (2020). Oxidative stress and dementia in Alzheimer’s patients: effects of synbiotic supplementation. *Oxidative medicine and cellular longevity*, *2020*.

Trzepacz, P. T., Hochstetler, H., Wang, S., Walker, B., Saykin, A. J., & Alzheimer’s Disease Neuroimaging Initiative. (2015). Relationship between the Montreal Cognitive Assessment and Mini-mental State Examination for assessment of mild cognitive impairment in older adults. *BMC geriatrics*, *15*, 1-9.

Tsavkelova, E. A., Botvinko, I. V., Kudrin, V. S., & Oleskin, A. V. (2000). Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. In *Doklady Biochemistry* (Vol. 372, No. 1-6, pp. 115-117). Общество с ограниченной ответственностью Международная академическая издательская компания" Наука/Интерпериодика".

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized,

controlled, double-blind, crossover intervention study. *The American journal of clinical nutrition*, *93*(1), 62-72.

Tzounis, X., Vulevic, J., Kuhnle, G. G., George, T., Leonczak, J., Gibson, G. R., ... & Spencer, J. P. (2008). Flavanol monomer-induced changes to the human faecal microflora. *British journal of nutrition*, *99*(4), 782-792.

Vakharia, K., & Hinson, J. P. (2005). Lipopolysaccharide directly stimulates cortisol secretion by human adrenal cells by a cyclooxygenase-dependent mechanism. *Endocrinology*, *146*(3), 1398-1402.

Valles-Colomer, M., Falony, G., Darzi, Y., Tigchelaar, E. F., Wang, J., Tito, R. Y., ... & Raes, J. (2019). The neuroactive potential of the human gut microbiota in quality of life and depression. *Nature microbiology*, *4*(4), 623-632.

Van de Wouw, M., Boehme, M., Lyte, J. M., Wiley, N., Strain, C., O'Sullivan, O., ... & Cryan, J. F. (2018). Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain–gut axis alterations. *The Journal of physiology*, *596*(20), 4923-4944.

Van den Abbeele, P., Roos, S., Eeckhaut, V., MacKenzie, D. A., Derde, M., Verstraete, W., ... & Van de Wiele, T. (2012). Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. *Microbial biotechnology*, *5*(1), 106-115.

Van der Does, A. J. W., & Williams, J. M. G. (2003). Leiden index of depression sensitivity–revised (LEIDS-R). *Leiden University*.

van Hemert, S., Verwer, J., & Schütz, B. (2013). Clinical studies evaluating effects of probiotics on parameters of intestinal barrier function.

Van Honk, J., Kessels, R. P. C., Putman, P., Jager, G., Koppeschaar, H. P. F., & Postma, A. (2003). Attentionally modulated effects of cortisol and mood on memory for emotional faces in healthy young males. *Psychoneuroendocrinology*, *28*(7), 941-948.

Vasiliu, O. (2023). Is fecal microbiota transplantation a useful therapeutic intervention for psychiatric disorders? A narrative review of clinical and preclinical evidence. *Current Medical Research and Opinion*, *39*(1), 161-177.

Vazey, E. M., & Aston-Jones, G. (2012). The emerging role of norepinephrine in cognitive dysfunctions of Parkinson's disease. Frontiers in behavioral neuroscience, 6, 48

Villegas, J. M., Brown, L., de Giori, G. S., & Hebert, E. M. (2016). Optimization of batch culture conditions for GABA production by Lactobacillus brevis CRL 1942, isolated from quinoa sourdough. *LWT-Food Science and Technology*, *67*, 22-26.

Vishwakarma, S., Goyal, R., Gupta, V., & Dhar, K. L. (2016). GABAergic effect of valeric acid from Valeriana wallichii in amelioration of ICV STZ induced dementia in rats. *Revista Brasileira de Farmacognosia*, *26*, 484-489.

Vogt, N. M., Kerby, R. L., Dill-McFarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., ... & Rey, F. E. (2017). Gut microbiome alterations in Alzheimer's disease. *Scientific reports*, *7*(1), 13537.

Volokh, O., Klimenko, N., Berezhnaya, Y., Tyakht, A., Nesterova, P., Popenko, A., & Alexeev, D. (2019). Human gut microbiome response induced by fermented dairy product intake in healthy volunteers. *Nutrients*, *11*(3), 547.

Walczak, K., Dąbrowski, W., Langner, E., Zgrajka, W., Piłat, J., Kocki, T., ... & Turski, W. A. (2011). Kynurenic acid synthesis and kynurenine aminotransferases expression in colon derived normal and cancer cells. *Scandinavian journal of gastroenterology*, *46*(7-8), 903-912.

Walker, A. W., Duncan, S. H., McWilliam Leitch, E. C., Child, M. W., & Flint, H. J. (2005). pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Applied and environmental microbiology*, *71*(7), 3692-3700.

Wallis, A., Ball, M., Butt, H., Lewis, D. P., McKechnie, S., Paull, P., ... & Bruck, D. (2018). Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: neuropsychological symptoms and sex comparisons. *Journal of Translational Medicine*, *16*, 1-16.

Wallner, G., Amann, R., & Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry: The Journal of the International Society for Analytical Cytology*, *14*(2), 136-143.

Walrath, T., Dyamenahalli, K. U., Hulsebus, H. J., McCullough, R. L., Idrovo, J. P., Boe, D. M., ... & Kovacs, E. J. (2021). Age-related changes in intestinal immunity and the microbiome. *Journal of Leucocyte Biology*, *109*(6), 1045-1061.

Walton, G. E., van den Heuvel, E. G., Kosters, M. H., Rastall, R. A., Tuohy, K. M., & Gibson, G. R. (2012). A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *British Journal of Nutrition*, *107*(10), 1466-1475.

Wang, F. B., & Powley, T. L. (2007). Vagal innervation of intestines: afferent pathways mapped with new en bloc horseradish peroxidase adaptation. *Cell and tissue research*, *329*(2), 221-230.

Wang, H., Braun, C., Murphy, E. F., & Enck, P. (2019). Bifidobacterium longum 1714™ strain modulates brain activity of healthy volunteers during social stress. *The American journal of gastroenterology*, *114*(7), 1152.

Wang, H., Lee, I. S., Braun, C., & Enck, P. (2016). Effect of probiotics on central nervous system functions in animals and humans: a systematic review. *Journal of neurogastroenterology and motility*, *22*(4), 589.

Wang, J., Ji, H., Wang, S., Liu, H., Zhang, W., Zhang, D., & Wang, Y. (2018). Probiotic Lactobacillus plantarum promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Frontiers in microbiology*, *9*, 1953.

Wang, L., Zhang, J., Guo, Z., Kwok, L., Ma, C., Zhang, W., ... & Zhang, H. (2014). Effect of oral consumption of probiotic Lactobacillus planatarum P-8 on fecal microbiota, SIgA, SCFAs, and TBAs of adults of different ages. *Nutrition*, *30*(7-8), 776-783.

Wang, X., & Gibson, G. R. (1993). Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *Journal of applied microbiology*, *75*(4), 373-380.

Wang, Y., Li, N., Yang, J. J., Zhao, D. M., Chen, B., Zhang, G. Q., ... & Gai, Z. T. (2020). Probiotics and fructo-oligosaccharide intervention modulate the microbiota-gut brain axis to improve autism spectrum reducing also the hyper-serotonergic state and the dopamine metabolism disorder. *Pharmacological research*, *157*, 104784.

Wang, Y., Pan, F., Xie, F., He, R., & Guo, Q. (2022). Correlation between urine formaldehyde and cognitive abilities in the clinical spectrum of Alzheimer's disease. *Frontiers in Aging Neuroscience*, *14*, 57.

Wasylyshyn, C., Verhaeghen, P., & Sliwinski, M. J. (2011). Aging and task switching: a meta-analysis. *Psychology and aging*, *26*(1), 15.

Watson, D., & Clark, L. A. (1994). The PANAS-X: Manual for the positive and negative affect schedule-expanded form.

Weaver Cargin, J., Maruff, P., Collie, A., Shafiq-Antonacci, R., & Masters, C. (2007). Decline in verbal memory in non-demented older adults. *Journal of clinical and experimental neuropsychology*, *29*(7), 706-718.

Wells, T. T., Beevers, C. G., & McGeary, J. E. (2010). Serotonin transporter and BDNF genetic variants interact to predict cognitive reactivity in healthy adults. *Journal of Affective Disorders*, *126*(1-2), 223-229.

White, J. S., Hoper, M., Parks, R. W., Clements, W. D. B., Diamond, T., & Bengmark, S. (2006). The probiotic bacterium Lactobacillus plantarum species 299 reduces intestinal permeability in experimental biliary obstruction. *Letters in applied microbiology*, *42*(1), 19-23.

Whyte, A. R., Cheng, N., Butler, L. T., Lamport, D. J., & Williams, C. M. (2019). Flavonoid-rich mixed berries maintain and improve cognitive function over a 6 h period in young healthy adults. *Nutrients*, *11*(11), 2685.

Whyte, A. R., Schafer, G., & Williams, C. M. (2017). The effect of cognitive demand on performance of an executive function task following wild blueberry supplementation in 7 to 10 years old children. *Food & Function*, *8*(11), 4129-4138.

Wieërs, G., Belkhir, L., Enaud, R., Leclercq, S., Philippart de Foy, J. M., Dequenne, I., ... & Cani, P. D. (2020). How probiotics affect the microbiota. *Frontiers in cellular and infection microbiology*, *9*, 454.

Wilcox, R. R. (1987). New designs in analysis of variance. Annual Review of Psychology, 38(1), 29–60.

Williams, C. F., Walton, G. E., Jiang, L., Plummer, S., Garaiova, I., & Gibson, G. R. (2015). Comparative analysis of intestinal tract models. *Annual review of food science and technology*, *6*, 329-350.

Williams, T. J., Reay, A. J., Whitwood, A. C., & Fairlamb, I. J. (2014). A mild and selective Pd-mediated methodology for the synthesis of highly fluorescent 2-arylated tryptophans and tryptophan-containing peptides: a catalytic role for Pd 0 nanoparticles?. *Chemical Communications*, *50*(23), 3052-3054.

Wilmanski, T., Diener, C., Rappaport, N., Patwardhan, S., Wiedrick, J., Lapidus, J., ... & Price, N. D. (2021). Gut microbiome pattern reflects healthy ageing and predicts survival in humans. *Nature metabolism*, *3*(2), 274-286.

Wirthgen, E., Hoeflich, A., Rebl, A., & Günther, J. (2018). Kynurenic acid: the Janus-faced role of an immunomodulatory tryptophan metabolite and its link to pathological conditions. *Frontiers in immunology*, *8*, 1957.

Woodmansey, E. J., McMurdo, M. E., Macfarlane, G. T., & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and environmental microbiology*, *70*(10), 6113-6122.

Wright, L., Lipszyc, J., Dupuis, A., Thayapararajah, S. W., & Schachar, R. (2014). Response inhibition and psychopathology: a meta-analysis of go/no-go task performance. *Journal of abnormal psychology*, *123*(2), 429.

Wu, L., Han, Y., Zheng, Z., Peng, G., Liu, P., Yue, S., ... & Wang, B. (2021). Altered gut microbial metabolites in amnestic mild cognitive impairment and Alzheimer's disease: signals in host–microbe interplay. *Nutrients*, *13*(1), 228.

Xiao, J., Katsumata, N., Bernier, F., Ohno, K., Yamauchi, Y., Odamaki, T., ... & Kaneko, T. (2020). Probiotic Bifidobacterium breve in improving cognitive functions of older adults with suspected mild cognitive impairment: a randomized, double-blind, placebo-controlled trial. *Journal of Alzheimer's Disease*, *77*(1), 139-147.

Xiao, W., Li, J., Gao, X., Yang, H., Su, J., Weng, R., ... & Gu, Y. (2022). Involvement of the gut-brain axis in vascular depression via tryptophan metabolism: A benefit of short chain fatty acids. *Experimental Neurology*, *358*, 114225.

Xu, M., Xu, X., Li, J., & Li, F. (2019). Association between gut microbiota and autism spectrum disorder: a systematic review and meta-analysis. *Frontiers in psychiatry*, *10*, 473.

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., ... & Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*, *161*(2), 264-276.

Yilmaz, A., Ugur, Z., Bisgin, H., Akyol, S., Bahado-Singh, R., Wilson, G., ... & Graham, S. F. (2020). Targeted metabolic profiling of urine highlights a potential biomarker panel for the diagnosis of Alzheimer's disease and mild cognitive impairment: a pilot study. *Metabolites*, *10*(9), 357.

Yoshikawa, K., Shimizu, M., Matsumura, T., & Sakuragawa, A. (2018). Determination of various inorganic anions and organic acids in the atmospheric particulate matter using capillary zone electrophoresis with indirect UV detection. *International Journal of Environmental Analytical Chemistry*, *98*(8), 789-798.

Yu, D., Xia, Y., Ge, L., Tan, B., & Chen, S. (2021). Effects of Lactococcus lactis on the intestinal functions in weaning piglets. *Frontiers in Nutrition*, *8*, 713256.

Zaragoza-Martí, A., Ruiz-Robledillo, N., Sánchez-SanSegundo, M., Albaladejo-Blázquez, N., Hurtado-Sánchez, J. A., & Ferrer-Cascales, R. (2020). Eating habits in older adults: compliance with the recommended daily intakes and its relationship with sociodemographic characteristics, clinical conditions, and lifestyles. *Nutrients*, *12*(2), 446.

Zaragozá, R. (2020). Transport of amino acids across the blood-brain barrier. *Frontiers in physiology*, *11*, 973.

Zareie, M., Johnson-Henry, K., Jury, J., Yang, P. C., Ngan, B. Y., McKay, D. M., ... & Sherman, P. M. (2006). Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. *Gut*, *55*(11), 1553-1560.

Zhao, Y., & Lukiw, W. J. (2018). Bacteroidetes neurotoxins and inflammatory neurodegeneration. *Molecular Neurobiology*, *55*, 9100-9107.

Zhao, Z., Wang, B., Mu, L., Wang, H., Luo, J., Yang, Y., ... & Tao, C. (2020). Long-term exposure to ceftriaxone sodium induces alteration of gut microbiota accompanied by abnormal behaviors in mice. *Frontiers in cellular and infection microbiology*, *10*, 258.

Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., ... & Xie, P. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Molecular psychiatry*, *21*(6), 786-796.

Zhu, F., Ju, Y., Wang, W., Wang, Q., Guo, R., Ma, Q., ... & Ma, X. (2020). Metagenome-wide association of gut microbiome features for schizophrenia. *Nature communications*, *11*(1), 1612.

Appendices

Appendix 1 – Quality Criteria Checklist from the Academy of Nutrition and Dietetics

Quality Criteria Checklist: Primary Research

Symbols Used

+ **Positive:** Indicates that the report has clearly addressed issues of inclusion/exclusion, bias, generalizability, and data collection and analysis.

-- **Negative:** Indicates that these issues have not been adequately addressed.

Ø **Neutral:** Indicates that the report is neither exceptionally strong nor exceptionally weak.

Quality Criteria Checklist: Primary Research

RELEVANCE QUESTIONS	
1. Would implementing the studied intervention or procedure (if found successful) result in improved outcomes for the patients/clients/population group? (NA for some Epi studies)	Yes No Unclear N/A
2. Did the authors study an outcome (dependent variable) or topic that the patients/clients/population group would care about?	Yes No Unclear N/A
3. Is the focus of the intervention or procedure (independent variable) or topic of study a common issue of concern to dietetics practice?	Yes No Unclear N/A
4. Is the intervention or procedure feasible? (NA for some epidemiological studies)	Yes No Unclear N/A
If the answers to all of the above relevance questions are "Yes," the report is eligible for designation with a plus (+) on the Evidence Quality Worksheet, depending on answers to the following validity questions.	
VALIDITY QUESTIONS	
1. Was the research question clearly stated?	Yes No Unclear N/A
1.1 Was the specific intervention(s) or procedure (independent variable(s)) identified?	
1.2 Was the outcome(s) (dependent variable(s)) clearly indicated?	
1.3 Were the target population and setting specified?	
2. Was the selection of study subjects/patients free from bias?	Yes No Unclear N/A
2.1 Were inclusion/exclusion criteria specified (e.g., risk, point in disease progression, diagnostic or prognosis criteria), and with sufficient detail and without omitting criteria critical to the study?	
2.2 Were criteria applied equally to all study groups?	
2.3 Were health, demographics, and other characteristics of subjects described?	
2.4 Were the subjects/patients a representative sample of the relevant population?	
3. Were study groups comparable?	Yes No Unclear N/A
3.1 Was the method of assigning subjects/patients to groups described and unbiased? (Method of randomization identified if RCT)	
3.2 Were distribution of disease status, prognostic factors, and other factors (e.g., demographics) similar across study groups at baseline?	
3.3 Were concurrent controls used? (Concurrent preferred over historical controls.)	
3.4 If cohort study or cross-sectional study, were groups comparable on important confounding factors and/or were preexisting differences accounted for by using appropriate adjustments in statistical analysis?	
3.5 If case control study, were potential confounding factors comparable for cases and controls? (If case series or trial with subjects serving as own control, this criterion is not applicable. Criterion may not be applicable in some cross-sectional studies.)	
3.6 If diagnostic test, was there an independent blind comparison with an appropriate reference standard (e.g., "gold standard")?	
4. Was method of handling withdrawals described?	Yes No Unclear N/A
4.1 Were follow up methods described and the same for all groups?	
4.2 Was the number, characteristics of withdrawals (i.e., dropouts, lost to follow up, attrition rate) and/or response rate (cross-sectional studies) described for each group? (Follow up goal for a strong study is 80%.)	
4.3 Were all enrolled subjects/patients (in the original sample) accounted for?	
4.4 Were reasons for withdrawals similar across groups?	
4.5 If diagnostic test, was decision to perform reference test not dependent on results of test under study?	
5. Was blinding used to prevent introduction of bias?	Yes No Unclear N/A
5.1 In intervention study, were subjects, clinicians/practitioners, and investigators blinded to treatment group, as appropriate?	

	5.2	Were data collectors blinded for outcomes assessment? (If outcome is measured using an objective test, such as a lab value, this criterion is assumed to be met.)	
	5.3	In cohort study or cross-sectional study, were measurements of outcomes and risk factors blinded?	
	5.4	In case control study, was case definition explicit and case ascertainment not influenced by exposure status?	
	5.5	In diagnostic study, were test results blinded to patient history and other test results?	
6.	**Were intervention/therapeutic regimens/exposure factor or procedure and any comparison(s) described in detail? Were intervening factors described?**		Yes No Unclear N/A
	6.1	In RCT or other intervention trial, were protocols described for all regimens studied?	
	6.2	n observational study, were interventions, study settings, and clinicians/provider described?	
	6.3	Was the intensity and duration of the intervention or exposure factor sufficient to produce a meaningful effect?	
	6.4	Was the amount of exposure and, if relevant, subject/patient compliance measured?	
	6.5	Were co-interventions (e.g., ancillary treatments, other therapies) described?	
	6.6	Were extra or unplanned treatments described?	
	6.7	Was the information for 6.4, 6.5, and 6.6 assessed the same way for all groups?	
	6.8	In diagnostic study, were details of test administration and replication sufficient?	
7.	**Were outcomes clearly defined and the measurements valid and reliable?**		Yes No Unclear N/A
	7.1	Were primary and secondary endpoints described and relevant to the question?	
	7.2	Were nutrition measures appropriate to question and outcomes of concern?	
	7.3	Was the period of follow-up long enough for important outcome(s) to occur?	
	7.4	Were the observations and measurements based on standard, valid, and reliable data collection instruments/tests/procedures?	
	7.5	Was the measurement of effect at an appropriate level of precision?	
	7.6	Were other factors accounted for (measured) that could affect outcomes?	
	7.7	Were the measurements conducted consistently across groups?	
8.	**Was the statistical analysis appropriate for the study design and type of outcome indicators?**		Yes No Unclear N/A
	8.1	Were statistical analyses adequately described the results reported appropriately?	
	8.2	Were correct statistical tests used and assumptions of test not violated?	
	8.3	Were statistics reported with levels of significance and/or confidence intervals?	
	8.4	Was "intent to treat" analysis of outcomes done (and as appropriate, was there an analysis of outcomes for those maximally exposed or a dose-response analysis)?	
	8.5	Were adequate adjustments made for effects of confounding factors that might have affected the outcomes (e.g., multivariate analyses)?	
	8.6	Was clinical significance as well as statistical significance reported?	
	8.7	If negative findings, was a power calculation reported to address type 2 error?	
9.	**Are conclusions supported by results with biases and limitations taken into consideration?**		Yes No Unclear N/A
	9.1	Is there a discussion of findings?	
	9.2	Are biases and study limitations identified and discussed?	
10.	**Is bias due to study's funding or sponsorship unlikely?**		Yes No Unclear N/A
	10.1	Were sources of funding and investigators' affiliations described?	
	10.2	Was there no apparent conflict of interest?	

MINUS/NEGATIVE (-)

If most (six or more) of the answers to the above validity questions are "No," the report should be designated with a minus (-) symbol on the Evidence Worksheet.

NEUTRAL (Ø)

If the answers to validity criteria questions 2, 3, 6, and 7 do not indicate that the study is exceptionally strong, the report should be designated with a neutral (∅) symbol on the Evidence Worksheet.

PLUS/POSITIVE (+)

If most of the answers to the above validity questions are "Yes" (including criteria 2, 3, 6, 7 and at least one additional "Yes"), the report should be designated with a plus symbol (+) on the Evidence Worksheet.

Appendix 2 – Data extraction template for primary research from the Academy of Nutrition and Dietetics

Academy of Nutrition and Dietetics
Evidence Analysis Library® Worksheet Template and Quality Criteria Checklist: Primary Research

Citation	
Study Design	
Class	
Quality Rating	☐ + (Positive) ☐ - (Negative) ☐ ⦸ (Neutral)
Research Purpose	
Inclusion Criteria	
Exclusion Criteria	
Description of Study Protocol	Recruitment: Design: Blinding used (if applicable): Intervention (if applicable): Statistical Analysis:
Data Collection Summary	Timing of Measurements: Dependent Variables: Independent Variables: Control Variables:
Description of Actual Data Sample	Initial: (Males Females) Attrition (final N): Age: Ethnicity: Other relevant demographics: Anthropometrics: Location:
Summary of Results	Key Findings: Other Findings:
Author Conclusion	
Reviewer Comments	
Funding Source	

Appendix 3 – Results of the Quality Criteria Checklist from the Academy of Nutrition and Dietetics

	Adikari et al., (2020)	Agahi et al., (2018)	Akar et al., (2017)	Akbari et al., (2016)	Allen et al., (2016)	Asaoka et al., (2022)	Bagga et al., (2018)	Bajaj et al., (2014)	Benton et al., (2007)	Ceccarelli et al., (2017a)	Ceccarelli et al., (2017b)	Chou et al., (2010)	Chung et al., (2014)	Czajeczny et al., (2021)	Firmansyah et al., (2011)	Hwang et al., (2019)	Inoue et al., (2018)	Jacobs et al., (2017)	Kelly et al., (2017)	Kim et al., (2021)	Kobayashi et al., (2019a)	Kobayashi et al., (2019b)	Lew et al., (2019)	Lunia et al., (2014)	Malaguamera et al., (2010)	Nobile et al., (2022)	Ohsawa et al., (2018)	Papalini et al., (2019)	Roman et al., (2018)	Román et al., (2019)	Rudki et al., (2019)	Sanborn et al., (2020)	Slykerman et al., (2018)	Tamtaji et al., (2018)	Tillisch et al., (2013)	Ton et al., (2020)	Wallis et al., (2018)	Xiao et al., (2020)
Overall quality rating	N/P	N	N	N	N	N/P	N	N	N	N	N	N	N	N	N/P	N	N	N	N	N/P	N	N	N	N	N	N	N/P	N/P	N/P	N	N/P	N/P	N	N	N	N	N	N/P
Class	A	A	A	A	C	A	A	A	A	D	C	A	A	C	A	A	A	A	A	A	D	A	A	A	A	D	A	A	A	A	A	A	A	A	A	D	D	
Relevance questions																																						
1. Would Implementing the studied intervention or procedure (if found successful) result in	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

improved outcomes for the patients/clients/population group?																																						
2. Did the authors study an outcome (dependent variable) or topic that the patients/clients/population would care about?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3. Is the focus of the intervention or procedure (independent variable) or topic of study a common issue of concern in dietetics practice?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
4. Is the intervention or procedure feasible?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Validity questions																																						
1. Was the research question clearly stated?	Y	U	U	Y	Y	Y	Y	U	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	U	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2. Was the selection of the study subjects/patients free from bias?	Y	Y	Y	Y	Y	Y	U	U	Y	U	Y	U	U	Y	U	U	Y	U	Y	Y	Y	Y	U	U	Y	U	U	U	Y	U	U	Y	U	U	Y	N	Y	Y
3. Were study groups comparable?	Y	N	Y	U	Y	Y	U	Y	Y	N/A	N	Y	Y	Y	Y	Y	Y	Y	U	Y	N/A	N	Y	Y	Y	N/A	Y	Y	Y	Y	Y	Y	Y	U	Y	N/A	N/A	Y
4. Was method of handling withdrawals described?	Y	Y	Y	Y	U	Y	U	U	Y	Y	Y	Y	Y	Y	Y	U	Y	U	U	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	U	Y	Y	U	U	U	Y	Y	Y
5. Was blinding used to prevent introduction to bias?	Y	Y	U	Y	N	Y	Y	Y	Y	N	N	U	Y	N	Y	Y	Y	Y	N	Y	N	Y	Y	N	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Y
6. Were intervention/therapeutic regimens/exposure factor or procedure and any	U	U	U	U	Y	Y	Y	U	U	Y	Y	Y	Y	Y	Y	Y	U	U	Y	Y	U	U	Y	U	U	U	U	Y	U	Y	Y	Y	U	U	Y	U	Y	U

comparison(s) described in detail? Were intervening factors described?																																						
7. Were outcomes clearly defined and the measurements valid and reliable?	U	Y	Y	Y	U	U	Y	U	U	Y	Y	U	U	Y	Y	Y	U	Y	Y	U	Y	U	Y	U	Y	Y	Y	Y	Y	Y	Y	U	U	Y	Y	Y	Y	Y
8. Was the statistical analysis appropriate for the study design and type of outcome indicators?	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	U	U	U	U	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	U	U	U	Y	Y	Y	Y	Y	Y	Y	U	U	Y	Y
9. Are conclusions supported by results with biases and limitations taken into consideration	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
10. Is bias due to study's	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

funding unlikely?																																						

Appendix 4 – Batch culture data (short-chain fatty acids, neurotransmitters and bacterial enumeration) presented by donor for comparison.

Short chain fatty acids (including positive control vessel)

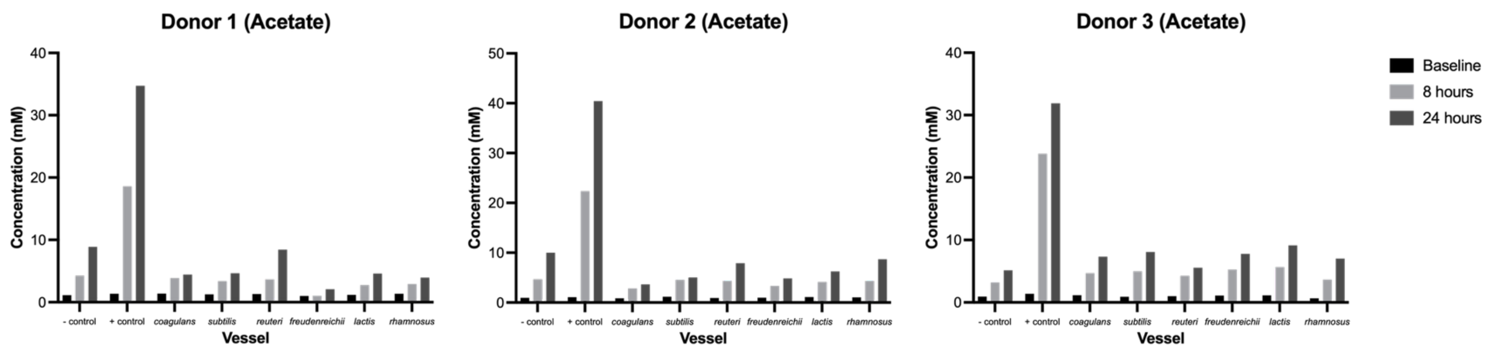


Figure 1 – Concentration (mM) of acetate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

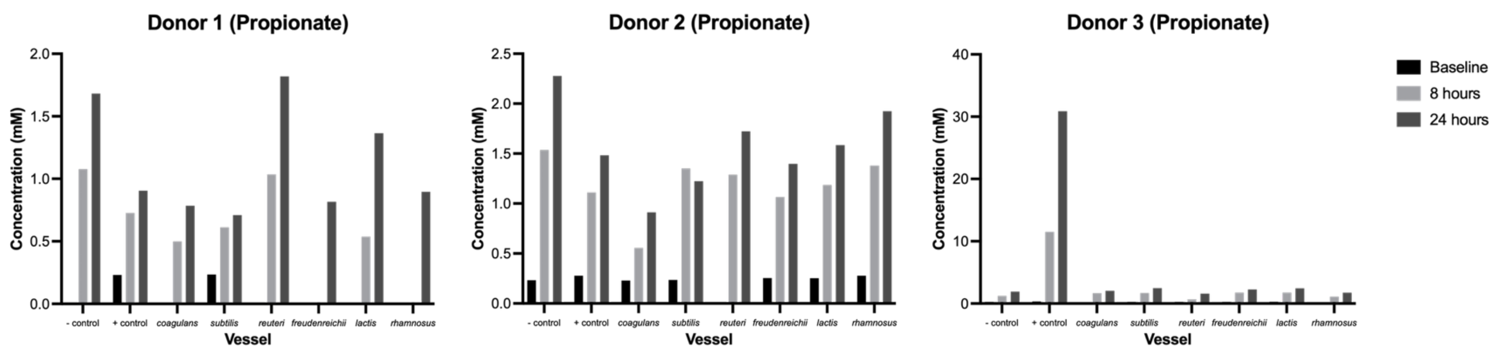


Figure 4 – Concentration (mM) of propionate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

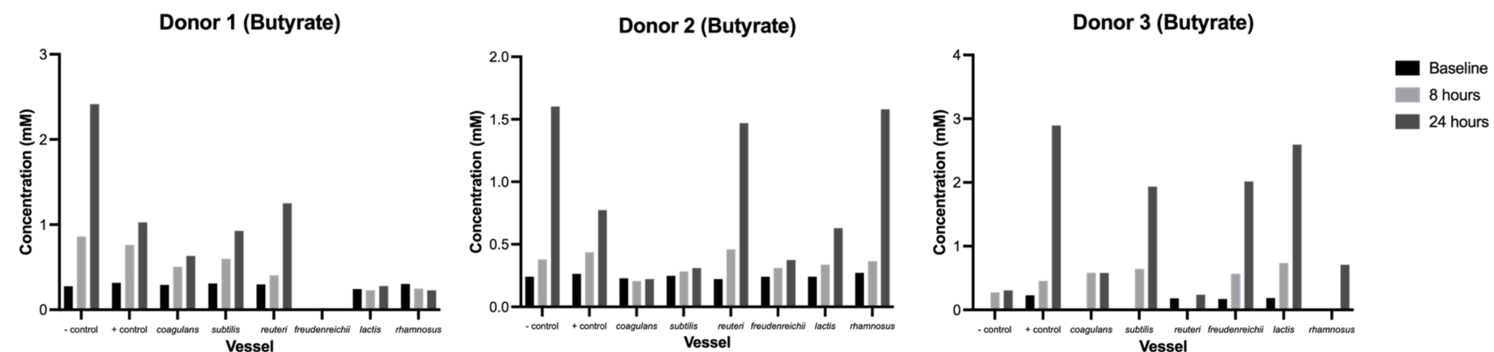


Figure 3 – Concentration (mM) of butyrate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

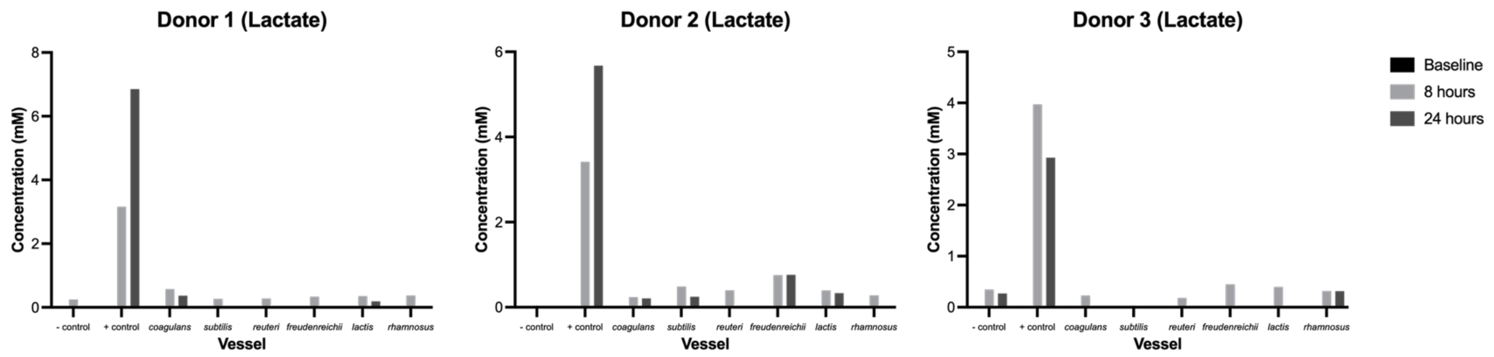


Figure 4 – Concentration (mM) of lactate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

Short-chain fatty acids (excluding positive control vessel)

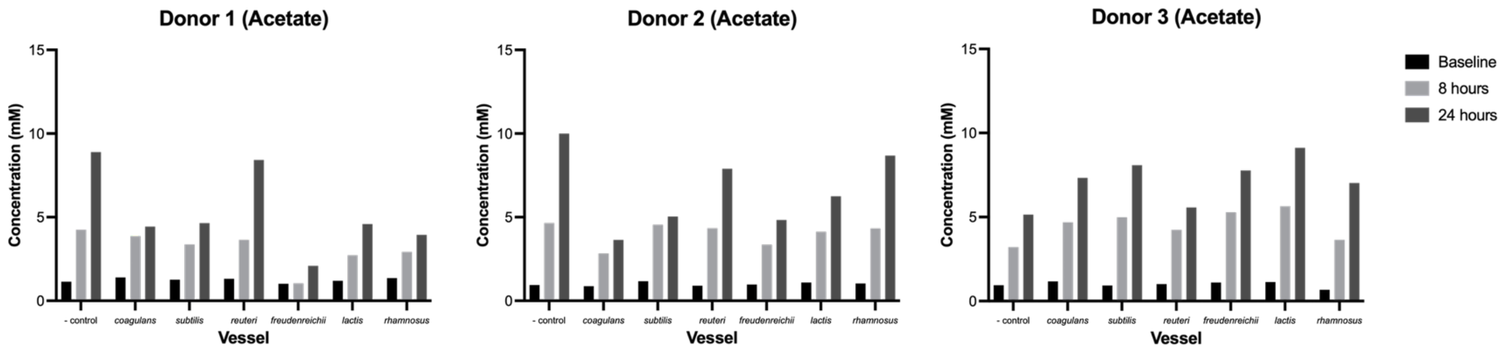


Figure 5 – Concentration (mM) of acetate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

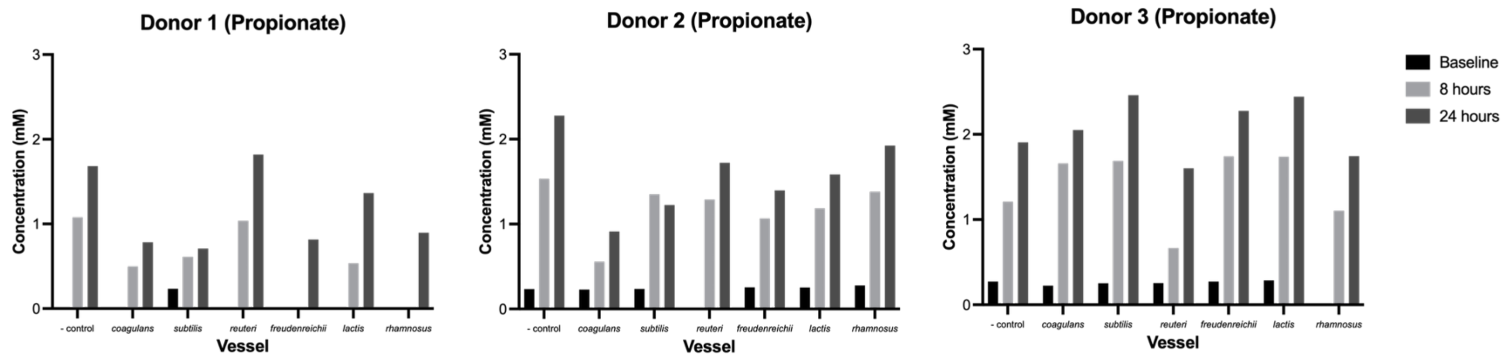


Figure 6 – Concentration (mM) of propionate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

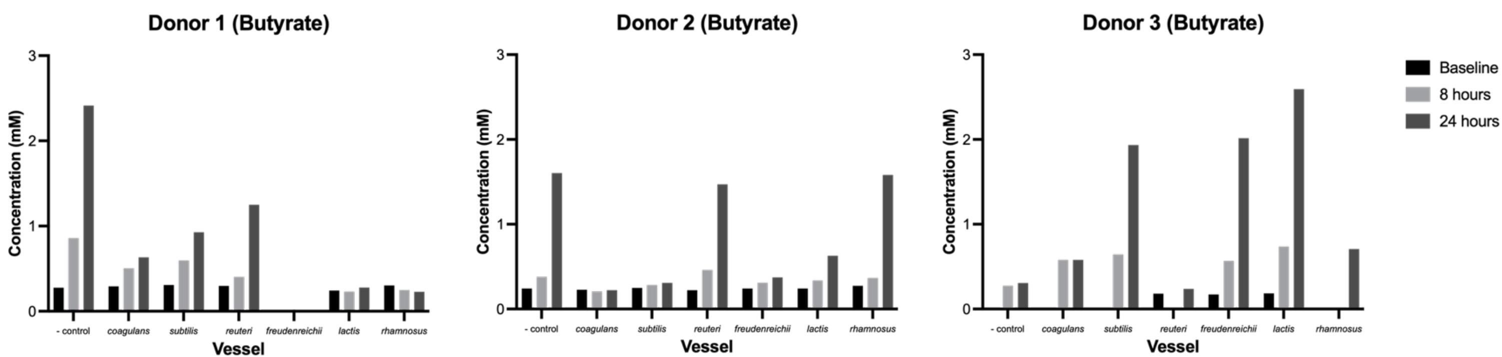


Figure 7 – Concentration (mM) of butyrate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

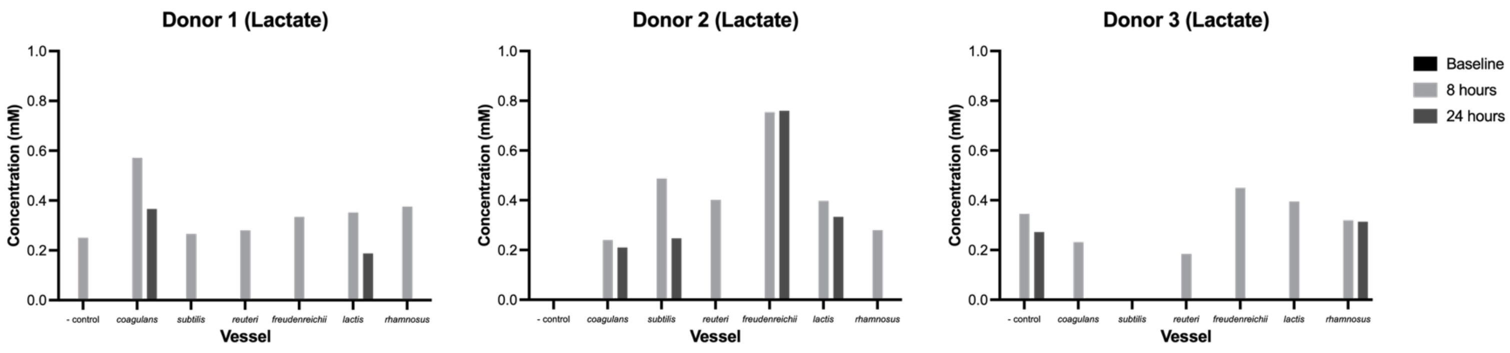


Figure 8 – Concentration (mM) of lactate at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

Neurotransmitters

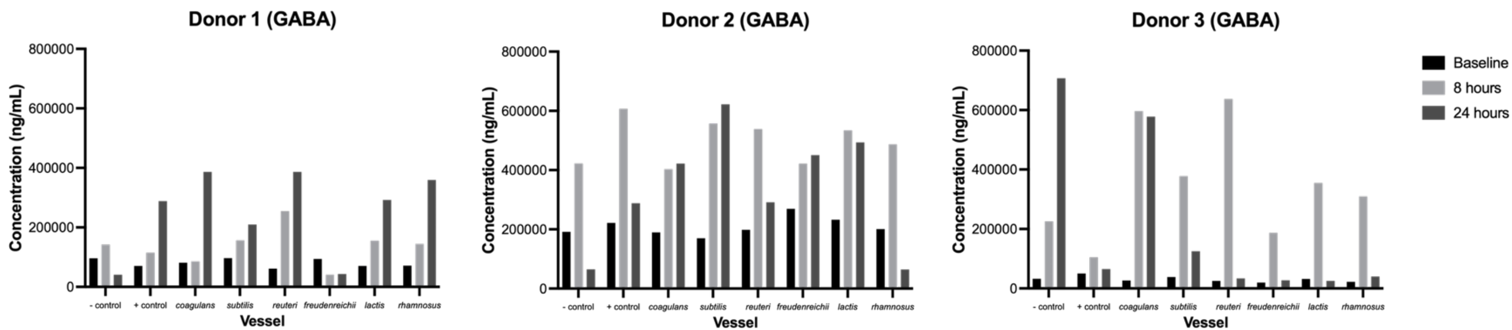


Figure 9 – Concentration (ng/mL) of GABA at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

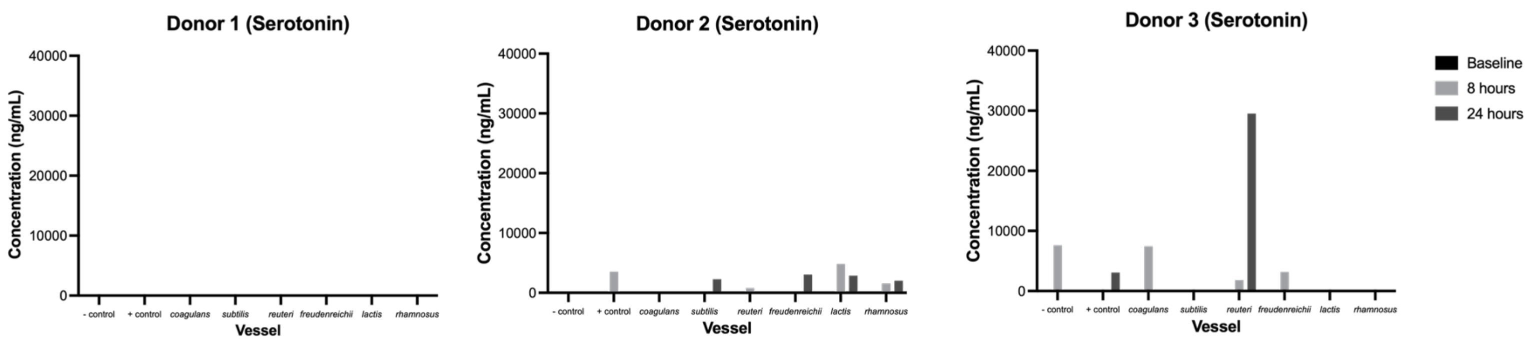


Figure 10 – Concentration (ng/mL) of serotonin at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

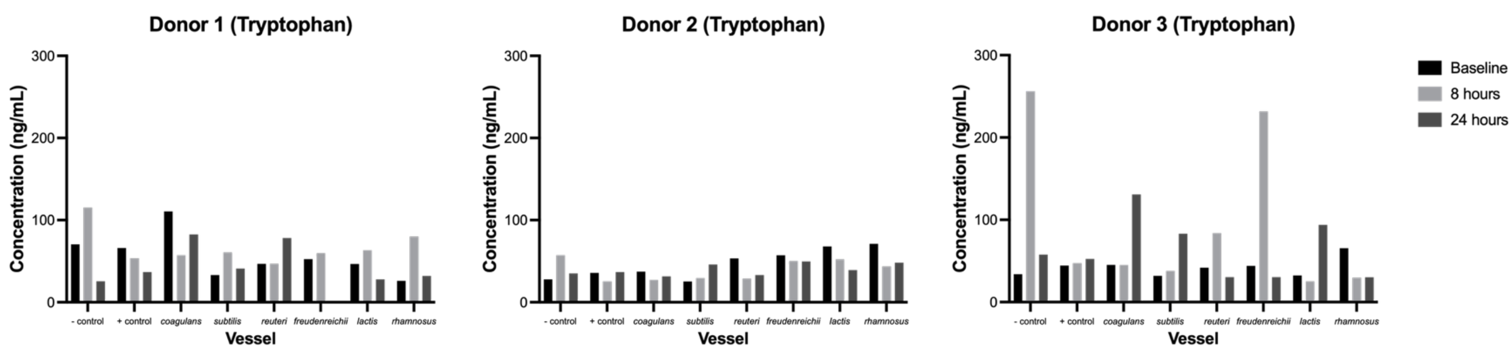


Figure 11 – Concentration (ng/mL) of tryptophan at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

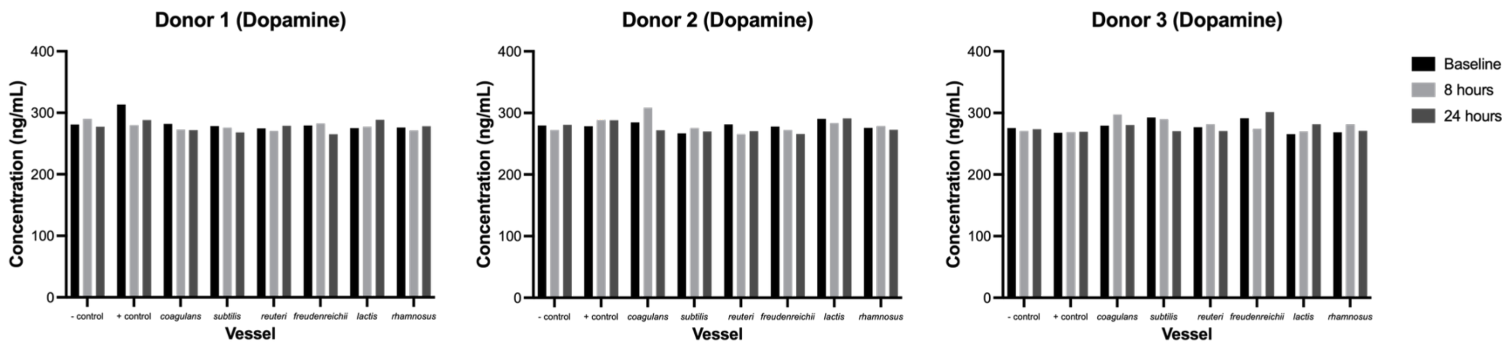


Figure 12 – Concentration (ng/mL) of dopamine at baseline and following 8 and 24 hours of fermentation per donors 1, 2 & 3 (left to right).

Table 1 – Donor 1, Enumeration of bacteria for by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS).

Vessel	Time (hours)	Bacterial groups detected by flow-FISH										
		Total bacteria	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Negative control	0	8.13	6.78	6.22	6.69	7.78	7.25	6.01	6.26	7.53	5.96	5.94
	8	7.91	6.74	5.82	6.75	7.46	6.46	6.02	6.27	7.21	3.91	5.49
	24	7.65	6.60	5.91	5.62	7.15	4.85	5.91	5.68	6.15	3.65	5.78
Positive control	0	8.18	6.61	6.40	6.74	7.82	7.39	6.12	6.32	7.58	5.96	5.43
	8	7.80	7.36	5.51	6.08	7.02	6.00	5.57	6.33	7.05	4.80	5.10
	24	7.49	7.01	5.54	5.02	6.28	4.53	5.88	5.75	6.64	4.34	4.10
B. coagulans	0	7.99	6.68	6.09	6.48	7.68	7.15	5.76	5.42	7.37	5.88	4.00
	8	7.78	6.84	5.39	6.27	7.33	6.46	5.47	6.09	7.10	3.78	4.74
	24	7.46	6.73	5.97	5.42	6.90	5.48	5.80	6.21	6.16	3.94	5.36
B. subtilis	0	8.04	6.69	5.93	6.63	7.71	7.14	5.04	6.24	7.44	5.77	5.82
	8	7.87	6.87	5.86	6.29	7.39	6.50	5.93	6.31	7.17	3.87	5.21
	24	7.46	6.73	5.78	5.19	6.84	5.56	5.97	5.86	6.13	3.46	5.76
L. reuteri	0	8.08	6.76	5.56	6.56	7.78	7.28	4.98	6.26	7.44	5.03	5.51
	8	7.79	6.70	5.83	6.67	7.33	6.66	5.67	6.38	7.12	4.75	5.30
	24	7.70	6.67	6.07	6.25	7.23	5.78	5.64	6.22	6.53	4.39	5.82
P. freudenreichii	0	8.06	6.82	6.02	6.17	7.71	6.90	5.24	6.22	7.33	4.67	5.18
	8	6.95	5.02	5.40	4.18	5.33	4.96	3.43	4.21	6.00	3.95	4.13
	24	6.52	4.65	5.14	3.52	4.97	3.98	5.64	5.08	5.19	4.21	4.51

Lc. lactis	0	8.04	6.74	6.23	6.57	7.74	7.12	5.75	6.42	7.44	5.04	6.18
	8	7.46	6.54	5.29	6.56	6.87	5.70	5.33	6.09	6.65	3.46	4.82
	24	7.19	6.44	4.82	5.70	6.13	4.90	5.33	6.21	5.47	3.19	3.89
L. rhamnosus	0	7.94	6.63	6.13	6.58	7.62	7.03	5.98	6.15	7.29	5.78	5.96
	8	7.93	6.62	6.08	6.93	7.57	6.82	4.83	6.10	7.36	4.61	5.27
	24	7.65	5.93	6.06	4.97	7.25	5.79	4.69	6.21	6.71	4.73	5.17

Table 2 – Donor 2, Enumeration of bacteria for by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS).

		Bacterial groups detected by flow-FISH										
Vessel	**Time (hours)**	**Total bacteria**	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Negative control	0	7.68	6.88	5.93	6.08	7.00	6.04	5.94	6.20	7.15	5.55	6.24
	8	7.55	6.61	4.75	5.82	6.43	5.25	5.83	6.02	6.89	3.55	5.08
	24	7.37	6.67	4.32	6.06	6.52	4.41	5.70	5.89	6.31	4.57	4.89
Positive control	0	7.63	6.60	4.63	5.85	6.87	5.1	5.44	5.85	7.09	4.41	6.13
	8	7.92	7.52	4.45	5.22	6.52	5.35	7.03	6.32	6.86	4.52	5.68
	24	8.11	8.02	5.11	5.46	6.48	5.66	6.41	6.10	6.28	5.16	5.23
B. coagulans	0	7.81	6.88	5.24	4.89	6.87	5.78	5.38	5.91	7.21	4.41	5.88
	8	7.13	6.42	4.45	5.47	5.63	3.43	6.02	5.87	5.89	3.61	4.28
	24	7.57	7.08	5.01	5.34	5.38	4.27	6.53	6.04	5.75	4.65	4.85
B. subtilis	0	7.67	6.79	4.84	5.20	6.86	5.54	5.30	5.83	7.05	4.27	5.45
	8	7.83	7.00	5.46	6.71	6.40	5.56	6.31	6.40	7.30	5.01	5.79
	24	7.63	6.97	4.88	5.60	6.04	4.63	6.32	6.04	6.77	3.92	4.09
L. reuteri	0	7.98	7.05	5.71	5.64	7.18	6.11	5.88	6.42	7.44	5.58	6.05
	8	7.97	7.18	5.42	6.81	6.81	5.66	6.65	6.57	7.34	4.87	5.68
	24	7.83	7.23	5.23	5.43	6.52	4.83	6.44	6.26	6.75	4.67	5.59
P. freudenreichii	0	7.90	6.89	5.22	5.82	7.11	6.00	5.77	6.19	7.34	4.81	5.18
	8	8.03	7.24	6.38	6.04	6.39	5.38	6.33	6.26	7.07	4.51	4.13
	24	7.98	7.13	6.82	5.38	6.30	4.83	6.61	6.29	6.76	4.68	4.51

Lc. lactis	0	7.93	6.94	5.49	5.53	7.20	5.84	5.51	6.11	7.36	4.53	6.07
	8	7.91	7.13	5.31	6.70	6.71	5.82	6.54	6.54	7.26	4.87	4.82
	24	7.88	7.34	5.11	5.55	6.59	4.48	6.77	6.30	6.83	4.48	3.89
L. rhamnosus	0	7.81	6.83	5.36	5.57	7.06	5.95	5.61	6.15	7.22	4.93	5.90
	8	7.97	7.18	5.42	6.81	6.81	5.66	6.65	6.57	7.34	4.87	5.68
	24	8.04	7.40	5.19	6.17	7.02	5.00	6.78	6.61	6.41	4.89	5.08

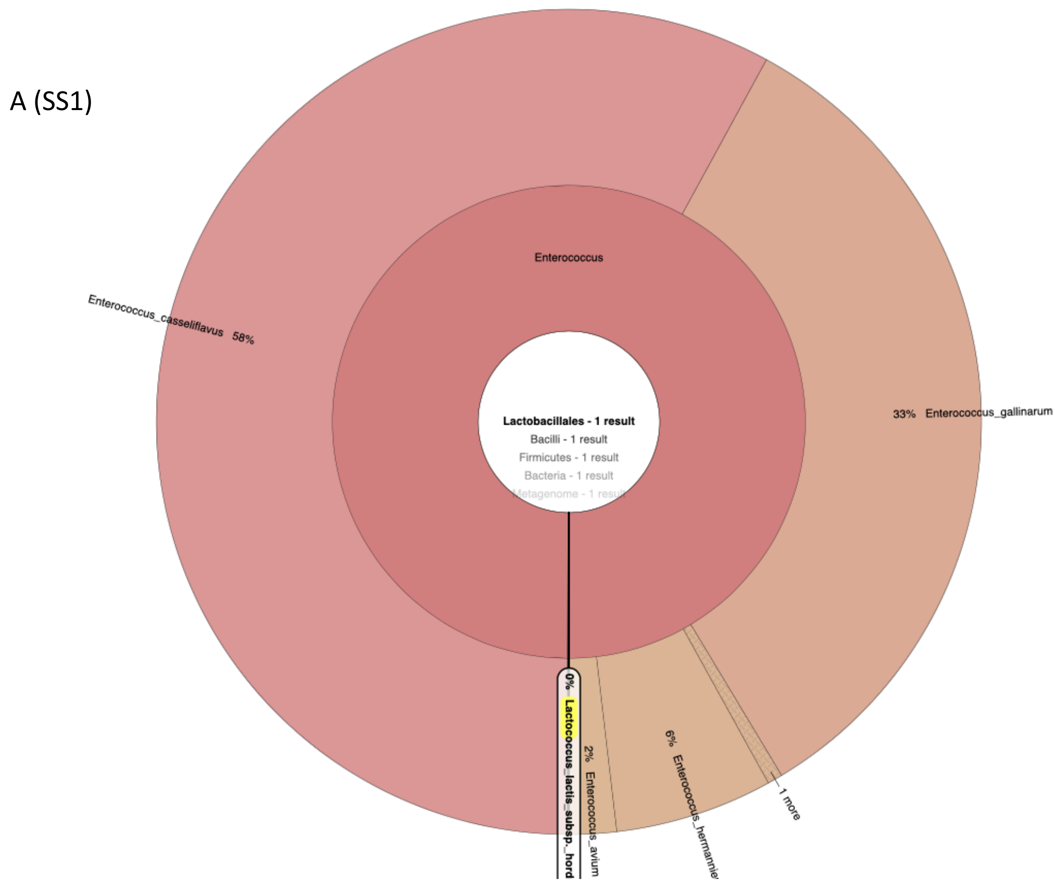
Table 3 – Donor 3, Enumeration of bacteria for by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS).

		Bacterial groups detected by flow-FISH										
Vessel	**Time (hours)**	**Total bacteria**	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Negative control	0	7.91	6.52	5.44	5.96	7.48	5.71	5.68	4.82	7.16	4.95	4.91
	8	7.33	5.98	4.54	6.05	6.70	5.06	6.28	4.96	6.77	4.23	4.03
	24	7.75	6.20	5.08	6.60	7.11	5.24	6.63	5.57	7.10	4.06	4.36
Positive control	0	7.98	6.67	5.26	5.78	7.56	6.08	5.75	5.44	7.21	4.46	4.68
	8	8.09	7.66	5.39	6.76	7.20	5.37	7.45	5.54	6.83	4.39	5.45
	24	7.60	6.94	4.20	7.10	6.32	4.07	6.60	5.32	6.14	3.6	3.6
B. coagulans	0	7.60	6.45	4.98	6.07	7.16	5.71	5.51	4.68	6.90	3.60	4.60
	8	7.80	5.00	5.05	6.59	7.31	5.34	6.46	5.20	7.03	3.80	4.75
	24	7.91	6.68	5.19	6.39	7.37	5.24	6.95	6.30	6.71	4.39	4.52
B. subtilis	0	7.81	6.47	5.08	5.71	7.35	5.77	5.65	5.17	7.15	4.85	4.65
	8	7.89	6.46	5.27	6.46	7.46	5.47	6.44	5.82	7.30	4.74	5.61
	24	7.66	6.29	4.77	6.36	7.09	5.09	6.43	5.62	6.77	4.13	4.56
L. reuteri	0	7.93	6.10	5.27	6.24	7.30	6.09	5.58	4.88	7.30	4.71	4.71
	8	7.76	5.87	5.62	6.44	4.24	5.68	5.38	5.06	7.11	4.46	4.76
	24	7.91	5.54	5.77	6.76	7.32	6.08	5.77	6.54	7.26	4.69	3.91
P. freudenreichii	0	7.93	6.21	5.23	6.14	7.38	5.98	5.52	4.63	7.31	5.25	5.18
	8	7.35	6.21	4.55	6.01	5.01	4.81	5.91	4.75	6.72	3.35	4.13
	24	7.49	6.07	5.02	5.79	7.06	3.97	6.32	6.30	6.34	4.09	4.09

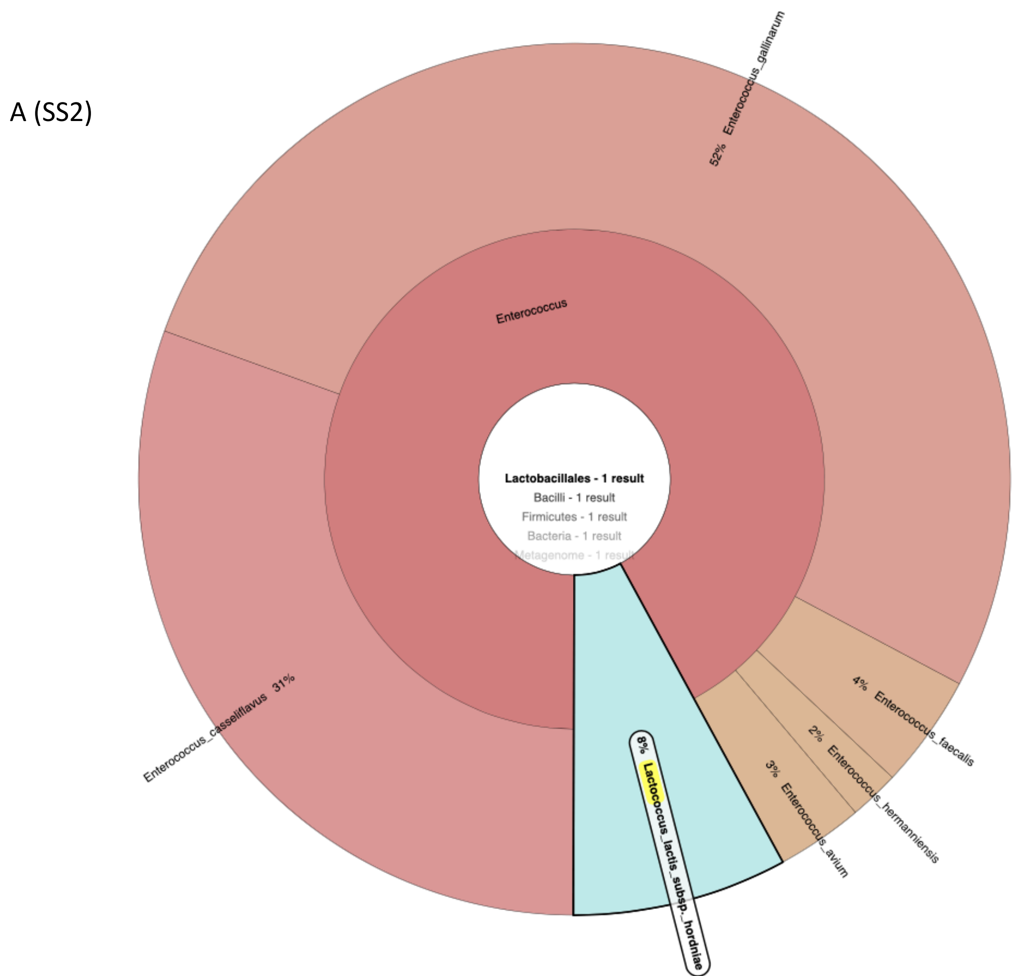
Lc. lactis	0	8.07	6.56	5.87	5.87	7.59	6.13	6.00	5.43	7.42	5.02	4.97
	8	7.61	6.35	5.56	6.34	7.13	5.21	6.21	5.48	6.86	4.21	4.69
	24	7.62	6.41	5.10	6.05	6.77	5.28	6.11	5.98	6.48	4.10	3.62
L. rhamnosus	0	7.81	6.32	5.35	5.44	7.32	5.84	5.27	5.22	7.11	4.99	4.71
	8	7.67	6.56	5.59	6.21	6.94	5.16	6.48	5.14	6.85	3.67	4.81
	24	7.53	6.36	5.37	5.94	6.77	4.81	6.02	6.73	6.48	3.83	3.83

Appendix 5- Krona charts representing change in relative abundance between steady state 1 and steady state 2 for *Lactococcus* (A) and *Roseburia* (B)

A (SS1)



A (SS2)



B (SS1)

B (SS2)

Appendix 6 – Gut model data (short-chain fatty acids, neurotransmitters and bacterial enumeration) presented by donor for comparison.

Short-chain fatty acids

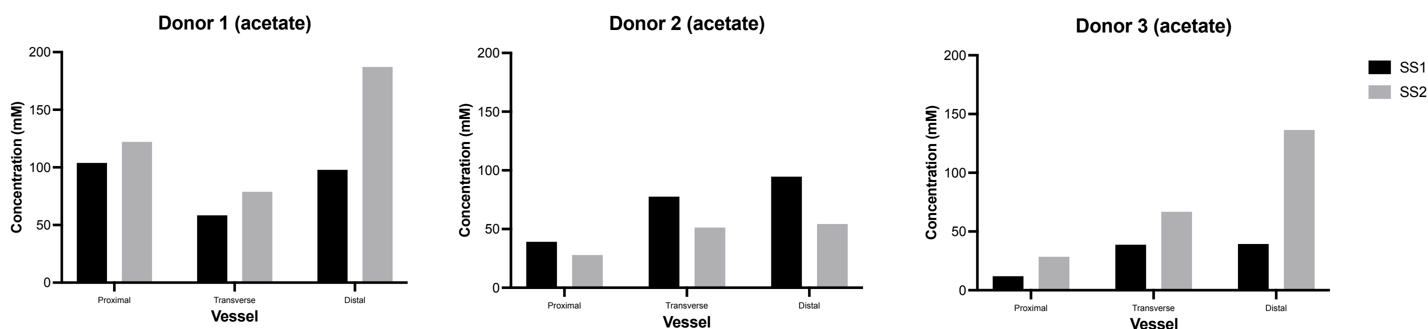


Figure 1 – Concentration (mM) of acetate at SS1 and SS2 per donors 1, 2 & 3 (left to right).

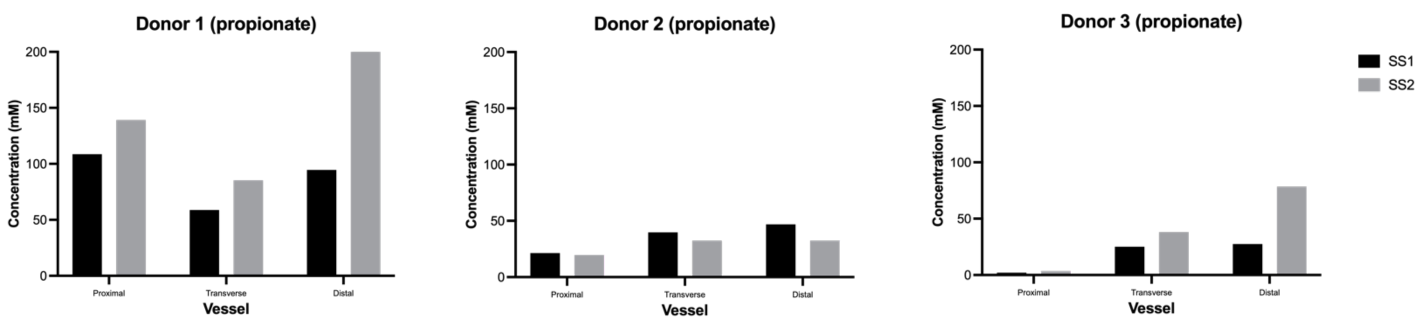


Figure 2 – Concentration (mM) of propionate at SS1 and SS2 per donors 1, 2 & 3 (left to right).

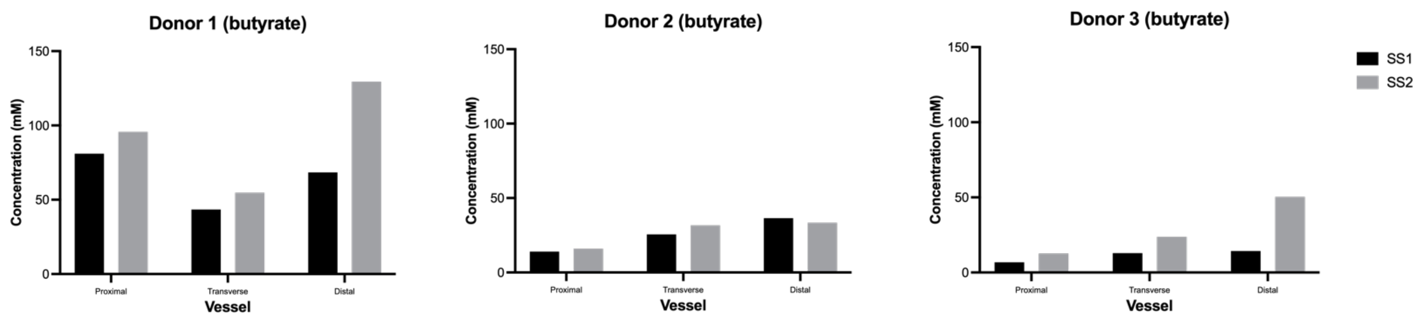


Figure 3 – Concentration (mM) of butyrate at SS1 and SS2 per donors 1, 2 & 3 (left to right).

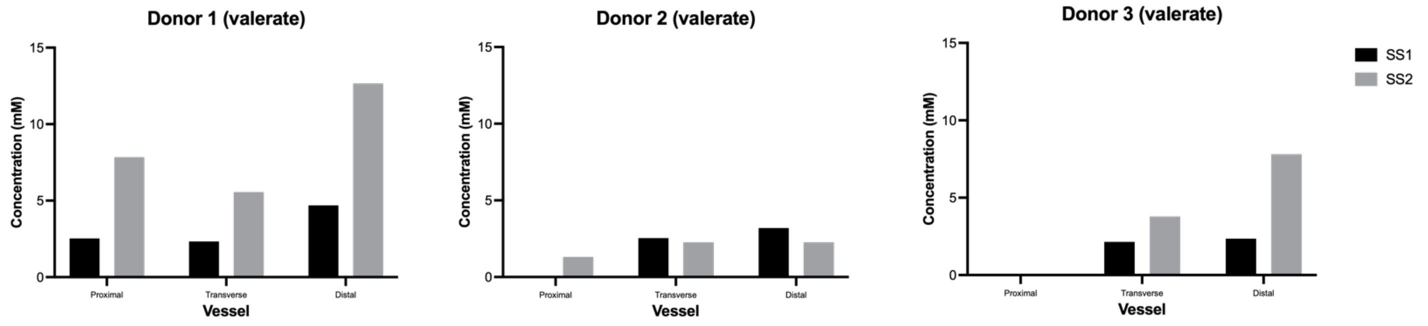


Figure 4 – Concentration (mM) of valerate at SS1 and SS2 per donors 1, 2 & 3 (left to right).

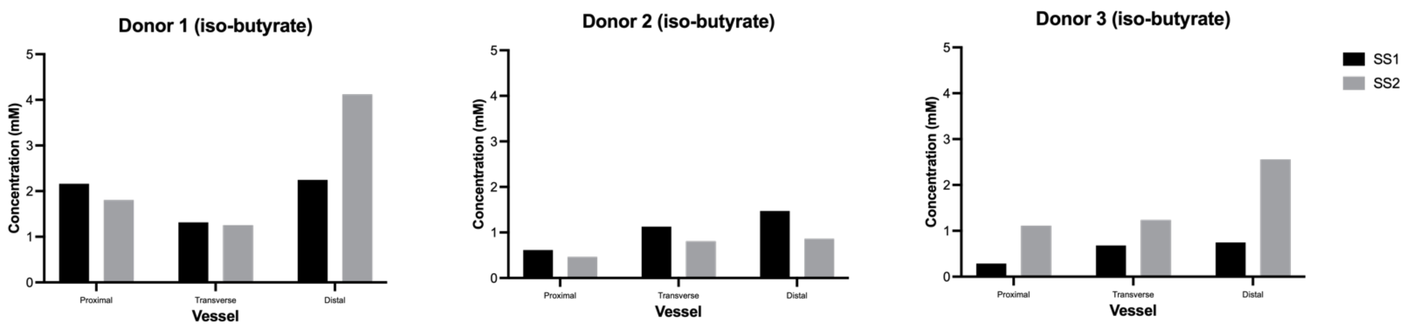


Figure 5 – Concentration (mM) of iso-butyrate at SS1 and SS2 per donors 1, 2 & 3 (left to right).

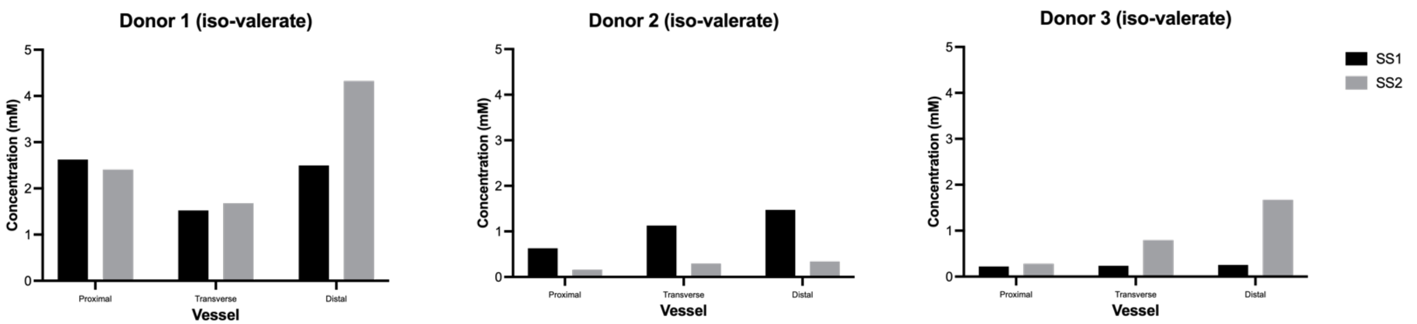


Figure 6 – Concentration (mM) of iso-valerate at SS1 and SS2 per donors 1, 2 & 3 (left to right).

Neurotransmitters

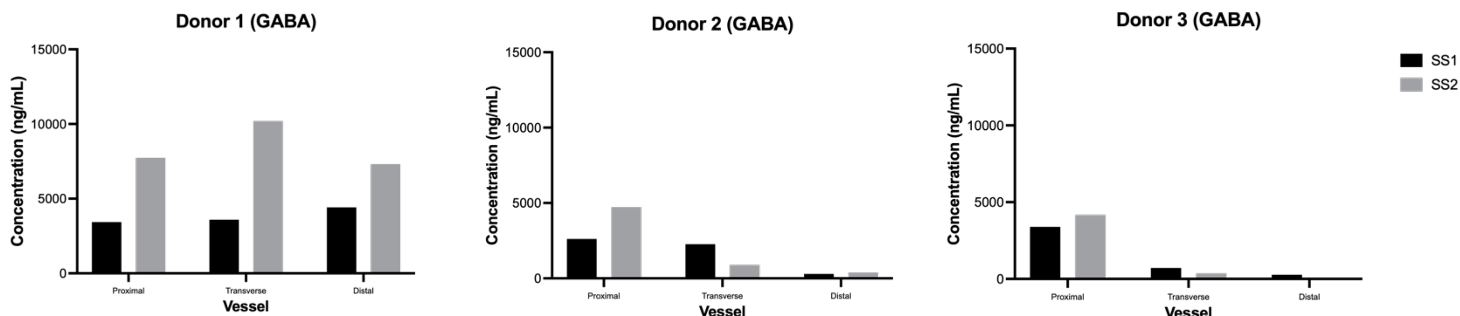


Figure 7 – Concentration (ng/mL) of GABA at SS1 and SS2 per donors 1, 2 & 3 (left to right).

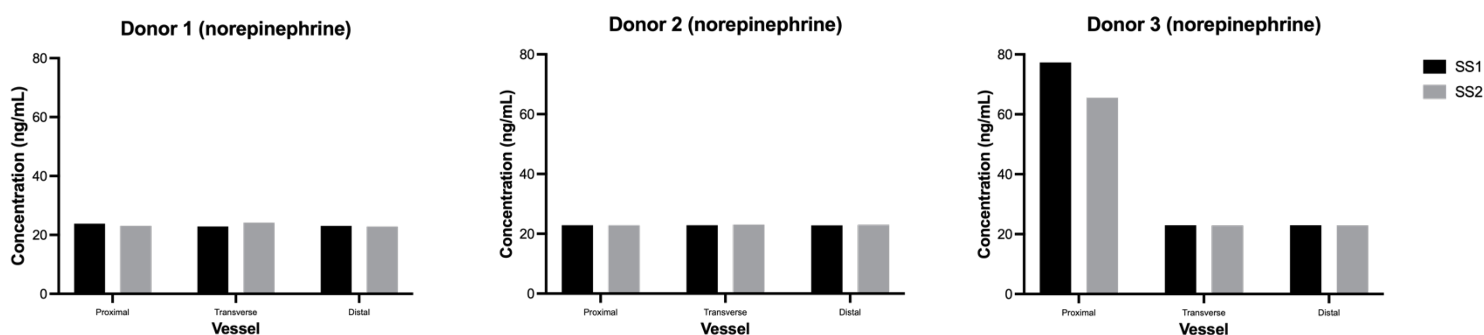


Figure 8 – Concentration (ng/mL) of norepinephrine at SS1 and SS2 per donors 1, 2 & 3 (left to right).

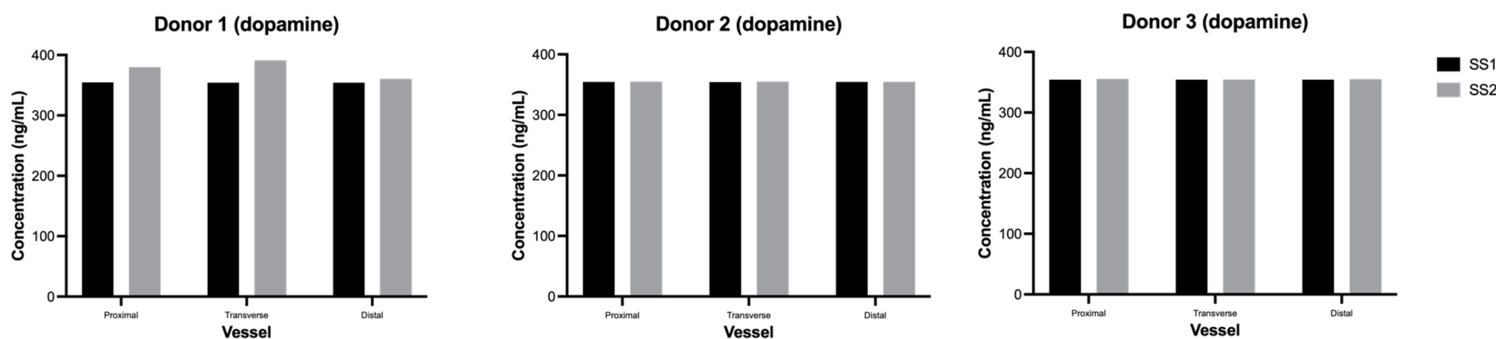


Figure 9 – Concentration (ng/mL) of dopamine at SS1 and SS2 per donors 1, 2 & 3 (left to right).

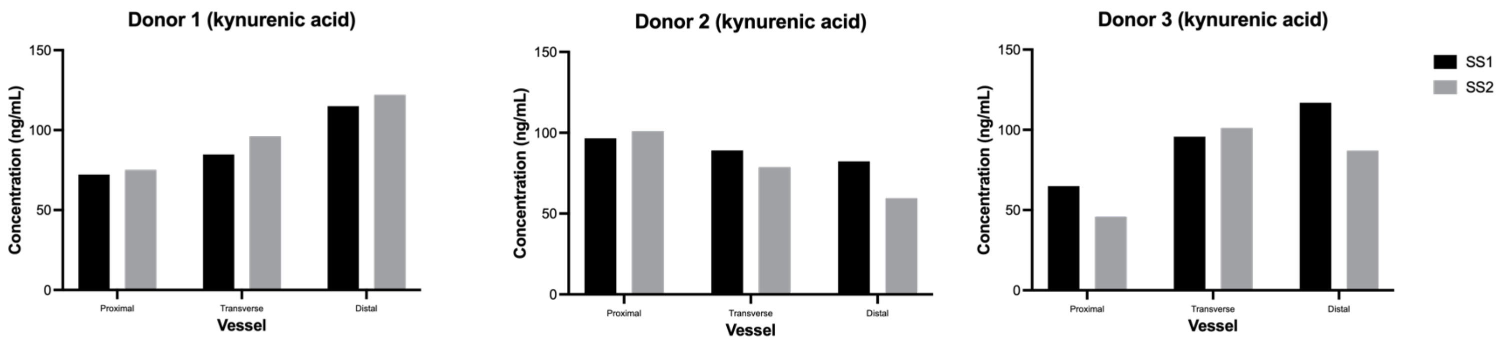


Figure 10 – Concentration (ng/mL) of kynurenic acid at SS1 and SS2 per donors 1, 2 & 3 (left to right).

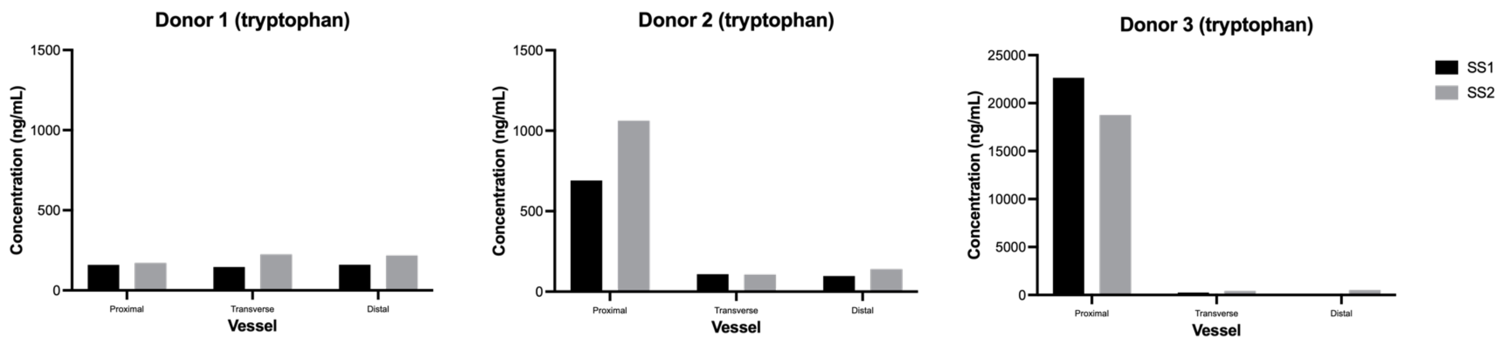


Figure 11 – Concentration (ng/mL) of tryptophan at SS1 and SS2 per donors 1, 2 & 3 (left to right).

Table 1 – Donor 1, Enumeration of bacteria for by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS).

		Bacterial groups detected by flow-FISH										
Vessel	**Steady State**	**Total bacteria**	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Proximal	SS1	8.46	4.50	5.31	8.23	7.84	6.40	5.74	7.05	7.69	5.31	5.54
	SS2	9.18	6.38	6.35	8.85	8.62	7.02	7.23	7.53	8.26	6.13	6.76
Transverse	SS1	8.30	8.30	7.42	7.81	7.72	5.48	6.11	7.29	7.56	4.91	4.78
	SS2	8.25	5.60	5.63	7.78	7.76	6.20	5.94	6.87	7.25	6.48	4.50
Distal	SS1	7.93	5.17	4.50	7.32	7.26	5.50	5.51	6.55	6.96	4.50	4.50
	SS2	7.55	4.50	4.50	6.62	7.16	6.09	6.29	6.66	6.23	4.50	4.50

Table 2 – Donor 2, Enumeration of bacteria for by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS).

		Bacterial groups detected by flow-FISH										
Vessel	**Steady State**	**Total bacteria**	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Proximal	SS1	9.07	6.76	6.28	8.71	8.62	6.94	6.25	7.66	6.77	4.50	8.27
	SS2	8.83	7.29	6.11	8.14	8.60	7.27	5.73	7.24	6.28	4.50	6.28
Transverse	SS1	8.76	6.44	6.73	7.87	8.28	6.73	6.40	7.33	6.04	4.50	8.01
	SS2	8.81	7.48	6.84	7.09	8.62	6.85	6.13	7.54	5.76	4.50	6.48
Distal	SS1	8.36	5.99	7.12	7.04	8.03	6.13	6.21	7.10	6.05	4.50	7.63
	SS2	8.79	7.73	6.91	6.07	8.54	6.25	6.02	7.54	5.74	4.50	6.39

Table 3 – Donor 3, Enumeration of bacteria for by Flow-FISH at baseline (0) and following 8 and 24 hours of fermentation within the negative control, positive control, and six probiotic vessels, represented as log10 cells/mL culture. Target bacteria: Bifidobacterium spp.(BIF), Lactobacillus spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), Faecalibacterium prausnitzii (FPRAU), Clostridium cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV) and Clostridium histolyticum (CHIS).

		Bacterial groups detected by flow-FISH										
Vessel	**Steady State**	**Total bacteria**	**BIF**	**LAB**	**BAC**	**EREC**	**RREC**	**ATO**	**PROP**	**FPRAU**	**DSV**	**CHIS**
Proximal	SS1	8.12	4.97	7.98	5.66	4.82	5.02	5.07	4.42	4.42	4.90	6.62
	SS2	8.69	5.47	8.51	6.72	4.50	5.87	5.69	6.17	6.01	5.84	7.29
Transverse	SS1	8.72	5.02	7.68	7.94	8.17	5.20	5.02	7.60	7.57	4.72	5.42
	SS2	8.72	5.02	7.07	8.20	8.34	6.08	5.20	7.24	7.76	4.72	5.86
Distal	SS1	8.49	4.49	7.70	7.33	4.490	4.79	4.49	7.34	7.07	5.09	4.49
	SS2	8.65	4.96	7.35	7.57	8.29	6.28	5.65	7.17	7.32	4.65	6.17

Appendix 7 – Study information document

School of Psychology and Clinical
Language Sciences
Earley Gate
Reading RG6 6BZ
+44 (0)118 378 8523

The effect of probiotics on gut and brain health

Participant Information sheet

What does the study involve?

Participation would involve a total of 7 visits over the course of 5 months to the department of Psychology at the University of Reading, where you will complete a number of questionnaires and cognitive tasks. Each study visit will take approx. 1.5 – 2 hours. We will provide breakfast (two plain croissants) on each of your study visits, and you will need to have fasted overnight (except for water) for 12 hours before each visit. We will also provide you with a probiotic supplement to take home and consume daily between your study visits.

As this study is looking at the effect of the supplement on gut bacteria, we will ask that you provide a stool sample at the beginning and end of each study arm (4 in total). We will also collect some information from you regarding your diet.

Who is running the study?

This study is being conducted by Jess Eastwood under the supervision of Associate Professor Daniel Lamport at the University of Reading. It has been reviewed by the University of Reading Research Ethics Committee and has been given favourable ethical opinion for conduct (UREC 20/17).

Who can take part?

We are looking for healthy adults aged 65 – 80. Unfortunately, you would be ineligible to take part in this particular study if any of the following apply to you:

- Coeliac disease
- Diabetes (type I or II)
- Epilepsy
- Gastrointestinal disorder, including irritable bowel disease (IBD) and irritable bowel syndrome (IBS)
- Regular consumer of probiotics, prebiotics or probiotic yoghurt
- Regular smoker
- Antibiotic treatment within the last 3 months
- Current diagnosis of and/or receiving treatment for mental health illness

What is the supplement?

The supplement we are using is a commercially available multi-strain probiotic. The supplement is stored as powder in sachets, which you will mix into a glass of water to consume. For more information, please see the ingredients list.

What can't I do while taking part in this study?

While we want you to be able to live as normal a life as possible during the course of the study, we do have some requirements that are important to maintaining the integrity of the research. We ask that you:

- Do not take other prebiotic, probiotic or synbiotic supplements (or live yoghurt/kefir drinks) other than the one we provide
- Do not change your diet or exercise routine drastically
- Do not eat within 12-hours of each study visit (overnight fasting), and no alcohol or caffeine the evening before a study visit

During each study visit (excluding the first familiarisation day), you will only be able to consume water and the breakfast that we provide.

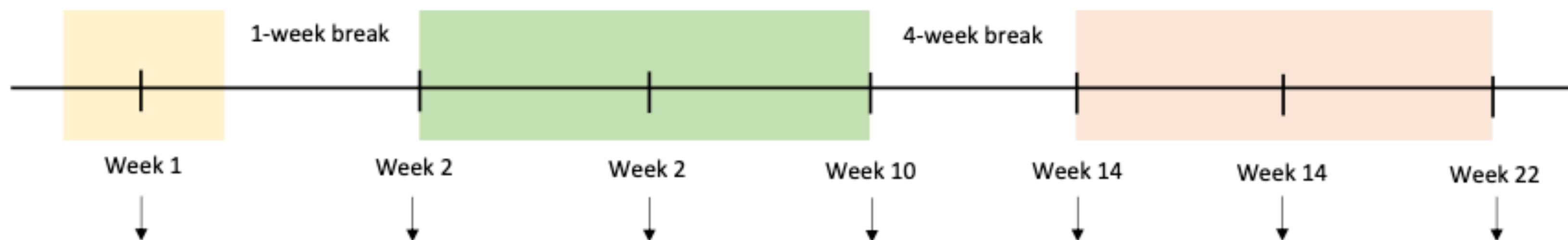
Will I get anything for taking part?

Yes! Upon completion of the study, you will receive £200 as a thank you for your time and dedication. If for some reason you have to drop out but have completed the first 9 weeks of the study, we will still compensate you for the time you have given.

Your participation is voluntary, and you are free to withdraw anytime without having to give an explanation.

If you have any questions, please feel free to contact the study manager.

Study manager: Jess Eastwood, j.r.eastwood@pgr.reading.ac.uk,
Principle Investigator: Associate Prof. Dan Lamport, daniel.lamport@reading.ac.uk, 0118 378 5032
Co-investigator: Dr Gemma Walton, g.e.walton@reading.ac.uk
Co-investigator: Prof Claire Williams, claire.williams@reading.ac.uk



Familiarisation session	Study visit 1 (baseline)	Study visit 2 (24 hours)	Study visit 3 (8 weeks)	Study visit 4 (baseline)	Study visit 5 (24 hours)	Study visit 6 (8 weeks)
Approximately 3 hours at the University one afternoon to confirm eligibility and complete 2 practice rounds of the cognitive task battery No fasting necessary Tea/coffee provided during the break	Bring stool sample with you to the University Breakfast provided (2 croissants) Mood measures & cognitive task battery (approx. 1 hour) Administration of supplement	Breakfast provided (2 croissants) Mood measures & cognitive task battery (approx. 1 hour) Provided with 8 weeks-worth of supplement and instructions for taking it	Bring stool sample with you to the University Breakfast provided (2 croissants) Mood measures & cognitive task battery (approx. 1 hour)	Bring stool sample with you to the University Breakfast provided (2 croissants) Mood measures & cognitive task battery (approx. 1 hour) Administration of supplement	Breakfast provided (2 croissants) Mood measures & cognitive task battery (approx. 1 hour) Provided with 8 weeks-worth of supplement and instructions for taking it	Bring stool sample with you to the University Breakfast provided (2 croissants) Mood measures & cognitive task battery (approx. 1 hour) Payment upon completion of study

Appendix 8 – Study consent form

The University of Reading

School of Psychology

Earley Gate, Whiteknights
PO Box 238, Reading RG6 6AL, UK

phone +44 (0)118 378 8523
fax+44 (0)118 378 6715

Consent form

Exploring the effects of probiotic supplementation on brain and gut outcomes in healthy older adults.

I have been given a copy of the study information sheet to read and I have understood the procedure of the study. ☐

I have been given the opportunity to ask any questions that I may have about the study and these have been answered to my satisfaction. ☐

I understand that my personal information will remain confidential to the researcher and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me. ☐

I agree the I have read the exclusion criteria carefully and I believe that I am eligible for the study. ☐

I have read the intervention information sheet and I understand what I'm being supplemented with. ☐

The procedure for collecting and storing stool samples has been explained to me and I consent to the researchers storing and analysing my samples in this way. ☐

I understand that my participation is voluntary, and I may withdraw at any time. ☐

I am happy to proceed with participation. ☐

Participant signature ....

Name (in capitals) ....

Date ....

Researcher Signature ....

Name (in capitals) ....

Date ....

Appendix 9 – UREC ethics committee approval

Coordinator for Quality Assurance in Research
Dr Mike Proven, BSc (Hons), PhD

Academic and Governance Services

Whiteknights House
Whiteknights, PO Box 217
Reading RG6 6AH

phone +44 (0)118 378 7119
email urec@reading.ac.uk

Dr Daniel Lamport
School of Psychology and Clinical Language Sciences
University of Reading
RG6 6AL

25 September 2020

Dear Daniel,

UREC 20/17: "An exploratory investigation into the acute effects of a probiotic (Ecologic Barrier©) on cognitive and mood outcomes. Amendment *favourable opinion AM012017*

Thank you for your email dated 23 September 2020 *(and including attachments refers*) requesting and outlining amendments to the above project *(which include the addition of a chronic element of supplementation to the existing intervention, and changes to the scope and conditions of participation, as a result of Covid-19).*

I can confirm that the UREC Chair has reviewed that request and is happy for the UREC-authorised project to continue.

Yours sincerely

Dr M J Proven
Coordinator for Quality Assurance in Research (UREC Secretary)
cc: Dr Andrew Glennerster (SREC Chair); Ms Liz White (SREC Administrator)

Appendix 10 – Intervention randomisation schedule

Subject ID	Arm 1	Arm 2
1	A	B
2	A	B
3	B	A
4	B	A
5	B	A
6	A	B
7	B	A
8	A	B
9	A	B
10	A	B
11	B	A
12	B	A
13	A	B
14	A	B
15	B	A
16	B	A
17	A	B
18	B	A
19	B	A
20	A	B
21	A	B
22	B	A
23	A	B
24	B	A
25	B	A
26	A	B
27	B	A
28	A	B
29	B	A
30	A	B
31	A	B
32	B	A
33	B	A

A = Placebo

B = Probiotic

Appendix 11 – Demographics questionnaire

Participant No:

Demographic Questionnaire (ProCog)

Full name: ____

Age at enrolment: ____

Ethnicity: ____

Height (cm): ____

Weight (kg): ____

BMI: ____

Highest level of education: _______________________________

Do you take any regular medication? Please write them in the box below.

Any other notes…

Appendix 12 – Montreal Cognitive Assessment

MoCA COGNITIVE ASSESSMENT — Version 8.1 English

Name:
Education:
Sex:
Date of birth:
DATE:

VISUOSPATIAL / EXECUTIVE — POINTS

(E) End, (A), (5), (B), (2), (1) Begin, (D), (4), (3), (C) — [ ]

Copy cube — [ ]

Draw CLOCK (Ten past eleven) (3 points) — [ ] Contour [ ] Numbers [ ] Hands

__/5

NAMING

[ ] [ ] [ ] __/3

MEMORY — Read list of words, subject must repeat them. Do 2 trials, even if 1st trial is successful. Do a recall after 5 minutes.

	FACE	VELVET	CHURCH	DAISY	RED
1ST TRIAL					
2ND TRIAL					

NO POINTS

ATTENTION — Read list of digits (1 digit/ sec.).

Subject has to repeat them in the forward order. [ ] 2 1 8 5 4

Subject has to repeat them in the backward order. [ ] 7 4 2

__/2

Read list of letters. The subject must tap with his hand at each letter A. No points if ≥ 2 errors

[ ] F B A C M N A A J K L B A F A K D E A A A J A M O F A A B

__/1

Serial 7 subtraction starting at 100. [ ] 93 [ ] 86 [ ] 79 [ ] 72 [ ] 65

4 or 5 correct subtractions: **3 pts**, 2 or 3 correct: **2 pts**, 1 correct: **1 pt**, 0 correct: **0**

__/3

LANGUAGE — Repeat: I only know that John is the one to help today. [ ]

The cat always hid under the couch when dogs were in the room. [ ]

__/2

Fluency: Name maximum number of words in one minute that begin with the letter F. [ ] ____ (N ≥ 11 words)

__/1

ABSTRACTION — Similarity between e.g. orange - banana = fruit [ ] train - bicycle [ ] watch - ruler

__/2

DELAYED RECALL

(MIS)	Has to recall words WITH NO CUE	FACE [ ]	VELVET [ ]	CHURCH [ ]	DAISY [ ]	RED [ ]	Points for UNCUED recall only
X3							
X2	Category cue						MIS = ____/15
X1	Multiple choice cue						

Memory Index Score (MIS)

__/5

ORIENTATION — [ ] Date [ ] Month [ ] Year [ ] Day [ ] Place [ ] City

__/6

© Z. Nasreddine MD **www.mocatest.org**

Training and Certification are required to ensure accuracy.

MIS: /15

(Normal ≥ 26/30)

Add 1 point if ≤ 12 yr edu

TOTAL __/30

Appendix 13 – Participant compliance diary

ProCog study compliance check sheet ppt no: _____________ arm no: ______________

This is a diary to keep track of when you take your probiotics. Please write the date in the left-hand corner of each box, and then write the time that you consumed your supplement each day. I will collect this from you at the end of the 8 weeks.

If you miss a day (try not to do this!), please leave that box blank. If you do miss a day, DO NOT take two sachets the next day. Instead, just have one the next day as normal.

Please try to keep the time of day that you take the supplement consistent.

Please DO NOT take the supplement on the day that you come in for your 8-week study session. This will interfere with the results for that day.

Thank you for your dedication to the study! Any problems or questions, please get in touch:

Jess Eastwood – 07947729494 – j.r.eastwood@pgr.reading.ac.uk

ProCog study compliance check sheet ppt no: _____________ arm no: ______________

☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐
☐	☐	☐	☐	☐	☐	☐

Appendix 14 – End of study questionnaire

End of study questionnaire

1. Did you notice a difference between the two phases of the study (phase one was the first 8-week period, and phase two was the second 8-week period)?

2. Did you experience any gastrointestinal side-effects of the supplement throughout the study?

   Yes / No

3. If yes, what were these, and did this differ between phase one and two of the study?

4. Do you believe that a probiotic supplement could improve your brain function?

   Yes / No

5. Do you know what the term 'placebo effect' means?

   Yes / No

6. If yes, do you believe the placebo effect to be real?

   Yes / No

Appendix 15 – Debrief

School of Psychology

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Debrief

Thank you for participating in our study "exploring the effects of probiotic supplementation on cognition and mood outcomes in healthy older adults". We really appreciate your time and commitment.

This is a novel study looking to explore whether one dose of probiotic supplement is sufficient to see any changes in cognitive ability or mood. Previous studies that looked at the effect of supplementation over a number of weeks have suggested that habitual use of this probiotic may reduce the risk of depression, and buffer against the negative impact of stress on brain function. This may be due to changes in neurotransmitter production in the gut, or reductions in inflammation. Based on our current knowledge it is unclear how just a single dose will interact with the gut and affect brain function, but should we see any effects, the timing of these may help us to understand a little more about how they occur.

We are also interested to see what areas of cognition are impacted by 8-weeks' worth of probiotic supplement, and whether this differs from person to person.

This study will also help us to understand how a person's diet or natural gut microbiota might have an influence over how effective the probiotic treatment is. It may be that those with a naturally more or less diverse gut microbiome respond better to probiotic intervention.

Until data analysis is complete, we will not know which order you consumed your probiotic and placebo supplements. If you are interested to know, we would be happy to share this and any results of the study with you upon completion. Please get in touch with the research team if you have any questions about the project.

Researcher:

Jessica Eastwood (Email: j.r.eastwood@pgr.reading.ac.uk)

Supervisors:

Dr Dan Lamport (Email: daniel.lamport@reading.ac.uk, Tel: 01183785032)

Dr Gemma Walton (Email: g.e.walton@reading.ac.uk, Tel: 01183786652)

Prof Claire Williams (Email: Claire.williams@reading.ac.uk, Tel: 01183787540)

Appendix 16 – Balanced Latin Square for alternative task battery randomisation

Balanced Latin Square for task battery versions

	Practice 1	Practice 2	Baseline	24 hours	8 weeks	Baseline (2)	24 hours (2)	8 weeks (2)
1	A	B	H	C	G	D	F	E
2	B	C	A	D	H	E	G	F
3	C	D	B	E	A	F	H	G
4	D	E	C	F	B	G	A	H
5	E	F	D	G	C	H	B	A
6	F	G	E	H	D	A	C	B
7	G	H	F	A	E	B	D	C
8	H	A	G	B	F	C	E	D
9	A	B	H	C	G	D	F	E
10	B	C	A	D	H	E	G	F
11	C	D	B	E	A	F	H	G
12	D	E	C	F	B	G	A	H
13	E	F	D	G	C	H	B	A
14	F	G	E	H	D	A	C	B
15	G	H	F	A	E	B	D	C
16	H	A	G	B	F	C	E	D
17	A	B	H	C	G	D	F	E
18	B	C	A	D	H	E	G	F
19	C	D	B	E	A	F	H	G
20	D	E	C	F	B	G	A	H
21	E	F	D	G	C	H	B	A
22	F	G	E	H	D	A	C	B
23	G	H	F	A	E	B	D	C
24	H	A	G	B	F	C	E	D
25	A	B	H	C	G	D	F	E
26	B	C	A	D	H	E	G	F
27	C	D	B	E	A	F	H	G
28	D	E	C	F	B	G	A	H
29	E	F	D	G	C	H	B	A
30	F	G	E	H	D	A	C	B
31	G	H	F	A	E	B	D	C
32	H	A	G	B	F	C	E	D
33	A	B	H	C	G	D	F	E
34	B	C	A	D	H	E	G	F
35	C	D	B	E	A	F	H	G
36	D	E	C	F	B	G	A	H
37	E	F	D	G	C	H	B	A
38	F	G	E	H	D	A	C	B
39	G	H	F	A	E	B	D	C
40	H	A	G	B	F	C	E	D

Appendix 17 – RAVLT word lists

A1 – Cane, Needle, Spike, Ferry, Owl, Package, Pearl, Bullet, Lung, Shoulder, Church, Jar, Picture, Dog, Bag

A2 – sword, pipe, vest, kennel, cliff, napkin, deck, lemon, frost, pepper, cloth, stone, mirror, seat, door

B1 – scout, palace, pit, lily, shell, gravel, blade, gravy, flag, wire, oil, tray, building, brush, book

B2 – beast, shield, crow, farmyard, heel, laundry, shark, movie, chin, uncle, throat, sleeve, pillow, lamp, girl

C1 – inn, doorway, barn, sardine, worm, slipper, soil, valley, stew, candy, coin, film, pocket, soap, men

C2 – wick, cheek, yacht, ruby, dart, knuckle, net, cherry, thread, dentist, wheel, heart, shower, hall, room

D1 – swamp, penny, fudge, woodland, sail, arrow, gun, kitten, board, rubber, pine, neck, oven, pot, fruit

D2 – herd, sheet, rake, camel, rod, mansion, mud, soldier, sweat, piano, rug, soup, ticket, sock, plant

E1 – clay, ceiling, bench, steeple, chain, meadow, stick, crystal, gate, elbow, chest, lake, woman, moon, bird

E2 – elm, purse, bow, pasture, palm, ribbon, cave, organ, wool, lettuce, skirt, fork, coffee, hill, skin

F1 – dew, forest, prune, dairy, knight, cotton, drain, iron, crown, teacher, nurse, broom, paper, leaf, knee

F2 – crane, pig, shrub, cellar, cone, daisy, pole, salad, stool, brother, beard, corn, bedroom, plate, box

G1 – hose, prison, tank, shepherd, seed, chapel, bone, jewel, card, hotel, wood, bin, toilet, clock, salt

G2 – lime, tack, calf, marble, oak, rifle, weed, tractor, van, office, blood, school, orange, rain, face

H1 – latch, metal, fox, armour, branch, measles, brick, linen, tool, chicken, star, ball, garden, meat, spoon

H2 – fawn, bowl, doll, beehive, duck, barrel, shore, hammer, bush, motor, land, glove, button, ring, tree

Appendix 18 – Linear mixed model results tables

Cognitive outcome	Model	Marginal R^2	Conditional R^2	Factor	Degrees of freedom	F statistic	P value
RAVLT							
Delayed recall (N)	1A	0.58	0.69	Session	(2,113.83)	9.63	<0.001
				Treatment	(1,32.18)	0.05	0.82
				Session x treatment	(2,113.48)	0.97	0.38
				Order	(1,51.16)	<0.01	0.97
				Sex	(1,49.29)	0.82	0.37
				MoCA	(4,51.56)	11.53	<0.001
				Age	(12,57.05)	9.94	<0.001
				Education	(1,53.99)	3.04	0.09
Immediate recall (N)	1B	0.29	0.54	Session	(2,105.51)	0.97	0.38
				Treatment	(1,27.72)	2.66	0.11
				Session x treatment	(2, 105.01)	1.37	0.26
				Order	(1,10.07)	<0.01	0.97
				Sex	(1.10.62)	<0.01	0.96
				MoCA	(4,10.23)	2.41	0.12
				Age	(12,10.74)	2.08	0.12
				Education	(1,10.23)	0.62	0.45
Amount learned (N)	1C	0.21	0.28	Session	(2,113.52)	0.18	0.83
				Treatment	(1,37.03)	0.37	0.55
				Session x treatment	(2,112.83)	0.09	0.92
				Order	(1,33.81)	0.63	0.43
				Sex	(1,33.72)	0.19	0.67
				MoCA	(4,34.39)	1.07	0.39
				Age	(12,36.16)	2.13	0.04
				Education	(1,34.38)	0.60	0.44
Total acquisition (N)	1D	0.40	0.74	Session	(2,101.26)	4.68	0.01
				Treatment	(1,27.55)	2.42	0.13
				Session x treatment	(2,101.07)	2.12	0.13
				Order	(1,10.02)	0.22	0.65
				Sex	(1,10.08)	0.73	0.41
				MoCA	(4,10.01)	1.63	0.24
				Age	(12,10.39)	1.77	0.18
				Education	(1,9.92)	0.40	0.54
Proactive interference (N)	1E	0.33	0.63	Session	(2,108.46)	0.56	0.57
				Treatment	(1,28.12)	0.99	0.33
				Session x treatment	(2,108.08)	1.65	0.20
				Order	(1,9.74)	3.91	0.08
				Sex	(1,9.88)	0.95	0.35

				MoCA	(4,9.67)	2.56	0.11
				Age	(12,10.01)	1.69	0.21
				Education	(1,9.64)	6.92	0.03
Total repetitions (N)	1G	0.40	0.74	Session	(2,114.66)	2.51	0.09
				Treatment	(1,96.09)	1.51	0.22
				Session x treatment	(2,114.89)	2.67	0.07
				Order	(1,9.14)	0.78	0.40
				Sex	(1,9.01)	1.26	0.17
				MoCA	(4,8.87)	0.73	0.59
				Age	(12,8.89)	0.90	0.58
				Education	(1,8.49)	0.70	0.42
Word recognition							
General accuracy (%)	1H	0.17	0.31	Session	(2,611.56)	0.99	0.37
				Treatment	(1,57.06)	0.001	0.97
				Session x treatment	(2,611.44)	0.75	0.47
				Order	(1,10.39)	3.59	0.09
				Sex	(1, 10.63)	0.003	0.96
				MoCA	(4,10.48)	2.93	0.07
				Age	(12,10.76)	2.31	0.09
				Education	(1,10.24)	1.40	0.26
Accurately identified items (List A) (%)	1I	0.35	0.61	Session	(2,101.59)	2.60	0.08
				Treatment	(1,25.88)	0.08	0.77836
				Session x treatment	(2, 101.51)	0.27	0.76
				Order	(1,10.08)	0.37	0.56
				Sex	(1,10.04)	5.20	0.05
				MoCA	(4,10.25)	0.69	0.62
				Age	(12,12.07)	2.32	0.08
				Education	(1, 10.51)	2.32	0.16
Accurately rejected items (List B) (%)	1J	0.45	0.78	Session	(2,104.52)	0.47	0.62
				Treatment	(1,25.10)	1.73	0.20
				Session x treatment	(2,104.48)	3.32	0.04
				Order	(1,9.77)	5.01	0.05
				Sex	(1,9.99)	0.13	0.73
				MoCA	(4,9.97)	3.21	0.06
				Age	(12,9.99)	3.04	0.04
				Education	(1,9.80)	2.77	0.13
Accurately rejected distractors (%)	1K	0.14	0.61	Session	(2,267.93)	0.72	0.49
				Treatment	(1,27.01)	1.73	0.20

				Session x treatment	(2,268.57)	0.09	0.92
				Order	(1,13.31)	0.25	0.62
				Sex	(1,13.55)	0.03	0.86
				MoCA	(4,13.39)	1.42	0.28
				Age	(12,13.16)	1.09	0.44
				Education	(1,12.87)	0.35	0.56
Accurately rejected distractors (semantic) (%)	1L	0.14	0.56	Session	(2,122.61)	0.28	0.75
				Treatment	(1,95.07)	0.48	0.49
				Session x treatment	(2,122.64)	0.39	0.68
				Order	(1,9.66)	1.73	0.22
				Sex	(1,9.95)	0.06	0.81
				MoCA	(4,9.90)	1.30	0.33
				Age	(12,10.10)	0.55	0.84
				Education	(1,9.63)	1.42	0.26
Accurately rejected distractors (phonological) (%)	1M	0.19	0.58	Session	(2, 101.67)	0.99	0.37
				Treatment	(1, 25.42)	1.33	0.26
				Session x treatment	(2, 101.70)	0.67	0.51
				Order	(1, 10.38)	2.21	0.17
				Sex	(1,10.37)	0.77	0.40
				MoCA	(4,10.23)	2.08	0.16
				Age	(12,10.30)	1.58	0.24
				Education	(1,9.48)	1.13	0.31
CBTT							
% of correct sequences	2A	0.77	0.82	Session	(2,2551.37)	3.80	0.02
				Treatment	(1,28.37)	1.57	0.22
				Blocks	(7,2543.85)	1496.67	<0.001
				Session x treatment	(2,2551.49)	1.84	0.16
				Session x block	(14,2542.60)	1.92	0.02
				Treatment x block	(7,2545.59)	2.27	0.03
				Session x treatment x block	(14,2543.02)	1.97	0.02
				Order	(1,9.77)	0.07	0.80
				Sex	(1,10.44)	2.51	0.14
				MoCA	(4,9.81)	2.63	0.10
				Age	(12,10)	1.55	0.25
				Education	(1,10.08)	1.39	0.27

% of correct blocks	2B	0.49	0.57	Session	(2,2599.16)	7.69	<0.01
				Treatment	(1,31.39)	0.09	0.76
				Blocks	(7,1276.92)	183.23	<0.001
				Session x treatment	(2,2599.22)	1.75	0.17
				Session x block	(14,2594.74)	3.30	<0.001
				Treatment x block	(7,2594.72)	3.22	<0.01
				Session x treatment x block	(14,2594.69)	0.99	0.46
				Order	(1,12.15)	2.60	0.13
				Sex	(1,12.28)	1.64	0.22
				MoCA	(4,12.10)	1.77	0.20
				Age	(12,12.25)	1.73	0.17
				Education	(1,10.03)	2.62	0.23
Switching task							
General accuracy	3A	0.05	0.43	Session	(2,602.08)	1.09	0.33
				Treatment	(1,25.09)	1.82	0.19
				Session x treatment	(2,602.91)	0.18	0.83
				Order	(1.9.97)	0.34	0.57
				Sex	(1,9.99)	0.16	0.70
				MoCA	(4,10)	0.15	0.96
				Age	(12,10.82)	0.21	0.99
				Education	(1,10.04)	<0.01	0.96
Accuracy by switch type	3B	0.16	0.54	Session	(2,595.73)	1.22	0.30
				Treatment	(1,25.49)	1.87	0.18
				Switch type	(1,590.97)	143.61	<0.001
				Session x treatment	(2,596.49)	0.19	0.83
				Session x switch type	(2,590.96)	2.23	0.11
				Treatment x switch type	(1,590.94)	0.99	0.32
				Session x treatment x switch type	(2,590.96)	0.51	0.60
				Order	(1,9.97)	0.35	0.57
				Sex	(1,10)	0.15	0.70
				MoCA	(4,10)	0.15	0.96
				Age	(12,10.77)	0.22	0.99
				Education	(1,9.97)	0.35	0.97
Accuracy by trial type	3C	0.07	0.45	Session	(2,596.22)	1.13	0.32
				Treatment	(1,25.17)	1.81	0.19
				Trial type	(1,592.62)	9.14	<0.01

				Session x treatment	(2, 597.08)	0.15	0.86
				Session x trial type	(2,592.04)	2.07	0.13
				Treatment x trial type	(1,592.52)	4.48	0.03
				Session x treatment x trial type	(2,592.04)	1.09	0.33
				Order	(1,9.97)	0.33	0.58
				Sex	(1,10)	0.15	0.70
				MoCA	(4,10)	0.14	0.96
				Age	(12,10.82)	0.21	0.99
				Education	(1,10.04)	<0.01	0.95
RT	3D	0.12	0.31	Session	(2,62390)	166.89	<0.001
				Treatment	(1,28)	2.85	0.10
				Session x treatment	(2,62390)	9.88	<0.001
				Order	(1,10)	1.09	0.32
				Sex	(1,10)	0.68	0.43
				MoCA	(4,10)	1.57	0.26
				Age	(12,10)	0.44	0.91
				Education	(1,10)	0.15	0.71
RT by switch type	3E	0.35	0.53	Session	(2,62377)	342.37	<0.001
				Treatment	(1,29)	4.05	0.05
				Switch type	(1,62365)	30055.41	<0.001
				Session x treatment	(2,62377)	26.51	<0.001
				Session x switch type	(2,62365)	93.47	<0.001
				Treatment x switch type	(1,62366)	8.24	<0.01
				Session x treatment x switch type	(2,62365)	14.74	<0.001
				Order	(1,10)	0.97	0.35
				Sex	(1,10)	0.76	0.40
				MoCA	(4,10)	1.57	0.26
				Age	(1,10)	0.44	0.91
				Education	(12,10)	0.16	0.69
RT by trial type	3F	0.12	0.31	Session	(2,62384)	166.87	<0.001
				Treatment	(1,28)	2.85	0.10
				Trial type	(1,62367)	0.37	0.55
				Session x treatment	(2,62384)	9.87	<0.001
				Session x trial type	(2,62366)	2.03	0.13
				Treatment x trial type	(1,62368)	0.24	0.62

				Session x treatment x trial type	(2,62366)	3.94	0.02
				Order	(1,10)	1.08	0.32
				Sex	(1,10)	0.68	0.43
				MoCA	(4,10)	1.57	0.26
				Age	(12,10)	0.44	0.91
				Education	(1,10)	0.15	0.71
Go/No-Go							
Commission errors (as a % of ‘no-go’ trials	4A	0.12	0.83	Session	(2,83.59)	5.27	0.007
				Treatment	(1,25.96)	0.04	0.84
				Session x treatment	(2,84.97)	0.18	0.84
				Order	(1,9.05)	0.14	0.73
				Sex	(1,9.18)	0.17	0.69
				MoCA	(4, 9.41)	0.43	0.79
				Age	(12,8.98)	0.23	0.99
				Education	(1,8.74)	0.52	0.49
Omission errors (as a % of ‘go’ trials)	4B	0.20	0.86	Session	(2,107.79)	4.92	0.01
				Treatment	(1,82.55)	<0.001	0.99
				Session x treatment	(2,107.97)	0.26	0.77
				Order	(1,8.97)	0.09	0.78
				Sex	(1,9.04)	<0.001	0.99
				MoCA	(4,9.13)	0.36	0.83
				Age	(11,9.05)	0.58	0.83
				Education	(1,9.12)	0.14	0.72
RT (ms)	4C	0.21	0.39	Session	(2,18524.60)	8.11	<0.001
				Treatment	(1,27.10)	0.17	0.69
				Session x treatment	(2,18547.70)	7.06	<0.001
				Order	(1,9.10)	0.02	0.90
				Sex	(1,8.90)	1.46	0.26
				MoCA	(4,9)	4.71	0.02
				Age	(12,9.7)	4.17	0.02
				Education	(1,9)	6.53	0.03

Mood outcome	Model	Marginal R^2	Conditional R^2	Factor	Degrees of freedom	F statistic	P value
PANAS							
Positive affect	5A	0.44	0.89	Session	(2,110.60)	0.33	0.72
				Treatment	(1,28.43)	2.91	0.10
				Session x treatment	(2,110.58)	0.07	0.93
				Order	(1,10.06)	0.69	0.43
				Sex	(1,9.93)	1.22	0.30
				MoCA	(4,9.98)	1.71	0.22
				Age	(12,10.24)	2.10	0.12
				Education	(1,10.16)	0.22	0.65
Negative affect	5B	0.25	0.61	Session	(2,96.25)	10.08	<0.001
				Treatment	(1,26.58)	0.34	0.56
				Session x treatment	(2,97.40)	0.18	0.84
				Order	(1,10.39)	0.05	0.83
				Sex	(1,9.74)	0.51	0.49
				MoCA	(4,10.20)	1.52	0.27
				Age	(12,10.25)	1.04	0.48
				Education	(1,10.29)	0.68	0.43
Sadness	5C	0.21	0.78	Session	(2,90.79)	1.25	0.29
				Treatment	(1,23.03)	1.28	0.27
				Session x treatment	(2,91.62)	2.03	0.14
				Order	(1,8.73)	0.39	0.55
				Sex	(1,8.67)	0.39	0.55
				MoCA	(4,8.66)	0.34	0.85
				Age	(11,8.65)	0.71	0.71
				Education	(1,8.51)	0.14	0.72
Hostility	5D	0.43	0.75	Session	(2,103.92)	0.77	0.47
				Treatment	(1,28.11)	1.49	0.23
				Session x treatment	(2,103.71)	0.02	0.98
				Order	(1,9.84)	1.73	0.22
				Sex	(1,9.95)	2.80	0.13
				MoCA	(4,9.86)	2.58	0.10
				Age	(12,9.90)	1.99	0.14
				Education	(1,9.85)	0.08	0.78
Fear	5E	0.19	0.58	Session	(2,102.65)	2.35	0.10
				Treatment	(1,26.05)	6.42	0.02
				Session x treatment	(2,102.66)	2.46	0.09
				Order	(1,8.76)	0.42	0.53
				Sex	(1,9.26)	0.16	0.70
				MoCA	(4,9.25)	1.34	0.33
				Age	(12,8.91)	0.47	0.89
				Education	(1, 9.01)	0.45	0.52
Joviality	5F	0.36	0.90	Session	(2,104.60)	0.03	0.97

				Treatment	(1,26.76)	2.34	0.14
				Session x treatment	(2,104.56)	0.19	0.83
				Order	(1,9.49)	0.73	0.41
				Sex	(1,9.32)	0.77	0.40
				MoCA	(4,9.44)	0.99	0.46
				Age	(12,9.73)	1.34	0.33
				Education	(1,9.57)	0.04	0.84
Attentiveness	5G	0.47	0.85	Session	(2,107.79)	2.64	0.08
				Treatment	(1,26.58)	2.82	0.10
				Session x treatment	(2,107.74)	0.12	0.89
				Order	(1,9.91)	0.46	0.52
				Sex	(1,9.78)	1.94	0.19
				MoCA	(4,9.84)	1.68	0.23
				Age	(12,10.18)	2.49	0.08
				Education	(1,9.99)	<0.01	0.98
Fatigue	5H	0.42	0.73	Session	(2,106.91)	0.36	0.70
				Treatment	(1,27.52)	0.94	0.34
				Session x treatment	(2,106.91)	2.00	0.14
				Order	(1,9.10)	2.33	0.16
				Sex	(1,9.12)	10.24	0.01
				MoCA	(4,9)	0.35	0.84
				Age	(12,9.05)	2.25	0.11
				Education	(1,8.97)	0.12	0.74
Serenity	5I	0.33	0.77	Session	(2,110.78)	1.19	0.31
				Treatment	(1,28.45)	2.81	0.10
				Session x treatment	(2,110.75)	0.49	0.61
				Order	(1,10.01)	0.06	0.81
				Sex	(1,10.11)	3.99	0.07
				MoCA	(4,10)	0.76	0.57
				Age	(12,10.02)	1.37	0.31
				Education	(1,10.02)	0.04	0.85
Self-assurance	5J	0.34	0.85	Session	(2,136.74)	0.03	0.97
				Treatment	(1,124.62)	3.87	0.05
				Session x treatment	(2,136.75)	0.34	0.71
				Order	(1,10.05)	0.07	0.80
				Sex	(1,10)	1.32	0.28
				MoCA	(4,10)	0.80	0.55
				Age	(12,10.09)	1.15	0.42
				Education	(1,10.03)	0.22	0.65
PSS	6A	0.20	0.80	Session	(2,107.15)	1.03	0.36
				Treatment	(1,27.65)	0.09	0.76
				Session x treatment	(2,107.55)	0.15	0.86
				Order	(1,9.90)	0.05	0.83
				Sex	(1,9.97)	0.46	0.52
				MoCA	(4,9.89)	0.57	0.69
				Age	(12,9.99)	0.57	0.82

				Education	(1,9.89)	0.15	0.70
STAI	7A	0.27	0.66	Session	(2,134.02)	0.57	0.57
				Treatment	(1,125.48)	3.69	0.06
				Session x treatment	(2,134.02)	1.57	0.21
				Order	(1,9.91)	0.31	0.59
				Sex	(1,9.91)	2.93	0.12
				MoCA	(4,9.96)	0.25	0.90
				Age	(12,10.21)	0.85	0.61
				Education	(1,9.97)	0.07	0.80
CESD	8A	0.25	0.79	Session	(1,79.53)	1.71	0.19
				Treatment	(1,69.91)	0.58	0.45
				Session x treatment	(1,79.52)	0.05	0.82
				Order	(1,8.88)	0.05	0.83
				Sex	(1,8.84)	1.51	0.25
				MoCA	(4,8.93)	0.60	0.67
				Age	(12,9.01)	0.50	0.87
				Education	(1,9.09)	0.09	0.77
LEIDS-r							
Total score	9A	0.31	0.91	Session	(2,109.32)	1.24	0.29
				Treatment	(1,26.80)	1.90	0.18
				Session x treatment	(2,109.29)	0.40	0.67
				Order	(1,10.03)	2.80	0.13
				Sex	(1,10.13)	3.81	0.08
				MoCA	(4,10.01)	1.58	0.25
				Age	(12,10.03)	0.76	0.68
				Education	(1,10.03)	0.30	0.60
Hopelessness	9B	0.25	0.95	Session	(2,110.30)	5.05	<0.01
				Treatment	(1,27.24)	0.06	0.80
				Session x treatment	(2,110.26)	2.65	0.08
				Order	(1,10.01)	0.28	0.61
				Sex	(1,10.01)	0.13	0.73
				MoCA	(4,10)	0.18	0.94
				Age	(12,10.02)	0.57	0.82
				Education	(1,10.01)	0.03	0.86
Acceptance	9C	0.21	0.81	Session	(2,111.25)	0.40	0.67
				Treatment	(1,28.14)	2.53	0.12
				Session x treatment	(2,111.22)	0.92	0.40
				Order	(1,10.04)	0.33	0.58
				Sex	(1,10.05)	0.24	0.63
				MoCA	(4,10.02)	0.32	0.86
				Age	(12,10.07)	0.46	0.90
				Education	(1,10.04)	0.02	0.89
Aggression	9D	0.32	0.89	Session	(2,111.53)	3.23	0.04
				Treatment	(1,28.61)	3.45	0.07
				Session x treatment	(2,111.50)	4.11	0.02
				Order	(1,10.02)	0.23	0.64

				Sex	(1,9.99)	0.57	0.47
				MoCA	(4,9.99)	0.55	0.71
				Age	(12,9.99)	1.00	0.51
				Education	(1,10.02)	0.20	0.66
Control	9E	0.29	0.78	Session	(2,109.50)	0.83	0.44
				Treatment	(1,26.86)	3.84	0.06
				Session x treatment	(2,109.48)	1.69	0.19
				Order	(1,10.02)	0.60	0.46
				Sex	(1,10.13)	0.16	0.70
				MoCA	(4,10)	0.63	0.66
				Age	(12,10.01)	0.74	0.69
				Education	(1,10.02)	0.04	0.84
Risk avoidance	9F	0.25	0.83	Session	(2,110.51)	0.43	0.65
				Treatment	(1,27.68)	0.61	0.44
				Session x treatment	(2,110.48)	0.51	0.60
				Order	(1,10.03)	0.26	0.62
				Sex	(1,10.14)	4.82	0.05
				MoCA	(4,10.02)	0.77	0.57
				Age	(12,10.02)	0.45	0.90
				Education	(1,10.03)	0.67	0.43
Rumination	9G	0.29	0.88	Session	(2,110.90)	3.39	0.04
				Treatment	(1,28.01)	0.94	0.34
				Session x treatment	(2,110.87)	1.81	0.17
				Order	(1,10.04)	2.16	0.17
				Sex	(1,10.16)	1.94	0.19
				MoCA	(4,10.02)	0.60	0.67
				Age	(12,10.03)	0.77	0.67
				Education	(1,10.04)	0.24	0.63