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National University of Ireland, Cork



Gut Microbes and Brain Function

Thesis presented by

Niamh Wiley, B.Sc.

for the degree of

Doctor of Philosophy

University College Cork

Department of Psychiatry and Neurobehavioural Science

Head of School/Department: Prof. Timothy G. Dinan

Supervisors: Prof. Catherine Stanton, Prof. Paul Ross, Prof. John F. Cryan and Prof. Timothy G. Dinan

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Author Contributions

All of the work herein was performed independently by the author, with the following exceptions:

Chapter 3

Amy Murphy, Teagasc, isolated *Lactobacillus* and *Bifidobacterium* strains from MyNewGut faecal samples to generate the biobank from which the isolates in this study were selected for screening and assisted with study design; Crystal Johnson and Kevin Linehan, Teagasc, isolated strains from fermented food products; Gerard Clarke and Francisca Villalobos, APC Microbiome Ireland, performed and assisted with interpretation of the HPLC analysis; Shane O'Donnell, Teagasc, assisted in preparing strains for whole genome sequencing.

Chapter 4

Nicolas Singewald, University of Innsbruck, designed the study; Anupam Sah, University of Innsbruck, designed the study, performed animal experiments, and collected behavioural data; Anna Golubeva and Thomaz Bastiaanssen, APC Microbiome Ireland, assisted with data analysis and interpretation; Fiona Fouhy, Teagasc, ran 16S sequences through Teagasc Moorepark pipeline to create OTU tables.

Chapter 5

Niamh Wiley performed DNA extractions and library preparation with assistance from Gabrielle Davidson and Crystal Johnson, analysed the data and wrote the manuscript with assistance from Gabrielle Davidson. Gabrielle Davidson, Amy Cooke and John Quinn, University College Cork, designed the experiment with input from Paul Ross and Catherine Stanton. Gabrielle Davidson and Amy Cooke ran the experiment with assistance from Michael Reichert, Ipek Kulachi, Ivan de la Hera and Jodie Crane. Fiona Fouhy carried out bioinformatics analysis.

Chapter 6

Elaine Patterson, Paul M. Ryan, and Niamh Wiley conducted research, analysed data, and drafted the manuscript; Ilaria Carafa, Eoin Sherwin and Gerard Moloney performed laboratory work, analysed data, and contributed to the writing of the manuscript. Elena Franciosi, Rupasri Mandal, David S. Wishart and Kieran Tuohy provided resources and intellectual input that supported the study.

Chapter 7

Kirsten Berding contributed to data analysis, writing and interpretation of data related to the stress and cognition analysis. Caitriona Long-Smith designed the intervention study, performed the participants visits and assisted with interpreting the stress and cognition data. Conall Strain contributed to biological analyses by running the SCFA samples on the Gas Chromatography machine. Carina Cabria contributed to the analysis of the psychological questionnaires and CANTAB data. Fiona Fouhy and Katríona Lyons ran 16S sequences through the Teagasc Moorepark pipeline to create OTU tables.



Niamh Wiley

Publications

First Author Publications

Niamh C. Wiley, Timothy G. Dinan, R. Paul Ross, Catherine Stanton, Gerard Clarke, John F. Cryan (2017). The microbiota-gut-brain axis as a key regulator of neural function and the stress response: implications for human and animal health. *Journal of Animal Science,* Volume 95, Issue 7, July 2017, Pages 3225–3246, https://doi.org/10.2527/jas.2016.1256.

Niamh C. Wiley, John F. Cryan, Timothy G. Dinan, R. Paul Ross, Catherine Stanton. Production of Psychoactive Metabolites by Gut Bacteria (2021). Microbes and the Mind. The Impact of the Microbiome on Mental Health. Mod Trends Psychiatry. Basel, Karger, 2021, vol 32 (DOI: 10.1159/000510419)

Co-Author Publications

Marcel Van De Wouw, Marcus Boehme, Joshua M. Lyte, **Niamh Wiley**, Conall Strain, Orla O'Sullivan, Gerard Clarke, Catherine Stanton, Timothy G. Dinan, John F. Cryan. Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain–gut axis alterations. *Journal of Physiology*, 596.20 (2018) pp 4923–4944 18. https://doi.org/10.1113/JP276431.

Elaine Patterson*, Paul M. Ryan*, **Niamh Wiley**, Ilaria Carafa, Eoin Sherwin, Gerard Moloney, Elena Franciosi, Rupasri Mandal, David S. Wishart, Kieran Tuohy, R. Paul Ross, John F. Cryan, Timothy G. Dinan, Catherine Stanton (2019). Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic

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*Joint first authors

Gabrielle L. Davidson, **Niamh Wiley**, Amy C. Cooke, Crystal N. Johnson, Fiona Fouhy, Michael S. Reichert, Ivan de la Hera, Jodie M. S. Crane, Ipek G. Kulahci, R. Paul Ross, C. Stanton, John L. Quinn. Diet induces parallel changes to the gut microbiota and problem solving performance in a wild bird. *Scientific Reports* **volume 10**, article number: 20783 (2020). https://www.nature.com/articles/s41598-020-77256-y.

Thomaz F. S. Bastiaanssen*, Anand Gururajan*, Marcel van de Wouw, Gerard M. Moloney, Nathaniel L. Ritz, Caitriona M. Long-Smith, **Niamh C. Wiley**, Amy B. Murphy, Joshua M. Lyte, Fiona Fouhy, Catherine Stanton, Marcus J. Claesson, Timothy G. Dinan, John F. Cryan. Volatility as a Concept to Understand the Impact of Stress on the Microbiome. *Psychoneuroendocrinology* volume 124, article number: 105047 (2020). https://doi.org/10.1016/j.psyneuen.2020.105047
*Joint first authors

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*Joint first authors

Conference Posters

Niamh C. Wiley, Timothy G. Dinan, John F. Cryan, Elaine Patterson, R. Paul Ross, Catherine Stanton (2017). *In vitro screening of human-derived lactobacilli for novel psychobiotic potential.* Presented at NeuroGASTRO 2017.

Abbreviations

5-HIAA 5-hydroxyindoleacetic acid

5-HT Serotonin

ACE Abundance-based coverage estimator

AD Alzheimer's disease

AHR Aryl hydrocarbon receptor

ASD Autism spectrum disorder

AUC Area under the curve

BBB Blood brain barrier

BCFA Branch chain fatty acids

BDNF Brain derived neurotrophic factor

BHI Brain heart infusion

CA Cholic acid

CAR Cortisol awakening response

CCA Canonical correspondence analysis

CFU Colony forming unit

CLA Conjugated linoleic acid

CNS Central nervous system

CORT Corticosterone

DCA Deoxycholic acid

DHA Docasahexaenoic acid

DLPC Dorsolateral prefrontal cortex

DMN Default mode network

DOPAC 3,4-dihydroxyphenylacetic acid

EAT Epididymal adipose tissue

ECL Enterochromaffin-like cells

ECs Enterochromaffin cells

ELISA Enzyme-linked immunosorbent assay

ENS Enteric nervous system

EPA Eicosapentaenoic acid

EPM Elevated plus maze

ERT Emotion recognition test

FDR False discovery rate

FFQ Food frequency questionnaire

FGFP Belgian Flemish gut flora project

FLASH Fast length adjustment of short reads to improve genome assembly

FMT Faecal microbiota transplantation

FOS Fructooligosaccharide

FST Forced swim test

GABA Gamma-aminobutyric acid

GAD Glutamic acid decarboxylase

GBM Gut brain module

GF Germ free

GIT Gastrointestinal tract

GOS Galactooligosaccharide

HAB High anxiety-related behaviour

HADS Hospital anxiety and depression scale

HF High fat

HFC High fat control

HPA Hypothalamic pituitary adrenal

HPLC High performance liquid chromatography

IDO Indoleamine-2,3-dioxygenase

IED Intra-extra dimensional set shift

IP-GTT Intraperitoneal glucose tolerance test

IP-ITT Intraperitoneal insulin tolerance test

LAB Lactic acid bacteria

LBS Lactobacillus Selection Agar

LCFA Long chain fatty acids

L-dopa Levodopa

LEfSe LDA effect size

LF Low fat

LFC Low fat control

LPS Lipopolysaccharide

MAT Mesenteric adipose tissue

MDD Major depressive disorder

MIA Maternal immune activation

MMG Mixed meal gavage

MOT Motor screening test

MRS de Man, Rogosa and Sharpe

MS Multiple sclerosis

MSG Monosodium glutamate

NAB Normal anxiety-related behaviour

NAS Negative affect schedule

NGS Next generation sequencing

NMR Nuclear magnetic resonance

NOR Novel object recognition

OF Open field

OTU Operational taxonomic unit

PAL Paired associates learning

PAS Positive affect schedule

PASA Primary appraisal secondary appraisal

PCA Principal component analysis

PD Parkinson's disease

PDX Polydextrose

PFC Prefrontal cortex

PNAS Positive negative affect schedule

PPARγ Proliferator-activated receptor gamma

PSQI Pittsburgh sleep quality index

PSS Perceived stress scale

PyNAST Python nearest alignment space termination

RDA Redundancy analysis

RVP Rapid visual information processing

SAT Subcutaneous adipose tissue

SCFA Short chain fatty acids

SECPT Socially evaluated cold pressor test

SEM Standard error of the mean

SITA Sitagliptin

SPF Specific pathogen free

sPLS-DA Sparse partial least square discriminant analysis

SS Spatial span

STAI State trait anxiety inventory

T1D Type 1 diabetes

T2D Type 2 diabetes

TDO Tryptophan-2,3-dioxygenase

TNF Tumour necrosis factor

tyrDC Tyrosine decarboxylase

VAS Visual analogue scale

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Abstract

The gut-brain-microbiota axis comprises an extensive communication network between the brain, the gut, and the microbiota residing there. If the composition or the diversity of the gut microbiota is impaired, this can have negative consequences for host health and has been associated with disorders such as obesity, diabetes, inflammatory diseases, and neuropsychiatric illnesses including anxiety and depression. Therefore, much research effort in recent years has focused on understanding the potential of targeting the intestinal microbiota to prevent and treat such disorders. Preclinical data strongly support the view that microbe manipulation using probiotics and prebiotics to enhance the host-microbe symbiotic relationship has great potential in the future prevention and treatment of such disorders. Therefore, this study aimed to identify novel probiotics by screening gut-derived lactobacilli for bioactive metabolite production, namely GABA and serotonin, identifying one *Lactobacillus plantarum* GABA producer and two *Lactobacillus paracasei* serotonin producers which were subsequently characterised and assessed for probiotic characteristics. Enhanced whole genome sequencing identified the *trp* operon involved in tryptophan and serotonin metabolic pathways, antimicrobial activity including Enterolysin A and Carnocin CP52, as well as genes promoting acid tolerance and adhesion capability such as *dlt* and *fbp*.

The work described here also explores the influence that environmental factors and diet have on the microbiome and the subsequent effect on anxiety and stress-related behaviour in mouse models and an ecologically relevant model, namely wild caught great tit birds. Chapter 4 explores the effect of environment on the gut microbiota by exposing high anxiety and normal anxiety phenotype mice to an enriched versus standard environment, revealing improved anxiety-like behaviour following environmental enrichment. However, no effect was observed on alpha diversity while differences in beta diversity were based on mouse phenotype and not environment. Chapter 5 investigated the impact of diet on the gut microbiome in wild caught great tit birds, and demonstrates that a diet low in fat, protein and

fibre results in lower alpha diversity compared to a high fat, protein and fibre content diet, while several compositional differences were also observed, including a higher abundance of Bacteroidetes and Proteobacteria following administration of an insect diet.

Furthermore, the data presented herein outline the potential of harnessing the gut microbiota to improve metabolic and neuropsychological health through probiotic and prebiotic intervention in preclinical and clinical trials, respectively. The data presented in Chapter 6 outline a potential role of metabolic- and neuroactive-microbial metabolite production in the modulation of diet-induced metabolic dysfunction, including abnormal behaviour. Intervention with GABA-producing *Lactobacillus brevis* strains attenuated several abnormalities associated with metabolic dysfunction, causing a reduction in the accumulation of mesenteric adipose tissue, increased insulin secretion following glucose challenge, improved plasma cholesterol clearance, and reduced despair-like behaviour and basal corticosterone production during the forced swim test. Chapter 7 investigated an alternative method of gut microbiota manipulation, namely prebiotic administration with polydextrose in adults for four weeks which resulted in a modest improvement in cognitive flexibility and an increased ability for sustained attention in this double-blind, randomised, placebo-controlled clinical trial. Although there was no change in microbial diversity, abundance of *Ruminiclostridium* 5 significantly increased after polydextrose supplementation compared to placebo. Therefore, the results presented herein indicate that probiotic and prebiotic intervention could modulate gut-to-brain communication and benefit metabolic and neuropsychological health.

1. The microbiota-gut-brain axis as a key regulator of neural function and the stress response: Implications for human and animal health[1,2]

Niamh C. Wiley*,†,‡, Timothy G. Dinan†,‡, R. Paul Ross†,§, Catherine Stanton*,†,‡, Gerard Clarke†,‡,3, John F. Cryan†,#

*Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland; †APC Microbiome Ireland[4], University College Cork, Cork, Ireland; ‡Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland; §College of Science, Engineering and Food Science, University College Cork, Cork, Ireland; and #Department of Anatomy and Neuroscience, University College Cork, Ireland

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[3] **Corresponding author:** g.clarke@ucc.ie

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1.1 Abstract

The brain-gut-microbiota axis comprises an extensive communication network between the brain, the gut, and the microbiota residing there. Development of a diverse gut microbiota is vital for multiple features of behaviour and physiology, as well as many fundamental aspects of brain structure and function. Appropriate early-life assembly of the gut microbiota is also believed to play a role in subsequent emotional and cognitive development. If the composition, diversity, or assembly of the gut microbiota is impaired, this can have a negative impact on host health and lead to disorders such as obesity, diabetes, inflammatory diseases, and even potentially neuropsychiatric illnesses including anxiety and depression. Therefore, much research effort in recent years has focused on understanding the potential of targeting the intestinal microbiota to prevent and treat such disorders. This review aims to explore the influence of the gut microbiota on host neural function and behaviour, particularly those of relevance to stress-related disorders. The involvement of microbiota in diverse neural functions such as myelination, microglia function, neuronal morphology, and blood brain barrier integrity across the lifespan, from early life to adolescence to old age will also be discussed. Nurturing an optimal gut microbiome may also prove beneficial in animal science as a means to manage stressful situations and to increase productivity of farm animals. The implications of these observations are manifold, and researchers are hopeful that this promising body of preclinical work can be successfully translated to the clinic and beyond.

1.2 Introduction

The term ‘microbiome’ refers to the total number of microorganisms and their genetic material. The average human body contains 100 trillion microbes, primarily residing in the gut (approximately 1 to 2 kg, the weight of the human brain). It is commonly cited that bacteria residing in the human body outnumber human cells by a factor of 10; however, new estimates indicate this figure is closer to 1.3:1 (Sender, Fuchs and Milo, 2016). In recent years, it has become more evident that

our microbiome is essential to health, impacting nearly every aspect of host physiology (Lyte, 2014; Shanahan, 2013). This has been driven by parallel advances in sequencing technology such as 16S rRNA gene sequencing and the recent findings implicating the gut microbiome in psychological and stress-related disorders (Foster *et al.*, 2015).

Hologenome theory takes account of the sum of the genetic information of the host and its microbiota (Bordenstein and Theis, 2015). This theory indicates that the genetic abundance of diverse microbial symbionts can contribute to adaptation and evolution of higher organisms (Zilber-Rosenberg and Rosenberg, 2008). We coexist with our gut microbiota as mutualists; we provide an environment and a constant source of nutrition for the microbiome, while the microbial genes supplement available genetic information such as metabolic pathways, and these host-microbe interactions modulate mammalian development and brain evolution (Stilling *et al.*, 2014; Theis *et al.*, 2016). In this review, we explore these concepts in the context of the influence of the gut microbiota on host neural function and behaviour, particularly those relevant to stress-related disorders such as anxiety and depression. We also consider the implications of these observations, mostly derived from a promising body of preclinical work, in the context of translational challenges.

1.3 Gut Microbiota Composition

Although the exact composition of the gut microbiome is still an open question, recent advances in metagenomic technologies have enhanced our knowledge. While early information regarding the intestinal microbiota was derived from culture-dependent techniques, which revealed only the minority of species amenable to cultivation, the advent of DNA-based analyses has provided information on the composition and function of previously uncultured microbiota (Power *et al.*, 2014). The human gastrointestinal (GI) tract is inhabited by 10^{13} to 10^{14} microorganisms, with estimates of possibly over 1,000 species and more than 7,000 strains (Grenham *et al.*, 2011) largely from approximately 14 ‘core’ genera

(Falony *et al.*, 2016). There is a significant variety of microbiota; approximately 160 species per person per faecal sample (Rodriguez *et al.*, 2015). It is predominantly comprised of anaerobic bacteria, but viruses, protozoa, archae, and fungi are also present. Recent estimates indicate that the dominant *Bacteroidetes* and *Firmicutes* phylotypes account for at least 70% of the composition (Zhernakova *et al.*, 2016), and *Proteobacteria, Actinobacteria, Fusobacteria*, and *Verrucomicrobia* phyla are present in relatively low abundance (Eckburg *et al.*, 2005). The gut microbiota of monogastric animals such as pigs, chickens and rabbits are dominated by *Bacteroides, Clostridium, Eubacterium, Lactobacillus, Enterocateriacae* and *Streptococcus*. The rumen of polygastric animals such as cows and sheep contains mostly fibre degrading species such as *Fibrobacter, Ruminococcus, Bacteroides*, and other groups including *Prevotella, Selenomonas, Streptococcus* and *Lactobacillus* (Simpson *et al.*, 2002).

1.4 Where Do We Get Our Microbiota From?

1.4.1 Programming the Infant Gut Microbiome

Although stable over long periods, the composition of the microbiome throughout life is influenced by genetic and environmental factors such as mode of delivery, age, diet, geographic location, and medical treatments (Belizario and Napolitano, 2015). Traditionally, it was believed that the first exposure to microbes occurs during birth. Recent evidence of bacteria found in umbilical cord blood (Jimenez *et al.*, 2005), amniotic fluid (Bearfield *et al.*, 2002) and fetal membranes (Rautava *et al.*, 2012) has challenged this sterile womb paradigm (Funkhouser and Bordenstein, 2013). However, it is important to consider that a recent study comparing placental samples with contamination controls found no evidence to indicate the presence of a distinct placental microbiota and the low bacterial biomass of placental samples allows the possibility that some or all of the bacterial DNA is derived from contamination in dust or commercial reagents (Lauder *et al.*, 2016).

Vaginally delivered infants are colonised by maternal vaginal and faecal bacteria,

whereas infants born via Caesarean section are not initially directly exposed to maternal microbes and are instead colonised with microbes associated with the maternal skin and the hospital environment. The microbiota composition of these infants may remain disturbed for months or even years with *Bacteroidetes* colonisation delayed (Jakobsson *et al.*, 2014). The microbiota begins to take on a more 'adult-like' composition from about 2 years of age (Rodriguez *et al.*, 2015).

A major source for colonisation of the infant gut is through bacteria in the mother's milk, which contains over 700 species of bacteria as well as antimicrobial compounds, immunoglobulins, cytokines, growth factors and leukocytes that transfer passive immunity to the infant (Stelwagen *et al.*, 2009; Fernandez *et al.*, 2013). Colonisation patterns fostered by breast milk are believed to play a major role in health status during childhood and limit the overgrowth of potentially pathogenic bacteria by maternal transfer of antibodies and immunoglobulins (Rodriguez *et al.*, 2015; Tsukumo *et al.*, 2015). The optimal conditions for neonatal microbiome development occur in infants delivered vaginally at full-term, who are breastfed and not treated with antibiotics (Alex *et al.*, 2013; Mueller *et al.*, 2015). The composition of the microbiota changes further with the introduction of solid foods, and a stable community similar to the adult microbiota becomes established post weaning (Power *et al.*, 2014).

The gut microbiota is also strongly influenced by diet because different bacterial species are better equipped to utilise different substrates (Sonnenburg *et al.*, 2016). Generally, bacteria favour carbohydrates as primary energy sources. De Filippo *et al.* (2010) examined the effect of diet on gut microbiota by comparing the faecal microbiota of European children consuming a Western diet with children in Burkina Faso who consume a plant-rich diet high in fibre. The Burkina Faso children had less abundance of bacteria of the phylum *Firmicutes* and a greater abundance of those of the phylum *Bacteroidetes* (mainly *Prevotella* and *Xylanibacter)* compared with the European children, who had greater levels of *Enterobacteriaceae* (De Filippo *et al.*, 2010). A study by Wu *et al.* (2011) found that enterotypes were strongly associated with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella)*.

The digestive system of horses is adapted to a high-fibre diet, however during training high-starch and low-fibre diets are usually fed, which is considered to be an alimentary stress. Destrez *et al.* (2015) investigated the microbiota composition changes that occur in response to a diet modified from high-fibre to a progressive low-fibre and high-starch diet. Significant increases of colonic total anaerobic bacteria, lactate-utilising bacteria and amylolytic bacteria concentrations were induced by this alimentary stress. Behavioural changes were also observed, indicating that behavioural cues may be useful as non-invasive indicators of alimentary stress, which would be useful to prevent intestinal pain of horses on farms (Destrez *et al.*, 2015).

Murphy *et al.* (2012) evaluated the effect of barley-based diets versus oat-based diets on levels of *Lactobacillus, Bifidobacterium* and *Enterobacterium* in the porcine GI tract. The oat-based diet gave rise to the most diverse populations of lactobacilli, along with greater lactobacilli counts in the cecum and colon. *Bifidobacterium* counts were also greater in the ileum, cecum and colon compared with the barley-based diet. Overall, oat-based diets were found to be more conducive to beneficial microorganisms and richness of the lactobacilli population in the porcine GI tract compared with a barley-based diet (Murphy *et al.*, 2012).

Small variations in diet can also influence microbiota composition. The gut microbiota of two groups of broiler chickens fed either a standard corn-soybean meal ration or a ration high in wheat middlings was characterised and it was found that this small variation in diet did not influence the distribution of microbial species throughout the digestive tract. However, the *Lactobacillus* strain composition was significantly altered. For example, *Lactobacillus agilis* R5 was the dominant type in birds fed a corn-soybean meal diet but was not detected in birds fed the diet high in wheat middlings. These birds harboured a different strain of *L. agilis* and several other strains were also specific to the particular diet (Hammons *et al.*, 2010).

Geographical location also impacts on the microbiota whereby people from Northern European countries have greater levels of *Bifidobacterium* spp. and some

Clostridium spp., whereas people from Southern Europe have a greater abundance of *Eubacteria, Lactobacillus,* and *Bacteroides.* Host genotype also influences microbiota as monozygotic twins show the greatest similarity in their microbiota (Rodriguez *et al.*, 2015; Murphy *et al.*, 2015).

1.4.2 Overlapping Development of Gut Microbiota and the Brain

Development of the nervous system, the microbiota, and the elements of the microbiota-gut-brain axis occur in parallel during interacting time windows, and prenatal, early postnatal and adolescence phases are particularly important for brain development. The developing brain is dependent on critical developmental processes including proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis (Rice and Barone Jr, 2000; Borre *et al.*, 2014). Brain growth continues into the second and third year of life while development of the brain continues into postnatal life and adolescence. Similarly, following initial colonisation during infancy and birth, the gut microbiota continues to develop throughout childhood and adolescence. Developmental events in both the brain and the microbiota are vulnerable to environmental disturbances such as premature birth, low birth weight, exposure to toxins, nutritional deficiencies, and maternal infection (Clarke *et al.*, 2014a). The neonatal brain is more vulnerable to circulating molecules, which may be directly or indirectly attributed to the metabolic activity of the gut microbiota (MacFabe, 2012), and it is during the postnatal period of gut microbiota development that the microbiota exerts significant influence over the developing brain (Clarke *et al.*, 2014a). Vagal innervation allows two-way communication between the brain and the gut and is essential for homeostasis. Therefore, developmental abnormalities of enteric vagal innervation may contribute to disorders of the brain-gut-microbiota axis (Ratcliffe, Farrar and Fox, 2011).

1.4.3 Gut Microbiota and Aging

Growing evidence indicates that the microbiota influences the aging process (O'Toole and Jeffery, 2015). Germ free (GF) mice live longer than conventional animals, although this is likely due to the absence of pathological infections as a result of being raised in sterile conditions (Tazume *et al.*, 1991). Human microbiota composition remains stable throughout adult life until old age, when the composition and quantity change gradually. The potential for the gut microbiota to affect health is particularly relevant at this time because the microbiota may modulate aging-related changes in innate immunity and cognitive function. There is greater inter-individual variation in the microbiota of the elderly than that of younger adults and pronounced differences between frail elderly subjects and healthy elderly subjects (Claesson *et al.*, 2011). Potentially pathogenic bacteria, such as *Enterobacteria* and *Clostridia*, increase with aging, whereas *Bifidobacterium* species, which contribute to the protection of the intestinal tract, decrease (Tsukumo *et al.*, 2015). There is also a shift towards a population comprised predominantly of *Bacteroidetes* (Claesson *et al.*, 2011). Lifestyle factors, particularly diet, are also involved because lower fibre intake leads to a decrease in microbiota diversity which has a negative impact on gut health and is associated with reduced cognitive performance (Flint *et al.*, 2012). Therefore, modulating the microbiota with dietary interventions may be a potential strategy for promoting healthier aging (O'Toole and Jeffery, 2015).

1.5 What is the Microbiome-Gut-Brain Axis?

1.5.1 Components and Signalling along the Microbiome-Gut-Brain Axis

There is extensive communication between the brain and the microbiota in the gut via the brain-gut-microbiome axis. This is composed of the central nervous system (CNS), the neuroendocrine and neuroimmune systems, sympathetic and parasympathetic branches of the autonomic nervous system, the enteric nervous system (ENS) and the intestinal microbiota (Sherwin *et al.*, 2016; Dinan and Cryan, 2016). Through this bidirectional communication system, signals from the brain can

influence the motor, sensory, and secretory functions of the gut, and visceral messages from the gut can influence brain function (Dinan *et al.*, 2015). Several routes of communication between the gut and the brain have been identified including activating afferent sensory fibers of the vagus nerve, neuroimmune pathways, neuroendocrine pathways, microbial metabolites such as short chain fatty acids (SCFA), and microbially-derived neurotransmitters (Cryan and Dinan, 2012).

The gut microbiota secretes neurotransmitters and neuromodulators, such as gamma-aminobutyric acid (GABA), serotonin, catecholamines, and acetylcholine, which impact host psychiatric health (Cryan and O'Mahony, 2011; Lyte, 2011). These neurotransmitters may induce epithelial cells to release molecules that modulate neural signalling within the ENS and, consequently, impact brain function and behaviour of the host (Wall *et al.*, 2014). Gamma-aminobutyric acid-producing bacteria in particular have the potential to influence brain function, behaviour and mental health, and communicate with the brain via the vagus nerve. Large milligram quantities are produced by many gut microbiota, including *Lactobacillus* and *Bifidobacterium*, during a stress response to protect the organism from the acidic environment of the stomach. *Lactobacillus brevis* DPC 6108 in particular is a highly efficient GABA producer, and has been shown to convert up to 90% of monosodium glutamate to GABA from the irreversible α-decarboxylation of L-glutamate (Barrett *et al.*, 2012). Bacteria that constitute the normal microbiome in mice are capable of the *in vivo* production of large quantities of noradrenaline (Asano *et al.*, 2012). Certain bacteria are capable of producing acetylcholine including *L. planatarum*, *E. coli* JCM 5491, *Staphylococcus aureus* JCM 2151 and *Bacillus subtilis* PCI 219 (Girvin and Stevenson, 1954; Horiuchi *et al.*, 2003). It is likely that a number of systems are involved in the transmission of information between the microbiota and the brain (Dinan, Borre and Cryan, 2014).

1.5.2 Microbial Regulation of Tryptophan Metabolism

Although there are a number of mechanisms of communication along the brain-gut-

microbiome axis, microbial regulation of tryptophan metabolism has recently received much attention. Serotonin is a metabolic product of this essential amino acid and plays an important role in the regulation of a number of bodily functions, including GI tract motility, secretion, sensation and mood. Over 90% of serotonin in the human body is synthesised from tryptophan by enterochromaffin cells of the gut (Gershon, 2013; Yano *et al.*, 2015). The rate-limiting enzyme in its synthesis is tryptophan hydroxylase, and it has been shown that gut microbiota promote its expression and serotonin production through stimulatory activities of SCFA on enterochromaffin cells (Reigstad *et al.*, 2015). This was further supported by (Yano *et al.*, 2015) who demonstrated that the gut microbiota plays a key role in regulating high levels of colon (64%) and blood (49%) serotonin concentrations by enhancing synthesis by host enterochromaffin cells. Furthermore, many species of bacteria are capable of producing serotonin including *Streptococcus, Escherichia, Enterococcus, Lactococcus* and *Lactobacillus* (Wall *et al.*, 2014). There is significant overlap between behaviours regulated by the serotonergic system and those that are influenced by the microbiota (O'Mahony *et al.*, 2015). Therefore, the gut microbiota may be a potential therapeutic target for serotonin-related brain-gut axis disorders. Tryptophan-regulating bacteria may have potential as antidepressants because the majority of antidepressant drugs result in increased serotonin levels in the brain.

The dominant metabolic pathway for tryptophan is along the kynurenine pathway, which also yields many interesting and relevant neuroactive molecules (Schwarcz *et al.*, 2012; Schwarcz and Stone, 2017). Circulating tryptophan availability, distribution, and subsequent kynurenine pathway metabolism are tightly regulated at all stages of life (Ruddick *et al.*, 2006). Microbiota depletion using both GF animals and antibiotic administration results in an increase in circulating total tryptophan levels, a reduction in kynurenine pathway metabolism, and increased hippocampal serotonin levels (Clarke *et al.*, 2013). Metabolomic profiling has revealed that plasma serotonin levels are 2.8-fold greater in conventional mice compared with GF mice (Wikoff *et al.*, 2009). Colonisation of GF animals normalises elevated tryptophan concentrations, again indicating that commensal bacteria may

have the ability to influence tryptophan metabolism (Clarke *et al.*, 2013).

The mechanism underlying this regulation is unclear but may involve control over degradation of tryptophan along the kynurenine pathway, which is the dominant pathway for tryptophan, accounting for around 90% of tryptophan metabolism. Increased metabolism of tryptophan along this pathway results in reduced availability for serotonin synthesis, along with increased production of neuroactive kynurenine pathway metabolites that can impact CNS and ENS function (Maes *et al.*, 2011). Direct microbial utilisation and metabolism of tryptophan also needs to be considered (Kennedy *et al.*, 2016). These include quinolinic and kynurenic acid that act on N-methyl-D-aspartate and α7-nicotinic receptors in the CNS and ENS (Forrest *et al.*, 2002). Kynurenic acid is an N-methyl-D-aspartate receptor antagonist in the CNS and is viewed as neuroprotective, whereas quinolinic acid is considered a neurotoxic N-methyl-D-aspartate receptor agonist (Stone and Darlington, 2013). The ability of the microbiota to regulate these neuroactive kynurenine metabolites and important precursors such as tryptophan and kynurenine is likely involved in its ability to influence CNS and ENS neurotransmission (Clarke *et al.*, 2014b).

Variable kynurenine pathway metabolism can be particularly detrimental during the first 1,000 days of life, leading to neurodevelopmental abnormalities. For example, prenatal inhibition of this pathway in rats results in altered hippocampal neuron morphology along with differences in neocortical and cerebellar protein expression that persist into adulthood (Khalil *et al.*, 2014) (Pisar *et al.*, 2014). Therefore, targeting the gut microbiota to modulate kynurenine pathway metabolism, particularly during early life, may provide a therapeutic strategy for CNS function (Kennedy *et al.*, 2016). Potential strategies include restoring intestinal permeability via the gut microbiota, promoting gut microbiota diversity during old age to improve health outcomes, and modulating microbiota through diet (Kelly *et al.*, 2015).

1.5.3 The Vagus Nerve

A predominant pathway in the microbiome-gut-brain axis is the vagus nerve. Certain *Lactobacillus* species communicate with the brain via this route and do not produce their CNS effects if an animal has been vagotomised (Bravo *et al.*, 2011). It is also recognised that the hypothalamic-pituitary-adrenal (HPA) axis and cytokines from the immune system play a role in microbiota-gut-brain axis communication (Jones *et al.*, 2006). The brain can communicate with the enteric microbiota directly by releasing signalling molecules into the gut lumen from enterochromaffin cells, neurons, and immune cells in the lamina propria. Indirect communication also occurs by altering intestinal permeability, GI motility and secretion through activation of the HPA axis, autonomic nervous system, and the neuroendocrine system (Rhee, Pothoulakis and Mayer, 2009; Mangiola *et al.*, 2016). Conversely, the enteric microbiota can communicate with the brain via epithelial cells, receptor-mediated signalling, and stimulation of cells of the lamina propria (O'Mahony *et al.*, 2011). Altered gut microbiota composition affects intestinal permeability, leading to the release of LPS into the bloodstream, which modulates the CNS by increasing the activity of areas involved in emotional control, such as the amygdala, and by leading to the production of inflammatory cytokines (Mangiola *et al.*, 2016).

1.5.4 Basic and Complex Functions of the Microbiome-Gut-Brain Axis

The gut microbiota plays a key role in various metabolic, nutritional, physiological, and immunological processes, but this relationship sometimes becomes pathological, as in obesity, diabetes, atherosclerosis, and inflammatory bowel diseases (Wu *et al.*, 2011). Therefore, research is being directed towards manipulating the microbiome to treat such disorders using probiotics, prebiotics, and faecal microbiota transplantation (FMT). Studies using GF mice have revealed that these animals have decreased activity of digestive enzymes, decreased activity of the mucosa-associated lymphoid tissue and reduction of muscle wall thickness, vascularisation and motility. The gut microbiota may be involved in the metabolism of potentially harmful substances such as cholesterol and bile acids (Barczynska *et*

al., 2015). Germ free mice require a greater caloric intake to maintain the same body weight as conventional animals, confirming that the microbiota is vital for energy and nutrient extraction. Microbiota produce health-beneficial bioactive metabolites or 'pharmabiotics' including bioactive lipids (such as SCFA and conjugated linoleic acid), antimicrobials, and exopolysaccharides (Patterson *et al.*, 2014; Neish, 2002).

The microbiome is essential for host immunity (Hooper, Littman and Macpherson, 2012) and GF mice robustly and reproducibly display defects in both immune development and the immune response (Tsukumo *et al.*, 2015). Microbiota stimulate the innate immune system, leading to the maturation of acquired immunity (Nell, Suerbaum and Josenhans, 2010). They also prevent the colonisation of pathogens by producing anti-microbial compounds and by competing for nutrients and receptors (Grenham *et al.*, 2011). Microbial colonisation has a profound impact on the formation of lymphoid tissues, as shown in GF mice that do not develop isolated lymphoid follicles in the small intestine, and as such are also deficient in secretory IgA (Hooper, Littman and Macpherson, 2012). The influence of the microbiota on the immune system extends beyond the intestinal lamina propria as it has also been shown to influence homeostatic T cell proliferation (Kieper *et al.*, 2005).

1.5.5 Strategies to Investigate the Role of the Microbiome-Gut-Brain Axis in Health and Disease

Germ free mice are extensively used to investigate the importance of the microbiota to host health, particularly behaviour. This model has been vital in uncovering the mechanisms of microbiota-gut-brain communication and the role of the microbiota in development and host health (Luczynski *et al.*, 2016a). These animals are delivered via Caesarean section to avoid contamination with the microbes on the mother's vagina and skin and are then raised in an aseptic isolator to ensure they never come into contact with any bacteria. Germ free mice display cognitive defects and altered behaviour in comparison with conventionalised mice

(Figure 1). The principal advantages of this model are in proof-of-principle studies and that a complete microbiota or specific strains of bacteria can be introduced at various developmental time-points to examine critical questions. Furthermore, a complete microbiota from conventionally colonised animals, animal models of disease or human patients can be transferred via faecal transplantation to determine if disease characteristics are transferrable via the microbiota (Luczynski *et al.*, 2016). However, the GF model may have permanent neurodevelopmental deficits that could deem it unsuitable for certain studies. Germ free mice are criticised for a lack of clinical relevance because humans are never in a GF state (Luczynski *et al.*, 2016a). Chronic antibiotic-antimicrobial administration may be a more realistic representation of natural microbiota perturbation than GF mice and many phenotypic features that are associated with the GF state are also evident, such as reduced anxiety-like behaviour (Bercik *et al.*, 2011). Furthermore, chronic antibiotic treatment can be used to investigate the impact of altered microbiota composition that first occurs later in life, which is not possible in GF mice. Antibiotic-treated mice, an alternative model that allows greater control over timing aspects of microbiota-deficiency (Luczynski *et al.*, 2016a; Hoban *et al.*, 2016a), can also be inoculated with a human microbiota to examine microbiota-gut-brain communication (Kelly *et al.*, 2016b).

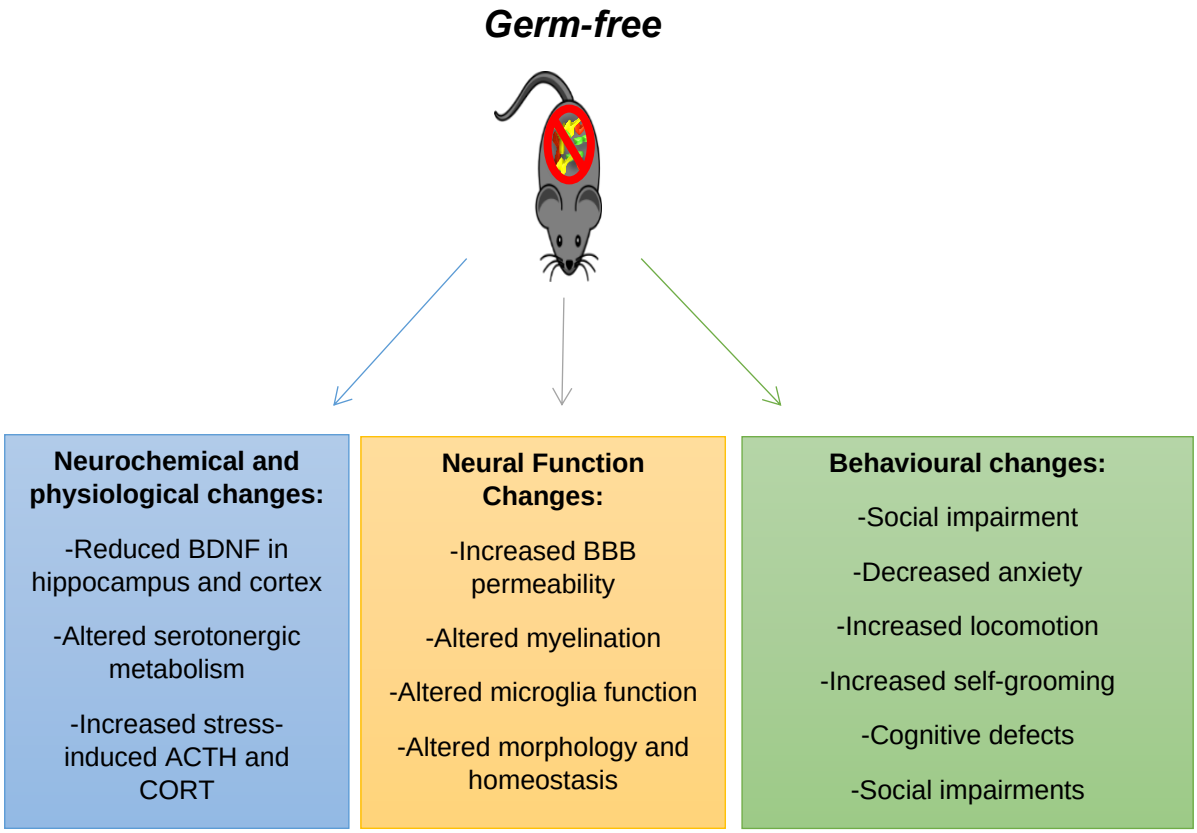


Figure 1. Neurochemical, neural function and behavioural changes present in GF mice. BDNF = brain-derived neurotrophic factor; CORT = corticosterone; BBB = blood brain barrier.

1.6 Stress and the Gut Microbiome

1.6.1 Impact of Stress on the Brain

Stress is a significant health issue, and the microbiome provides a potential therapeutic target for modulation of the stress response. The major response to stress is activation of the HPA axis, resulting in the release of glucocorticoid hormones that leads to a range of biological effects including immune modulation and energy metabolism (McEwen, 1998). Although acute stress is an important adaptive response that allows an organism to respond to stimuli in its external

environment, chronic stress is maladaptive and can lead to many adverse effects for brain and behaviour (Lupien *et al.*, 2009). Permanently increased glucocorticoid levels are associated with reduced hippocampal volume and impairments in cognitive domains, such as memory, perception, and attention (Jameison and Dinan, 2001; Newcomer *et al.*, 1999). In rodents, chronic stress has been shown to induce alteration in various neurotransmitter systems (Popoli *et al.*, 2012), influence oxidative stress (Madrigal *et al.*, 2001), and affect inflammatory cytokine expression (Shintani *et al.*, 1995).

1.6.2 The Impact of Stress on the Gut Microbiome

Stress has negative implications for the gut microbiota, host health and behaviour from the beginning of life. Prenatal stress is associated with a greater prevalence of neurodevelopmental, cardiovascular and metabolic disorders in offspring. Golubeva *et al.* (2015) investigated the impact of late gestational stress on physiological outcomes in adulthood in a rat model of prenatal stress. Affected offspring displayed an exaggerated HPA axis response to stress, elevated blood pressure, impaired GI development and function and impaired cognitive function. Furthermore, prenatal stress resulted in long-lasting alterations in intestinal microbiota composition where *lactobacilli* were decreased (Golubeva *et al.*, 2015).

Maternal separation is an increasingly utilised method for studying the impact of early life stress on the GI microbiota, host health, and behaviour (O'Mahony *et al.*, 2011). Maternally separated animals display many features relevant to psychiatric and functional GI disorders including increased plasma corticosterone, behavioural deficits, decreased noradrenaline in the brain, increased visceral sensitivity, and increased immune response (Desbonnet *et al.*, 2010; O'Mahony *et al.*, 2009). Maternal separation studies have also shown that neonatal stress results in long-term alterations in both the diversity and composition of gut microbiota, which may play a role in the long-term alterations in stress-related behaviour in these animals (O'Mahony *et al.*, 2009). Evidence indicates that supplementation of a mixture of eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) n-3 polyunsaturated acids

could restore the disturbed microbiota composition following early life maternal separation (Pusceddu *et al.*, 2015).

Exposure to a social stressor has been shown to reduce microbial diversity whereby the relative abundance of *Bacteroides* and *Lactobacillus* spp. decreased, while bacteria in the genus *Clostridium* increased (Bailey *et al.*, 2011). Behavioural analysis and microbial profiling of rats exposed to chronic social defeat revealed behavioural changes that were associated with reduced diversity of the gut microbiota. Defeated mice exhibited a lower prevalence of pathways involved in the synthesis and metabolism of neurotransmitter precursors and SCFA along with sustained alterations in dendritic cell activation. The authors suggest that perturbed gut microbiota could predict the onset of neurocognitive deficits that occur following exposure to severe stressors (Bharwani *et al.*, 2016). Mice exposed to chronic restraint stress displayed markedly altered mucosa-associated and luminally-associated microbiota compared with controls. Restraint stress altered the relative abundance of various groups of bacteria, for example there was a significant reduction in the *Lactobacillus* genus in colonic mucosa (Galley *et al.*, 2014). Environmental stress such as restraint stress causes gut dysmotility. However, *L. rhamnosus* administration restored motor function to unstressed levels within minutes of application, indicating a potential for certain bacteria as treatment for stress-induced dysmotility (West *et al.*, 2017). Studies have shown that stressful situations also alter intestinal microbial profiles in humans (Knowles, Nelson and Palombo, 2008). Reber *et al.* (2016) assessed the potential benefits of a heat-killed preparation of *Mycobacterium vaccae* and found that it was protective against some of the adverse effects of a chronic psychosocial stressor.

1.6.3 Stress and Microbiota-Gut-Brain Axis Signalling

Both HPA axis programming in early life and stress reactivity over the lifespan are influenced by the gut microbiota (Moloney *et al.*, 2014). Development of the stress response system occurs in the postnatal period, which coincides with colonisation of the intestinal microbiota (Foster and McVey Neufeld, 2013). A seminal study by

Sudo *et al.* (2004) confirmed that the microbiota is essential in the development of an appropriate stress response. A mild restraint stressor induced an exaggerated release of corticosterone and ACTH in GF mice, which was reversed following monoassociation with *B. infantis.* However, monoassociation with enteropathogenic *Escherichia coli* enhanced the stress response, indicating that there are bacterial strain-specific effects on stress responsivity (Luczynski *et al.*, 2016a). The enhanced HPA response of GF mice was partly corrected by specific pathogen free faecal transplantation at an early stage but not at later stages. Therefore, these results indicate that exposure to microbes at an early developmental stage is necessary for HPA system function (Sudo *et al.*, 2004).

The role of microbiota in stress is being increasingly recognised because key findings have shown that stress influences the composition of the gut microbiota, and that bidirectional communication between microbiota and the CNS influences stress reactivity (Foster and McVey Neufeld, 2013). It has emerged that the gut microbiota mediates the interaction between stress, the HPA axis, and the immune system (De Palma *et al.*, 2014). For example, infants of mothers with high cortisol levels during pregnancy displayed altered microbiota composition and exhibited a greater level of GI symptoms and allergic reactions (Zijlmans *et al.*, 2015). The microbiota has also been shown to play an indirect role in the stress response. Stress increases intestinal permeability, which allows bacteria to translocate across the intestinal mucosa and access immune cells and neuronal cells of the ENS, thereby allowing the microbiota to influence the CNS (Gareau, Silva and Perdue, 2008). Intestinal microbiota can also activate stress circuits through activation of vagal pathways in addition to HPA axis modulation (Lyte *et al.*, 2006).

1.7 The Gut Microbiome and Behaviour

Several models of microbiota manipulation including GF mice, antibiotic administration, FMT, probiotics and prebiotics have demonstrated the important role that the gut microbiota plays in behaviour. Hsiao *et al.* (2013) demonstrated that the maternal immune activation model of autism displayed a perturbed gut

microbiota and GI barrier defects. *Bacteroides fragilis* corrected these disturbances and improved defects in communicative, stereotypic, anxiety-like, and sensorimotor behaviours, all of which are common features of autism spectrum disorders (Vuong and Hsiao, 2016). Germ free mice have been shown to have significant social impairments in the three-chambered sociability test. These behaviours were partially normalised upon bacterial colonisation, confirming the importance of the gut microbiota in social behaviour (Desbonnet *et al.*, 2014). Evidence has shown that the microbiota is required for normal cognitive function. Gareau *et al.* (2011) examined learning and memory in GF mice and found that these animals spent less time exploring novel objects and environments compared with controls, indicating the presence of non-spatial, hippocampal mediated and working memory deficits.

Several studies have shown reduced anxiety-like behaviour in GF mice using a variety of paradigms (Diaz Heijtz *et al.*, 2011). It may seem strange that GF mice exhibit reduced anxiety-like behaviour while showing exaggerated endocrinological stress responses. However, anxiety-like behaviours in the elevated plus maze (EPM) may dissociate from stress-induced corticosterone production in some instances (Rodgers *et al.*, 1999). Interestingly, GF rats display increased anxiety-like behaviour, confirming in a different species the involvement of the gut microbiota in this behaviour, albeit in a different direction (Crumeyrolle-Arias *et al.*, 2014). Neufeld *et al.* (2011) also demonstrated an altered behavioral phenotype in the absence of intestinal microbiota. They compared basal behaviour of GF with specific pathogen-free mice in the EPM paradigm and found that GF mice exhibited reduced-anxiety like basal behaviour. Analysis of brain mRNA transcription of genes implicated in anxiety and stress-reactivity revealed increased brain derived neurotrophic factor (BDNF) and decreased serotonin 5HT1A receptor expression (Neufeld *et al.*, 2011). These findings are of relevance to stress behaviour, as BDNF expression in the hippocampus is associated with CNS stress circuitry and stress-related behaviours (Berton *et al.*, 2006; Bergami *et al.*, 2008).

The 5-HT1A receptor is the most commonly linked of the serotonin receptors to anxiety and emotional behaviour (Kalynchuk, Pinel and Meaney, 2006).

Reconstitution of microbiota post-weaning normalises anxiety-like behaviour in GF mice in some (Clarke *et al.*, 2013) but not all studies depending on timing of colonisation (Diaz Heijtz *et al.*, 2011; Neufeld *et al.*, 2011). Results observed in GF mice have been replicated using a cocktail of antibiotics from weaning onwards, resulting in decreased anxiety-like behaviour and cognitive deficits (Desbonnet *et al.*, 2015). Further evidence of the role of microbiota in anxiety-like behaviour was provided when GF male Swiss Webster mice were colonised with microbiota from specific pathogen free Balb/C mice. The GF mice exhibited increased anxiety-like behaviour, reflecting the behavioural phenotype of Balb/C mice and vice versa (Bercik *et al.*, 2011). These and other recent experiments confirm that behavioural phenotypes, including anxiety and features of depression, are transferrable via microbiota (Kelly *et al.*, 2016; Zheng *et al.*, 2016).

1.8 The Gut Microbiome and Brain Function Across the Lifespan

1.8.1 Abnormal Brain Development

The microbiome contributes to maintaining normal neural function in numerous ways. For example, the serotonergic system does not develop properly without microbes (Clarke *et al.*, 2013). It is hypothesised that cognitive impairments may be due to a reduction in hippocampal activity, as GF mice have less c-Fos-positive CA1 hippocampal cells following memory testing (Gareau *et al.*, 2011). Microglia are the macrophages of the brain and are crucial for maintaining homeostasis and for scavenging pathogens. Erny and colleagues (2015) found substantial contributions of the host microbiota towards microglia homeostasis and this was supported by the fact that the immature phenotype and morphological changes were also observed upon antibiotic administration. Moreover, normal microglia features were partially restored by introduction of a complex microbiota or by supplementation with SCFA.

A recent study indicated that appropriate cortical myelination is dependent on the presence of a diverse microbiota during critical windows of development. The

alterations in myelination, present at both the transcriptional and ultrastructural level, were only evident in the prefrontal cortex, a key region in anxiety and social behaviour. These changes in myelin structure and gene expression could be reversed by colonisation with a conventional microbiota (Hoban *et al.*, 2016). Transcriptional alterations in myelin genes were also evident following alternative microbiota-manipulation strategies (Gacias *et al.*, 2016).

Morphological changes have also been detected in dendrites of the basolateral amygdala and the hippocampus in GF mice. For example, basolateral amygdala pyramidal neurons were shorter, less branched and contained fewer stubby and mushroom spines in GF mice. This study indicates that the microbiota is required for the normal gross morphology and ultrastructure of the amygdala and hippocampus with the possibility that this neural remodelling may contribute to the abnormal behaviour and stress response observed in GF mice (Luczynski *et al.*, 2016). Stilling *et al.* (2015) also found significant differences in the expression of genes involved in important brain physiological processes such as neurotransmission, neuronal plasticity, metabolism, and morphological development. These brain regions are collectively important for cognitive, emotional, and social behaviour, all of which are impaired in GF mice.

The blood brain barrier (BBB) is a vital structure for maintaining brain homeostasis and for protection from harmful toxins and bacteria circulating in the bloodstream. Evidence highlights the gut microbiota as a potential regulator of BBB integrity, as GF mice display increased BBB permeability compared with pathogen-free mice. This increased BBB permeability was associated with diffuse and disorganised tight junctions, and reduced expression of occludin and claudin-5, which are important regulators of barrier function in endothelial cells. Blood brain barrier integrity can be reinforced through faecal transplantation from pathogen-free adult mice or by the administration of SCFA-producing bacteria (Braniste *et al.*, 2014).

1.8.2 Nutrition, the Gut Microbiota, and Brain Development

The production of SCFA is important for brain health and behaviour, and bacteria capable of producing them include *Bacteroides, Bifidobacterium, Propionibacterium, Eubacterium, Lactobacillus, Clostridium*, *Roseburia*, and *Prevotella* (Macfarlane and Macfarlane, 2012). Butyrate in particular contributes significantly to host health, and has been implicated in aspects of brain function and behaviour (Stilling *et al.*, 2016). Administration of sodium butyrate elicits an antidepressant effect in the murine brain by inducing transient histone acetylation in the frontal cortex and hippocampus while altering BDNF expression (Schroeder *et al.*, 2007). Propionate has anti-inflammatory potential, plays an important role in satiety, and improves insulin sensitivity (Vipperla and O'Keefe, 2012). However, excessive propionate may have negative effects on psychological health and behaviour because intraventricular infusion in rats impaired social behaviour and induced brain abnormalities similar to those detected in autism (El-Ansary, Ben Bacha and Kotb, 2012).

Long-chain fatty acids are involved in many important functions including neurogenesis, neurotransmission, and neurological development, as well as having structural and signalling roles. Furthermore, they are believed to be crucial for mental health across the lifespan (Sinn, Milte and Howe, 2010). It has been reported that mice receiving a *Bifidobacterium breve* strain, *B. breve* NCIMB702258, exhibited significantly greater concentrations of arachidonic acid and docosahexaenoic acid in the brain when compared with unsupplemented mice (Wall *et al.*, 2012). Conjugated linoleic acid was shown to reduce cerebral prostaglandin E_2 in the peripheral and central nervous system, exert anti-angiogenic actions in the brain and protect cortical neurons from excitotoxicity (Nakanishi *et al.*, 2003; Sikorski, Hebert and Swain, 2008; Hunt *et al.*, 2010). Lactobacilli and bifidobacteria, particularly *B. breve* and *L. plantarum,* have been shown to produce CLA (Barrett *et al.*, 2007). Therefore, these bacteria have the potential to be used as probiotics for brain health.

1.8.3 Early Life Microbiota Disruption

Disruption of bacterial colonisation during the early postnatal period, either due to Caesarean section delivery (Cardwell *et al.*, 2008) or antibiotic administration (Shaw, Blanchard and Bernstein, 2010), has been repeatedly linked to adverse health outcomes including neurodevelopmental disorders and the onset of psychiatric disorders later in life (Borre *et al.*, 2014). In a previous study from our group, administration of the antibiotic vancomycin to rat pups resulted in visceral hypersensitivity in adulthood. A transient disruption to the microbiota occurred during a critical neurodevelopmental window that had a lasting effect, because although there were no differences in the gut microbiota during adulthood, there were still differences in visceral hypersensitivity. There was no significant effect on anxiety responses, cognitive performance, or immune and stress-related psychological responses (O'Mahony *et al.*, 2014); however, whether this altered microbial composition is causal or consequential of disease state, remains unclear.

Research shows that infants delivered by Caesarean section have abnormal short-term immune responses and a greater risk of developing immune diseases in the long-term (Dominguez-Bello *et al.*, 2010). A population study in Western Australia has shown that individuals with autism spectrum disorder were more likely to have been born by Caesarean section (Glasson *et al.*, 2004) but more recent studies are less clear-cut and indicate the possibility of familial confounding by genetic and/or environmental factors (Curran *et al.*, 2015a; Curran *et al.*, 2015b; Curran *et al.*, 2016). When interpreting results from such studies, it is important to consider the high rates of antibiotic usage and diets that differ from those consumed by healthy populations (Cryan and Dinan, 2012).

1.8.4 Adolescent Microbiota

The brain is highly plastic but also vulnerable during the adolescent period (McCormick and Mathews, 2007). Various structural, neurochemical, and molecular changes occur including synaptic pruning and the growth and modification of

intracortical circuits (Paus, Keshavan and Giedd, 2008). This is accompanied by a less diverse microbiota compared with adults, and this period is also associated with hormonal changes, although the effects of puberty on microbiota development are unclear (Agans *et al.*, 2011). It is also the peak period for the onset of psychiatric disorders such as schizophrenia. Men and women have different susceptibility to autism, schizophrenia (Jacquemont *et al.*, 2014), and mood disorders (McHenry *et al.*, 2014), possibly due hormonal changes that have differential effects on the microbiota of males and females. It therefore appears that an unstable and immature gut microbiota in childhood and adolescence susceptible to environmental factors such as antibiotics, stress, diet, and infection could have a negative impact on neurodevelopment and mental health later in life (Borre *et al.*, 2014; Neufeld *et al.*, 2016).

1.8.5 Stress, the Microbiome and the Aging Brain

Aging and stress are comparable on both cellular and behavioural levels, because the aged brain resembles the stressed brain (Prenderville *et al.*, 2015) and chronic stress can exacerbate cognitive defects during aging (Sterlemann *et al.*, 2010). Repeated exposure to stress also leads to behavioural alterations such as increased anxiety- and depressive-like behaviour and reduced social behaviour (McEwen, 2007). Data from rodent studies indicate that aging is accompanied by increased anxiety-like behaviour, and indeed the prevalence of anxiety disorders in elderly adults is considerably greater than in younger persons (Bryant, Jackson and Ames, 2008). Similarly, increased depressive-like behaviour is associated with aging in rodent studies, and 1 to 4% of elderly adults suffer from major depressive disorder, although this is highly comorbid with medical and neurological disorders (Blazer, 2003). Therefore, chronic stress is undoubtedly destructive for the aging brain and can accelerate declining brain function associated with the aging process. As discussed previously the gut microbiota composition also changes with aging and the ELDERMET (Elder Metagenome; http://eldermet.ucc.ie/) consortium found that this age-related change in gut microbiota composition was related to a number of

health measures such as depression, cognition, and inflammatory markers (Claesson *et al.*, 2012).

1.9 Altered Gut Microbiome and Neuropsychiatric and Neurological Disorders

Preclinical studies provide promising evidence indicating an important role for the gut microbiota in the pathogenesis of depression (Kelly *et al.*, 2016b). In addition to the information taken from GF studies outlined above, animal models of depression including the olfactory bulbectomised mouse and the maternal separation model are associated with significant alterations in microbial composition (Park *et al.*, 2013; Bailey and Coe, 1999; O'Mahony *et al.*, 2009). Various microbiota-directed inventions, including probiotics such as *L. rhamnosus* and *B. infantis*, reduce depressive-like behaviours (Bravo *et al.*, 2011; Desbonnet *et al.*, 2008). These and other studies provided the impetus for the assessment of the gut microbiota in clinical psychiatric populations.

Jiang and colleagues (2015) examined changes in the faecal microbiota of major depressive disorder patients and found significant differences compared with controls. *Bacteroidetes, Proteobacteria*, and *Actinobacteria* were increased whereas *Firmicutes* was markedly reduced in major depressive disorder patients. A negative correlation was observed between *Faecalibacterium* and the severity of depressive symptoms (Jiang *et al.*, 2015). Although a consistent microbial signature of depression has not yet been documented, a number of recent publications have also reported alterations in aspects of composition or diversity (Kelly *et al.*, 2016b; Zheng *et al.*, 2016).

The possibility of a causal role for the gut microbiome in the expression of the cardinal symptoms of depression has also been addressed in translational studies. A faecal microbiota transplant from major depressive disorder patients to GF mice resulted in depression-like behaviours compared with colonisation with microbiota from controls (Zheng *et al.*, 2016). Kelly *et al.* (2016b) also recently demonstrated that FMT from depressed patients and controls to a microbiota-deficient rat model

induced behavioural and physiological changes characteristic of depression, including anhedonia, anxiety-like behaviours, and alterations in tryptophan metabolism.

There is preliminary evidence of a role for microbiota in neurological disorders because recent studies have demonstrated that GF mice are protected from traits associated with multiple sclerosis (MS) (Ascherio, 2013) and Alzheimer's disease (Harach *et al.*, 2015). The experimental autoimmune encephalomyelitis animal model is used to study MS. Interestingly, GF mice are highly resistant to experimental autoimmune encephalomyelitis but responsiveness is restored upon colonisation (Lee *et al.*, 2011) and some reports indicate that patients with MS have a distinct gut microbiota compared with healthy controls (Chen *et al.*, 2016). Therefore, the microbiota may be a potential therapeutic target for MS (Luczynski *et al.*, 2016a). A recent preclinical study generated a GF mouse model of Alzheimer's disease and found a significant decrease in cerebral Aβ amyloid pathology when compared with controls. Furthermore, an increase in Aβ-degrading enzymes and a reduction in neuroinflammation were detected. Colonisation of these GF models with microbiota from conventionally raised Alzheimer's disease mice drastically increased cerebral Aβ amyloid pathology. These results indicate that a distinct microbial constitution may play a role in the development of Alzheimer's disease pathology (Harach *et al.*, 2015).

1.10 Therapeutic Targeting of the Gut Microbiome

1.10.1 Opportunities

Dietary supplementation with probiotics and prebiotics is the most widely used dietary adjuncts to modulate the gut microbiota (Sandhu *et al.*, 2017). Probiotics are 'live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001; Hill *et al.*, 2014) and are used either by healthy individuals to maintain a healthy state and reduce the risk of illness, or as treatment for particular diseases. They have proven to be effective in treating

some aspects of GI disorders such as irritable bowel syndrome and inflammatory bowel disease (Distrutti *et al.*, 2016; Matijasic *et al.*, 2016; Clarke *et al.*, 2012). Psychobiotics are live microorganisms or factors that positively influence the microbiome to confer potential benefits for neuropsychiatric illnesses (Dinan, Stanton and Cryan, 2013; Sarkar *et al.*, 2016), and accumulating preclinical evidence points towards the possibilities of their use in disorders such as depression and anxiety (Bravo *et al.*, 2011; Desbonnet *et al.*, 2014; Savignac *et al.*, 2014). The potential for psychobiotics to treat such disorders cannot be ignored given the fact that commensal bacteria are capable of not only altering the HPA axis, but also key neurotransmitters that are relevant to the etiology of depression (Dinan and Cryan, 2013). Probiotics that are capable of producing neuroactive compounds *in vitro* and that show behavioural effects in animal studies are worth testing for psychobiotic potential, particularly in stress-related disorders such as anxiety and depression (Dinan, Stanton and Cryan, 2013). To be suitable for use as psychobiotics, probiotic strains could act as delivery vehicles for neuroactive compounds, decrease proinflammatory cytokines, and reduce HPA activity as well as showing behavioural effects preclinically (Dinan, Stanton and Cryan, 2013).

Prebiotics are "non-digestible food ingredients that promote the growth of beneficial microorganisms in the intestine" (Glenn and Roberfroid, 1995). They are not hydrolysed and absorbed in the upper GI tract and, therefore, reach the large intestine unchanged where they are nutrients for beneficial colonic bacteria. Prebiotic treatment has been found to alter the gut microbiota, improve metabolic function, and decrease gut permeability and metabolic endotoxemia, while improving insulin sensitivity and low-grade inflammation (Cani and Delzenne, 2011) (Bindels *et al.*, 2015). Examples of prebiotic substances include fructo-oligosaccharides, gluco-oligosaccharides, and lactulose (Barczynska *et al.*, 2015). Prebiotics also have the potential to treat psychological disorders because evidence shows that prebiotics impact brain neurochemistry and behaviour. For example, galactooligosaccharide (GOS) has been shown to increase hippocampal neurotrophin levels (Savignac *et al.*, 2013). Furthermore, a combination of GOS and polydextrose improved anxiety-like behaviour in rats as well as altering gut

microbiota composition (Mika *et al.*, 2014).

Faecal microbiota transplantation is also an effective method of modifying intestinal microbiota to improve host health. It can potentially re-establish healthy microbiota in the recipient and preserve approximately 1,150 bacterial species transferred by a healthy donor. It is increasingly utilised for treatment of recurrent infections in the human gut, such as *Clostridium difficile* infection (Bojanova and Bordenstein, 2016). Studies have shown that following infusion of microbiota from lean human donors to obese recipients, the insulin sensitivity of the recipients improved (Vrieze *et al.*, 2012). However, viable bacteria may make up a small fraction of total faecal matter as other components include colonocytes, archae, viruses, fungi, protists, and metabolites. Therefore, the contribution of non-bacterial matter to changes in the host cannot be ignored, and the effects and interactions of each component need to be separated out (Bojanova and Bordenstein, 2016).

1.10.2 Clinical Studies

In addition to preclinical studies, there is also now support from studies in healthy humans to indicate that clinical trials with probiotics or psychobiotics in depression are worth conducting. The translational potential of *B. longum* 1714 was recently demonstrated in healthy volunteers where it modulated stress, electrophysiology, and neurocognition (Allen *et al.*, 2016), although *L. rhamnosus* JB1 did not demonstrate benefits in a similar population (Kelly *et al.*, 2016a). In a double-blind, placebo-controlled, randomised clinical trial, subjects were fed a probiotic-containing milk drink or placebo for three weeks before mood and cognition assessment. Significant improvements were found in subjects who initially scored in the lowest third for depressed mood (Benton, Williams and Brown, 2007). A randomised, double-blind, placebo-controlled trial administered a probiotic capsule consisting of *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium bifidum* to patients with major depressive disorder. Following eight weeks of intervention, patients who received the probiotic had significantly decreased Beck

Depression Inventory compared with the placebo group (Akkasheh *et al.*, 2016). Table 1 summarises health benefits of probiotics and prebiotics that have been found in preclinical and clinical trials.

Table 1. Health benefits of probiotics and prebiotics in preclinical and clinical trials.

Probiotic/Prebiotic	**Findings**[1]	**Reference**
B. infantis	Reversed abnormal stress response in GF mice	(Sudo *et al*., 2004)
	Anti-depressant effects in rats Normalised immune response Restored noradrenaline levels in the brainstem	(Desbonnet *et al*., 2008)
L. rhamnosus (JB-1)	Anti-depressant effects and enhanced memory to an aversive cue in mice.	(Bravo *et al*., 2011)
	Not superior to placebo in modifying stress-related measures, HPA response, inflammation or cognitive performance in healthy male participants.	(Kelly *et al.*, 2016a)

B. longum 1714	BALB/c mice displayed anti-depressant effects in the tail suspension test. Reduced stress and improved visuospatial memory in healthy volunteers.	(Savignac *et al*., 2014) (Allen *et al.*, 2016)
B. breve 1205	BALB/c mice displayed reduced anxiety in EPM	(Savignac *et al*., 2014)
Mycobacterium vaccae NCTC 11659	Reduced anxiety in EPM after chronic psychosocial stress. Prevented stress-induced spontaneous colitis and aggravation of colitis in a model of IBD.	(Reber *et al.,* 2016)
B. fragilis NCTC 9343	Improved autism symptoms in MIA mouse model.	(Hsiao *et al*., 2013)
L. helveticus R0052 and *B. longum* R0175 probiotic mixture	Reduced psychosocial distress and cortisol output in human volunteers.	(Messaoudi *et al.*, 2011)
L. casei shirota	Fewer anxiety symptoms in chronic fatigue syndrome	(Rao *et al.*, 2009)

Fermented milk product with probiotic (*B. animalis* subsp. *lactis* CNCM I-2494; *Streptococcus thermophiles* CNCM I-1630; *L. bulgaris* CNCM I-1632, I-1519; *Lactococcus lactis* subsp. *lactis* CNCM I-1631).	patients. Altered brain activity in regions relevant to cognitive performance in healthy humans.	(Tillisch *et al.*, 2013)
Galactooligosaccharide	Increased hippocampal neurotrophin levels in rats. Reversed abnormal stress response in mice.	(Savignac *et al*., 2013) (Sudo *et al*., 2004)
Galactooligosaccharide and polydextrose	Promoted stress resistance in juvenile rats.	(Mika *et al*., 2014)
B-galactooligosaccharide	Decreased waking cortisol reactivity and increased attention to positive stimuli in healthy volunteers.	(Schmidt *et al.*, 2015)

[1] GF, germ free; HPA, hypothalamic pituitary adrenal; EPM, elevated plus maze; IBD, irritable bowel disease; MIA, maternal immune activation.

1.10.3 Translational Challenges

Although preclinical studies have generated promising results, it is important to consider the obstacles that might prevent successful translation to the clinic. These

challenges are neatly illustrated by the divergent results with two different candidate psychobiotics recently (Kelly *et al.*, 2016a; Allen *et al.*, 2016). Mice have become the model of choice in the field of microbiota-gut-brain axis research and have been vital in understanding signalling pathways and the consequences of perturbed gut microbiota. Concerns with their use include how informative they are in capturing aspects of human brain disorders. It is important that differences between murine and human systems are considered in translational studies (Mayer, Tillisch and Gupta, 2015).

The mammalian digestive tract is largely conserved among species, with divergences likely driven by diet. However, humans have evolved towards a smaller caecum and colon and a longer small intestine compared with the murine system, and morphological differences also exist between mouse and human colons. Therefore, care must be taken when comparing microbiota composition because host-microbe co-evolution may have been influenced by these anatomical divergences (Nguyen *et al.*, 2015). The gut microbiota of both humans and mice are dominated by *Bacteroidetes* and *Firmicutes*; however, 85% of bacterial genera found in the mouse gut microbiota are absent in humans (Ley *et al.*, 2005). Furthermore, differences in research techniques hinder the comparison of the murine and human microbiome. Human gut microbiota studies typically use faecal samples whereas caecal contents are more frequently used to study the mouse microbiome. The composition of the human gut microbiota has been examined using both metagenomic and 16S rDNA sequencing approaches (Qin *et al.*, 2010), whereas mouse studies involve predominantly 16S rDNA sequencing (Riboulet-Bisson *et al.*, 2012).

Multiple factors such as model of delivery, diet, and genetics contribute to shaping the human microbiome, but these factors are absent in mouse models, implying that they cannot reflect all relevant influences on the human gut microbiota. Humanised gnotobiotic mice, which are GF mice that have been inoculated with a human gut microbiota sample, are widely employed because they recapitulate a significant portion of the human gut microbiota composition (Nguyen *et al.*, 2015). Rats are proposed to be more representative of the human microbiota because the

gut bacterial communities of humanised rats more closely resemble the gut microbiota of human donors (Wos-Oxley *et al.*, 2012).

Despite their effectiveness, a concern associated with using probiotics is the vast number of active proteins associated with them, which makes defining their mechanisms of action difficult. Moreover, host effects are strain specific and not all strains are beneficial for all disorders or all individuals. To be used as a probiotic, microorganisms must display high resistance to gastric acids, lack any transferable antibiotic resistance gene, be non-pathogenic, and be non-toxic (Montalban-Arques *et al.*, 2015). It is likely that the mechanism of action of probiotics is multifactorial and strain specific (Power *et al.*, 2014). Concerns regarding FMT include the absence of safety measures, transfer of pathogenic bacteria and lack of donor screening. Using synthetically generated FMT material, if this approach turns out to achieve comparable efficacy to current approaches, may overcome these limitations and provide an enhanced safety profile and reproducibility (Aguirre and Venema, 2015).

1.11 The Gut Microbiome and Animal Science

As well as having significant potential for treating human disorders, the role of gut microbiota in stress has implications for animal science. Stress is an unavoidable aspect of animal production systems and can lead to reduced feed intake and decreased activity along with hormonal, physiological, and immunologic deficiencies. It is clear that the gut microbiota is involved in regulating the stress response; therefore, modulation of the gut microbiota, either through diet or probiotic administration, is a potential strategy to manage stress in farm and domestic animals. Antibiotic use has been restricted in the EU due to food safety concerns and the promotion of antibiotic resistance, therefore, probiotics are now more widely used as alternatives to antibiotics to maintain homeostasis and promote growth.

The most significant effects of probiotics have been observed when administered

during stressful periods such as weaning and the beginning of the lactation period (Chaucheyras-Durand and Durand, 2009). However, it is important to consider the different GI tract in ruminants, monogastric animals, and poultry when considering the relevance of the data discussed in this review as well as when selecting probiotic strains. For example, although lactobacilli are beneficial to postgastric microbiota, their proliferation in the rumen is a predominant feature of ruminal acidosis (Hook *et al.*, 2011). For optimal effect, probiotic administration should be based on mechanistically established, health-promoting host-microbe interactions (Yeoman and White, 2014).

Probiotics that target the rumen compartment are used in adult ruminants, and the most common marketed products are live yeasts, which have been shown to improve performance and increase dry matter intake and milk production (Stella *et al.*, 2007). Daily yeast supplementation has also been shown to improve growth performance (Lesmeister, Heinrichs and Gabler, 2004). Probiotic distribution on farms is a practical strategy to limit the risk of foodborne infections in humans because certain strains of *Lactobacillus acidophilus* were shown to decrease *E. coli* O157 in cattle faeces (Tabe *et al.*, 2008).

The most common probiotics for monogastric animals are yeasts and bacteria, such as *Lactobacillus* spp. and *Enterococcus* spp. (Chaucheyras-Durand and Durand, 2009). Administration of probiotics to pigs results in amelioration of GI disorders and reduction of pathogenic load (Gaggìa, Mattarelli and Biavati, 2010), whereas administration to gestating sows results in beneficial effects on feed intake and weight (Böhmer, Kramer and Roth-Maier, 2006). Stress is a particular problem in modern broiler production and can lead to a change in gut homeostasis and a weakened immune system, which increases the risk for colonisation by pathogenic bacteria (O'Callaghan *et al.*, 2016). Competitive exclusion is a common approach for probiotic administration in poultry and has been shown to reduce colonisation by pathogenic bacteria such as *Salmonella*, which chickens are particularly vulnerable to during the first weeks of life (Schneitz, 2005). Administration of 12 *Lactobacillus* strains had beneficial effects on bodyweight gain and reduced abdominal fat deposition (Kalavathy *et al.*, 2003). Probiotics and other microbiota interventions

need now to be considered beyond the traditional applications of weight gain and stress reduction.

1.12 Summary and Conclusion

It is impossible to ignore the impact that the microbiome has on health and disease. Preclinical data strongly support the view that microbe manipulation using probiotics and prebiotics to enhance the host-microbe symbiotic relationship has great potential in the future prevention and treatment of metabolic, immune, and even stress-related psychiatric disorders (Patterson *et al.*, 2014). The microbiome also has the potential to act as a biomarker, because alterations to microbial composition often precede disorders such as Parkinson's disease (Keshavarzian *et al.*, 2015). The microbiome is highly plastic and can alter rapidly in response to diet. Therefore, with increased understanding of the specific bacteria involved in particular disorders, it may be possible to use probiotics and other approaches such as dietary modulation to treat or prevent metabolic syndrome and stress-related disorders (Clarke *et al.*, 2014b).

Before microbe manipulation could be used to treat metabolic and stress-related disorders, a number of challenges must be addressed. Most findings have come from GF mice, which are not a realistic representation of the narrowing of gut microbiota diversity that has been most frequently reported in clinical populations. Despite advances in sequencing technologies, the ideal 'healthy' microbiota profile remains to be definitively established (Tsukumo *et al.*, 2015). Many microbes are not yet characterised, due to the difficulty of comparing data between studies because sample processing and analysis vary, and the due to the inability to culture all members of the microbiome. Our knowledge regarding microbiota temporospatial composition is limited due to the difficulty of obtaining samples from different regions of the intestine. Consequently, most studies have been performed using faecal samples, which are not representative of the microbial distribution or gradients within the GI tract as a whole (Cani and Delzenne, 2011).

Future research, based on microbiota-directed interventions, requires adequately

controlled, well-designed, large, randomised, double-blind placebo-controlled clinical trials. Research also needs to progress beyond the microbiota composition in various disease states and investigate potential mechanisms by which altered microbiota composition contributes to pathophysiology of disease, and by which potential probiotic bacteria exert beneficial effects on host health (Kelly *et al.*, 2015). Culturomics, metatranscriptomics, and metabolomics will likely aid in identifying such mechanisms (O'Callaghan *et al.*, 2016). The development of novel *in vitro* models of human intestinal epithelium such as bioreactors and organoids will enhance understanding of host-microbe interactions (Power *et al.*, 2014). To fully utilise the microbiota to improve health, it is vital not only to know which microbiota are present but also their function and how these functions interact with the host (Rodriguez *et al.*, 2015). These and other surmountable challenges go hand in hand with the opportunities presented. As we contemplate the important implications for many stress-related neuropsychiatric disorders and beyond, it is clear that this is just the beginning of an exciting new research paradigm that can inform future healthcare policy and yield novel medicinal strategies based on therapeutic targeting of the gut microbiome.

1.13 References

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2. Production of Psychoactive Metabolites by Gut Bacteria

Niamh C. Wiley[1,2], John F. Cryan[1,3], Timothy G. Dinan[1,4], R. Paul Ross[1,5] and Catherine Stanton[1,2,*]

[1]APC Microbiome Ireland, University College Cork, Cork, Ireland

[2]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[3]Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[4]Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[5]School of Microbiology, University College Cork, Cork, Ireland

**Corresponding author:* Professor Catherine Stanton: APC Microbiome Ireland, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

Tel: +353-25-42606; E-mail: Catherine.stanton@teagasc.ie.

2.1 Abstract

The gut microbiome plays a vital role in numerous aspects of physiology, including functions related to metabolism, the immune system, behaviour, brain structure and function. Furthermore, it is now becoming increasingly clear that alterations in microbial composition or diversity are implicated in several disease states including anxiety, depression, Autism Spectrum Disorder (ASD), Alzheimer's disease (AD), Parkinson's disease (PD), obesity and diabetes. Therefore, therapeutic targeting of the gut microbiota has the potential to be useful in the treatment of both stress-related disorders and metabolic diseases. An important method by which the gut microbiome can influence the gut-brain axis is through microbial production of psychoactive metabolites. Several bacteria have been shown to produce metabolites which can impact host health such as short chain fatty acids (SCFA), conjugated linoleic acid (CLA), antimicrobials, exopolysaccharides, and vitamins. Furthermore, several molecules with neuroactive functions, including serotonin, gamma-aminobutyric acid (GABA), catecholamines and acetylcholine have been isolated from bacteria within the human gut. This review aims to explore the psychoactive metabolites reported to be produced by gut bacteria, particularly those of relevance to stress-related disorders. Screening methods for psychoactive metabolite production, as well as the challenges and limitations of this research will also be addressed. Finally, the implications of metabolite production for neuropsychiatric disorders such as depression, anxiety, and stress; behavioural disorders such as ASD; and neurodegenerative disorders such as AD and PD; will be discussed.

2.2 Introduction

The gut microbiota is essential for host health, performing several vital structural, protective and metabolic functions (Clarke *et al.*, 2014), including food processing and energy extraction, digestion of complex indigestible polysaccharides, synthesis of vitamins and pathogen displacement (Neish, 2014; Trompette *et al.*, 2014; Grenham *et al.*, 2011), as well as regulating immune system development and

function (Rooks and Garrett, 2016). Alterations in microbial composition and diversity are evident in several disease states, with multiple conditions being studied for correlative and causative associations with the microbiome (Weinstock, 2012). For example, the gut microbiota has been implicated in intestinal motility disorders (Ge *et al.*, 2017), visceral pain (Luczynski *et al.*, 2017), ischemic stroke (Benakis *et al.*, 2016), multiple sclerosis (Berer *et al.*, 2017) and psychological disorders such as anxiety (De Palma *et al.*, 2017) and depression (Kelly *et al.*, 2016), as well as in neurodevelopmental and neurodegenerative diseases, including AD (Minter *et al.*, 2016), PD (Sampson *et al.*, 2016), and symptomologies of ASD (Hsiao *et al.*, 2013; Fung, Olson and Hsiao, 2017). It is also believed to play a role in emotional and cognitive development (Cryan and Dinan, 2012). A review by Clarke *et al.* (2014) suggested that the gut microbiota serves as a virtual endocrine organ due to its metabolic capacity to produce and regulate multiple compounds that reach the circulation and influence the function of distal organs and systems. Host-microbe interactions can co-metabolise dietary components to produce a wide variety of molecules which benefit host health. These health-beneficial bioactive metabolites include SCFA, CLA, antimicrobials, exopolysaccharides, vitamins B and K, and neurotransmitters such as GABA and serotonin (Patterson *et al.*, 2014). Such metabolite production and modulation confer great potential for therapeutic targeting of the gut microbiota in both stress-related disorders and metabolic diseases.

2.2.1 Potential Mechanisms for Influence of Metabolites on the Gut-Brain Axis

Modulation of the central nervous system (CNS) by the gut microbiome occurs principally through neuroimmune and neuroendocrine mechanisms and often involves the vagus nerve, according to current evidence (Bravo *et al.*, 2011; Tolhurst *et al.*, 2012; Wang *et al.*, 2014; Singh *et al.*, 2016; Martin *et al.*, 2018). This bidirectional communication between the gut microbiome and the CNS is mediated by microbially-derived metabolites which interact with enteroendocrine cells, enterochromaffin cells, the mucosal immune system, the hypothalamic-pituitary-

adrenal (HPA) axis and the vagus nerve (Figure 1). Bacteria communicate with each other using quorum sensing (Bassler, 1999), which is defined as the control of gene expression in response to cell density, to regulate a variety of physiological functions. Quorum signalling involves the production and detection of hormone-like extracellular signalling molecules called autoinducers and is a highly specific communication system which is adapted for multiple uses by varying the design of extracellular signals, the signal detection apparatus, and the biochemical mechanisms of signal relay. Recent evidence suggests that it modulates both intra- and inter- species cell to cell communication, which is vital in the formation of complex community structures, and also facilitates communication between bacteria and the host, known as inter-kingdom signalling (Bassler, 1999). Noradrenaline is hypothesised to have a role in this (Sperandio *et al.*, 2003) and catecholamines are also potential signalling molecules in the gut (Sudo, 2019). Furthermore, some metabolites such as SCFA, CLA, vitamins and serotonin are capable of crossing the intestinal barrier where they enter systemic circulation and may cross the blood brain barrier (Yano *et al.*, 2015; Samuel *et al.*, 2008; Haghikia *et al.*, 2015). However, it remains to be elucidated whether these metabolites reach brain sites directly or signal via vagal and spinal afferents to induce central responses (Bravo *et al.*, 2011; Goehler *et al.*, 2005). Metabolites produced by the gut microbiota may stimulate epithelial cells to release molecules which modulate neural signalling within the enteric nervous system (ENS), thereby influencing brain function and behaviour of the host (Figure 1) (Wall *et al.*, 2014). Additionally, gut neuropeptides, which are strong neuroimmune modulators with antimicrobial properties, are also emerging as important mediators of communication between commensal bacteria and the host (Sanz and El Aidy, 2016).

Recent evidence also suggests a role for the microbiota in neuronal programming for the regulation of intestinal physiology. Obata *et al.* (2020) demonstrated that microbiota-induced expression of the transcription factor aryl hydrocarbon receptor (AHR) in distal gastrointestinal tract neurons facilitates response to the luminal environment. Deletion of AHR results in reduced peristaltic activity of the colon in a manner similar to microbiota-depleted mice. Gao *et al.* (2019) increased

hindgut carbohydrate availability which promoted hypothalamic neurotransmitter synthesis, suggesting aromatic amino acids are potential mediators between hindgut microbiota and brain neurochemistry. Additionally, Torres-Fuentes *et al.* (2019) described a novel route of communication between the gut microbiota and the host via modulation of the ghrelin receptor GHSR-1a, which plays a vital role in maintaining energy balance, metabolism, modulating food intake, motivation, reward, and mood. They demonstrated that SCFAs, lactate and bacterial supernatants, including *Bifidobacterium* and *Lactobacillus* genera, can attenuate ghrelin-mediated signalling through the GHSR-1a receptor (Torres-Fuentes *et al.*, 2019). Evidence of the control that the gut microbiota exerts over the HPA axis can be found in studies involving germ free (GF) animals who display exaggerated responses to psychological stress, which can be normalised after monocolonisation by certain bacterial species including *Bifidobacterium infantis* (Sudo et al., 2004), which has since been identified as a GABA producer (Barrett *et al.*, 2012c). Therefore, psychoactive metabolite-producing probiotics may have the potential to treat certain neurological and neuropsychiatric conditions.

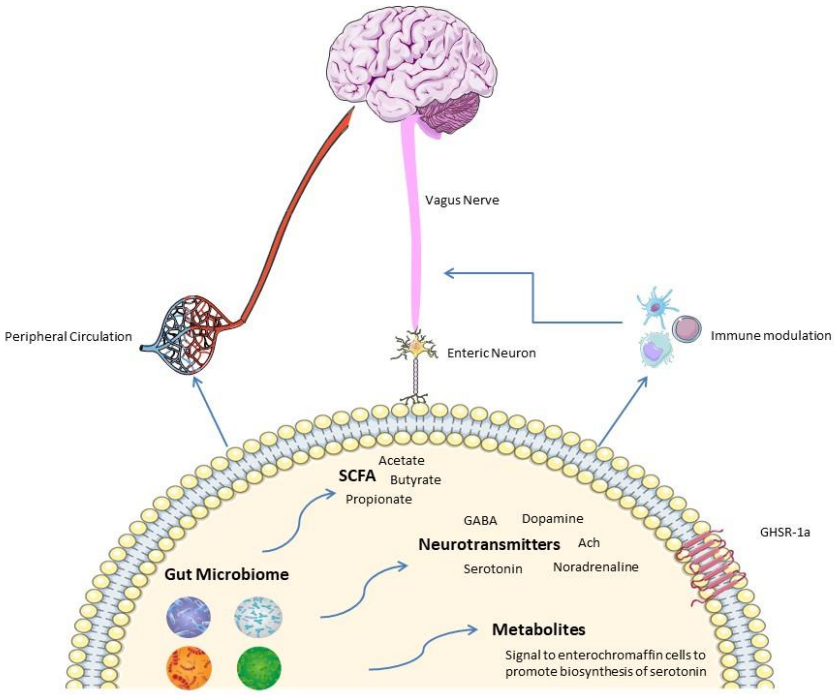


Figure 1: Modulation of the gut-brain axis by microbial production of psychoactive metabolites. Direct and indirect routes of communication between the gut microbiota and the brain include neuronal, circulatory, endocrine, immune-mediated, and metabolite-mediated pathways. Metabolites produced by the gut microbiome include SCFA and neurotransmitters which can affect the gut-brain axis through neuroimmune and neuroendocrine mechanisms, often involving the vagus nerve. Microbially-derived metabolites interact with enteroendocrine cells, enterochromaffin cells and the mucosal immune system, while some metabolites are capable of crossing the intestinal barrier where they enter systemic circulation and may cross the blood brain barrier.

2.3 Microbial Metabolite Production

Several species of bacteria isolated from within the human gut have been found to produce psychoactive metabolites; a summary of these metabolites can be found in Table 1. *Lactobacillus* spp. and *Bifidobacterium* spp. have been found to produce GABA; *Lactococcus* spp., *Streptococcus* spp., *Escherichia* spp., and *Enterococcus* spp. have been reported to produce serotonin; *Escherichia* spp. and *Bacillus* spp. have been identified as noradrenaline producers; *Bacillus* spp. have been reported to produce dopamine; and *Lactobacillus* spp. are described as acetylcholine and histamine synthesisers (Barrett *et al.*, 2012; Thomas *et al.*, 2012; Kawashima *et al.*, 2007; Marquardt and Spitznagel, 1959; Tsavkelova *et al.*, 2000). SCFAs, long chain fatty acids (LCFAs) and CLA are examples of other microbially-produced metabolites with neuroactive functions. CLA isomers have been found to provide health beneficial effects for cancer, atherosclerosis, obesity, and diabetes (Kelley, Hubbard and Erickson, 2007; Benjamin and Spener, 2009; Silveira *et al.*, 2007). *Bifidobacterium breve* is a highly efficient CLA producer and has been shown to modulate tissue fatty acid composition in the liver of pigs and mice, suggesting microbiota manipulation may represent a therapeutic strategy for inflammatory disorders (Coakley *et al.*, 2003; Wall *et al.*, 2009). Several vitamins are also synthesised by the gut microbiota, including vitamin K2 and many of the water-

soluble B vitamins (LeBlanc *et al.*, 2013). Many *Bifidobacterium* strains in particular exhibit vitamin production capabilities (Deguchi, Morishita and Mutai, 1985). Vitamin K is important for host health, regulating blood clotting, bone and vascular health and deficiencies are associated with low bone mineral density (Knapen *et al.*, 1998), increased risk of fracture (Luukinen *et al.*, 2000) and cardiovascular disease (Geleijnse *et al.*, 2004). Vitamin B12 is solely synthesised by some bacteria and archea, and *Propionibacteria freudenreichii* is used for its commercial production (Roessner *et al.*, 2002).

Table 1: Microbial Metabolite Production by Gut Bacteria

Neurochemical	Bacterial Strain	Reference
Serotonin	Streptococcus Thermophiles	(Özoğul *et al.*, 2012)
	E. coli K-12	(Shishov *et al.*, 2009)
	Lactococcus lactis	(Özoğul *et al.*, 2012)
	Lactococcus cremoris	(Özoğul *et al.*, 2012)
	Lactobacillus plantarum	(Özoğul *et al.*, 2012)
	Morganella morganii	(Özoğul, 2004)
	Klebsiella pneumonia	(Özoğul, 2004)
	Hafnia alvei	(Özoğul, 2004)
GABA	Lactobacillus brevis DPC6108	(Barrett *et al.*, 2012)
	Lactobacillus rhamnosus	(Lin, 2013)
	Bifidobacterium adolescentis	(Lin, 2013)
	Bifidobacterium dentium	(Lin, 2013)
	Bifidobacterium infantis	(Lin, 2013)
	Streptococcus Thermophilus	(Linares *et al.*, 2016)
Noradrenaline	E. coli K-12	(Shishov *et al.*, 2009)
	Bacillus subtilis	(Tsavkelova *et al.*, 2000)
	Bacillus mycoides	(Tsavkelova *et al.*, 2000)
	Proteus vulgaris	(Tsavkelova *et al.*, 2000)
	Serratia marcescens	(Tsavkelova *et al.*, 2000)
Dopamine	E. coli K-12	(Shishov *et al.*, 2009)
	Morganella morganii	(Özoğul, 2004)

	Klebsiella pneumonia	(Özoğul, 2004)
	Hafnia alvei	(Özoğul, 2004)
	Bacillus cereus	(Tsavkelova *et al.*, 2000)
	Bacillus mycoides	(Tsavkelova *et al.*, 2000)
	Bacillus subtilis	(Tsavkelova *et al.*, 2000)
	Proteus vulgaris	(Tsavkelova *et al.*, 2000)
	Serratia marcescens	(Tsavkelova *et al.*, 2000)
	Staphylococcus aureus	(Tsavkelova *et al.*, 2000)
Acetylcholine	Lactobacillus plantarum	(Girvin and Stevenson, 1954)
	Bacillus subtilis	(Kawashima *et al.*, 2007)
	Staphylococcus aureus	(Horiuchi *et al.*, 2003)
Histamine	Lactococcus lactis	(Coton *et al.*, 1998)
	Lactobacillus reuteri	(Thomas *et al.*, 2012)
	Streptococcus thermophilus	(Özoğul *et al.*, 2012)
	Lactobacillus plantarum	(Özoğul *et al.*, 2012)
	Streptococcus thermophiles	(Özoğul *et al.*, 2012)
	Bacillus subtilis	(Özoğul *et al.*, 2012)
	Morganella morganii	(Özoğul, 2004)
	Klebsiella pneumonia	(Özoğul, 2004)
	Hafnia alvei	(Özoğul, 2004)
CLA	Lactobacillus rhamnosus	(Lee *et al.*, 2006)
	Lactobacillus plantarum	(Lee *et al.*, 2007)
	Bifidobacterium breve	(Wall *et al.*, 2009)
	Bifidobacterium longum	(Gao *et al.*, 2020)
	Bifidobacterium bifidum	(Gorissen *et al.*, 2010)
SCFA	Bifidobacterium breve	(Wall *et al.*, 2012)
	Bifidobacterium longum	(LeBlanc *et al.*, 2017)
	Bifidobacterium bifidum	(LeBlanc *et al.*, 2017)
	Lactobacillus salivarius	(Meimandipour *et al.*, 2010)
	Lactobacillus gasseri	(LeBlanc *et al.*, 2017)
Vitamin B12	Propionibacteria freudenreichii	(Roessner *et al.*, 2002)
	Lactobacillus reuteri	(Saulnier *et al.*, 2011)

Water-soluble B group vitamins (Thiamine, Nicotinic acid, Folic acid and Pyridoxine)	Bifidobacterium bifidum Bifidobacterium infantis Bifidobacterium breve Bifidobacterium longum	(Deguchi, Morishita and Mutai, 1985) (Deguchi, Morishita and Mutai, 1985)
Vitamin K	Bacteroides fragilis Eubacterium lentum Propionibacterium E. coli	(Hill, 1997) (Hill, 1997) (Suttie, 1995) (Suttie, 1995)

2.3.1 GABA

GABA is the major inhibitory neurotransmitter in the CNS and therefore plays an important role in regulating many psychological and physiological functions. These include neurotransmission, diuretic effects and anxiolytic effects (Hayakawa *et al.*, 2004; Linares *et al.*, 2016). It is involved in the regulation of cardiovascular functions such as blood pressure and heart rate, and also plays a role in pain sensation and anxiety (Mody *et al.*, 1994). An altered GABAergic profile has been linked to CNS disorders such as anxiety and depression (Cryan and Kaupmann, 2005), as well as disruptions of ENS functions including intestinal motility, nociception, gastric emptying and acid secretion (Hyland and Cryan, 2010). Some pharmacological treatments for anxiety, such as benzodiazepines, act on $GABA_A$ receptors, highlighting the role of GABA in anxiety disorders (Foster and Kemp, 2006). GABA levels are reduced in the temporal cortex, occipital cortex and cerebellum of patients with AD (Seidl *et al.*, 2001). GABA has consequently been classified as a health-promoting bioactive component in foods and pharmaceuticals (Li and Cao, 2010). Several commercial formulations of GABA are available which claim to have a calming and tranquilising effect, for example, PharmaGABA™ has been approved by the Food and Drug Administration (FDA) as a food ingredient (Linares *et al.*, 2016).

It has been reported that GABA levels are significantly decreased in both the stool and blood of GF mice (Matsumoto *et al.*, 2017). Similarly, antibiotics can modify faecal GABA levels in specific pathogen free (SPF) mice (Fujisaka *et al.*, 2018), which

indicates that the microbiota may contribute to circulating levels of GABA. Certain strains of *Lactobacillus* and *Bifidobacterium* are capable of GABA production via the same biosynthetic pathway as in neuronal tissue, which involves conversion of glutamate by the action of the enzyme glutamate decarboxylase and vitamin co-factor pyridoxal phosphate (Komatsuzaki *et al.*, 2008). The ability to biosynthesise GABA is a strain-dependent attribute not present in all strains belonging to one species and is genetically linked to the genes *gadB* and *gadC* (Tajabadi *et al.*, 2015). Furthermore, the efficiency and yield of GABA production varies significantly between GAD^+ strains (Dhakal, Bajpai and Baek, 2012). This ability to produce GABA likely serves to protect the organism from the acidic environment of the stomach, as its synthesis involves proton exchange for the uptake of glutamate (Higuchi, Hayashi and Abe, 1997). GABA-producing bacteria have great potential to influence brain function and behaviour by communicating with the brain via the vagus nerve. Barrett *et al.* (2012) screened 91 strains of human-derived bifidobacteria and lactobacilli and found five strains capable of GABA production, with *Lactobacillus brevis* DPC6108 and *Bifidobacterium dentium* being the most efficient producers. *Lactobacillus brevis* DPC6108 is a highly efficient producer and has been shown to convert up to 90% of glutamate to GABA, while also retaining the capability to produce GABA in the presence of other gut-derived bacteria (Barrett *et al.*, 2012). Transcriptome analysis of human stool samples from healthy individuals has shown that *Bacteroides*, *Parabacteroides,* and *Escherichia* species actively express GABA-producing pathways. Furthermore, it was found that *Bacteroides* spp. and *Parabacteroides* spp. could produce GABA within the pH range of the human large intestine (Fallingborg, 1999).

Many studies have reported the presence of a *gad* gene in lactic acid bacteria (LAB) and several GABA-enriched fermented food products have been developed using starter cultures with GABA-producing capabilities (Siragusa *et al.*, 2007). Consumption of a ketogenic diet was shown to increase GABA levels in the cerebrospinal fluid of children with refractory epilepsy, which was accompanied by an improvement of symptoms (Dahlin *et al.*, 2005). Consuming such GABA-enriched food products is one approach to increasing GABA levels, however harnessing the

production capability of the intestinal microbiota or consuming probiotic bacteria that can utilise dietary monosodium glutamate to synthesise GABA is an additional possibility (Barrett *et al.*, 2012) which could have the potential to improve both neuropsychiatric and metabolic disorders. Intervention with *L. brevis* strains has been shown to attenuate several abnormalities associated with metabolic dysfunction, causing a reduction in the accumulation of mesenteric adipose tissue, increased insulin secretion following glucose challenge, improved plasma cholesterol clearance and reduced despair-like behaviour and basal corticosterone production during the forced swim test (Patterson *et al.*, 2019). A study by Ko *et al.* (2013) found that GABA production by *Lactobacillus brevis* FPA3709 in black soybean milk resulted in an antidepressant effect similar to fluoxetine but without the side effects of appetite loss and decreased weight. At gene expression level, ingestion of *L. rhamnosus* JB-1, one of the most cited GABA producers, altered the mRNA expression of both $GABA_A$ and $GABA_B$ receptors, which have been implicated in anxiety and depression and are expressed in regions responsible for maintaining normal mood and fear responses (Bravo *et al.*, 2011).

2.3.2 Serotonin

Serotonin is a monoamine neurotransmitter which is biochemically derived from tryptophan. It is primarily found in the gastrointestinal tract, blood platelets, and CNS of humans. It plays a key role in the microbiota-gut-brain axis at both the ENS (Mawe and Hoffman, 2013) and CNS (Ruddick *et al.*, 2006) levels. In the gastrointestinal tract (GIT), serotonin is synthesised independently by specialised endocrine cells, called enterochromaffin cells (ECs), as well as mucosal mast cells and myenteric neurons (Gershon and Tack, 2007), which supply serotonin to the mucosa, lumen and circulating platelets. High concentrations of SCFAs and glucose are also reported to stimulate serotonin release from ECs (Hsiao and Yano, 2016). In addition to its role as a neurotransmitter, serotonin is an important regulatory factor in other organ systems such as the GIT where over 90% of the body's serotonin is synthesised. Here it activates as many as 14 different serotonin

receptor subtypes (Gershon and Tack, 2007) located on enteric neurons (Mawe and Hoffman, 2013), enterocytes (Hoffman *et al.*, 2012), and immune cells (Baganz and Blakely, 2012). In addition, circulating platelets withdraw serotonin from the GIT and distribute it to various body sites to promote haemostasis (Amireault, Sibon and Côté, 2012). Therefore, gut-derived serotonin regulates diverse functions, including enteric motor and secretory reflexes (Gershon and Tack, 2007), platelet aggregation (Mercado *et al.*, 2013), blood pressure regulation (Watts *et al.*, 2012), immune responses (Baganz and Blakely, 2012), bone development (Chabbi-Achengli *et al.*, 2012; Yadav *et al.*, 2008), and cardiac function (Côté *et al.*, 2003). Furthermore, disruption of peripheral serotonin is evident in several disease states, including irritable bowel syndrome (IBS) (Stasi *et al.*, 2014), cardiovascular disease (Ramage and Villalón, 2008), and osteoporosis (Ducy and Karsenty, 2010).

Microbiota play a role in regulating serotonin levels, as studies have shown decreased serum, plasma, colonic and faecal serotonin concentrations in GF compared to SPF mice (Sjögren *et al.*, 2012; Wikoff *et al.*, 2009). No such deficit has been observed in the small intestine which indicates a role for the microbiota in regulating colonic serotonin because the mammalian colon harbours a far greater abundance and diversity of microbes than does the small intestine (Yano *et al.*, 2015). Studies in GF mice indicate that the gut microbiota regulate the peripheral availability of circulating tryptophan, which is critical for serotonin synthesis (Clarke *et al.*, 2013). The mechanism underlying microbial regulation of circulating tryptophan concentrations is unknown but is hypothesised to involve control over tryptophan degradation along an alternative metabolic pathway, the kynurenine pathway (Schwarcz *et al.*, 2012; Stone, Stoy and Darlington, 2013). An additional potential mechanism underlying the gut microbiota's influence over serotonin production was described by Yano *et al.* (2015) involving microbial biotransformation of cholic acid (CA) into deoxycholic acid (DCA). Restoration of peripheral serotonin levels in the colon and serum of GF mice occurred when luminal concentrations of DCA were raised, which correlated with elevations in colonic TPH1 expression, the tryptophan hydroxylase isoenzyme which mediates non-neuronal serotonin biosynthesis (Yano *et al.*, 2015; Gheorghe *et al.*, 2019).

Additionally, strains such as *Clostridium sporogenes* and *Ruminococcus gnavus* are capable of tryptamine synthesis, which plays a role in signalling and inducing the release of serotonin from enterochromaffin cells, by the decarboxylation of tryptophan (Williams *et al.*, 2014). Furthermore, enteric neurons can take up tryptamine, displacing serotonin in intracellular synaptic vesicles, resulting in serotonin release which may affect gastrointestinal motility (Israelyan *et al.*, 2019). Therefore, the ability of the microbiota to regulate influential precursors such as tryptophan, tryptamine and kynurenine likely contributes significantly to its ability to influence CNS and ENS neurotransmission (Gheorghe *et al.*, 2019). Moreover, intestinal ECs are morphologically larger in GF vs. SPF rats, indicating that host-microbe interactions can impact the development and/or function of serotonin-producing cells (Uribe *et al.*, 1994).

It was hypothesised that specific subsets of gut microbes are responsible for influencing host serotonin pathways. Monocolonisation with *Bacteroides fragilis* or with Segmented Filamentous Bacteria results in serum serotonin deficits which are similar to those observed in GF mice (Yano *et al.*, 2015). The relative abundance of spore forming bacteria which have previously been reported to promote host serotonin biosynthesis increases when intestinal luminal serotonin is elevated by oral supplementation or genetic deficiency in the host serotonin transporter SERT. In particular, *Turicibacter sanguinis* expresses a neurotransmitter sodium symporter-related protein with structure and sequence homology to mammalian SERT which it uses to import serotonin via a mechanism that can be inhibited by the selective serotonin reuptake inhibitor fluoxetine (Fung *et al.*, 2019). These results provide further evidence for bidirectional signalling of indigenous gut bacteria with the host serotonergic system to enhance their fitness in the intestine. Labus *et al.* (2019) hypothesised that the abundance of serotonin modulating *Clostridiales* microbes was associated with functional connectivity of somatosensory brain regions and GI sensorimotor function. They explored this relationship in human controls versus IBS patients and found associations between GI sensorimotor function and gut microbes in control samples but not in IBS patients, specifically in the *Lachnospiraceae incertae sedis*, *Clostridium XIVa* and *Coproccus* subnetworks

mediated by cortical and subcortical brain regions (Labus *et al.*, 2019). Certain bacteria, such as the probiotic *Bifidobacterium infantis* (Desbonnet et al., 2008), are capable of tryptophan biosynthesis via enzymes which include tryptophan synthase (Raboni, Bettati and Mozzarelli, 2009; Yanofsky, 2007), while some strains also have the ability to produce serotonin from tryptophan (Lyte, 2011; Jiménez *et al.*, 2013; Shishov *et al.*, 2009). The microbiome also influences the immuno-sensitive and stress-sensitive enzymes responsible for the initial conversion of l-tryptophan to l-kynurenine-indoleamine-2,3-deoxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) (O'Mahony *et al.*, 2015; Gheorghe *et al.*, 2019).

2.3.3 Catecholamines

Catecholamines such as noradrenaline and dopamine are important neurotransmitters which mediate multiple CNS functions including cognition, memory processing, emotion, motor control and endocrine regulation (Kobayashi, 2001). Noradrenaline in particular plays a role in arousal and alertness as well as sensory signal detection (Borodovitsyna, Flamini and Chandler, 2017). Catecholamine neurotransmission dysfunction has been implicated in neurological and neuropsychiatric disorders such as Parkinson's disease (Calabresi *et al.*, 2013), Alzheimer's disease (Robertson, 2013) and major depressive disorders (Hamon and Blier, 2013). Bacteria also respond to catecholamines, for example dopamine and noradrenaline have been shown to increase the growth rate of pathogenic *E. coli* (Freestone *et al.*, 2002), which also exhibits increased motility, biofilm formation and virulence in the presence of noradrenaline (Bansal *et al.*, 2007). Similarly, the presence of noradrenaline has also been shown to improve the growth of the pathogens *Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae, Shigella sonnei*, and *Staphylococcus aureus in vitro*, which may be due to iron acquisition (O'Donnell *et al.*, 2006). Furthermore, certain microbes have also been identified as catecholamine producers. Both noradrenaline and dopamine were identified in the biomass of certain bacteria *in vitro* by Tsavkelova *et al.* (2000) Dopamine was found in the biomass of *Bacillus cereus, Bacillus mycoides, Bacillus subtilis, Proteus vulgaris, Serratia marcescens, Staphylococcus aureus* and *E. coli* in

concentrations from 0.45-2.13 mmol/L. Noradrenaline was detected at concentrations from 0.21-1.87 mmol/L in *Bacillus mycoides, Bacillus subtilis, Proteus vulgaris* and *Serratia marcescens* (Tsavkelova *et al.*, 2000). As mentioned previously, it is hypothesised that noradrenaline is produced as a quorum sensing molecule in bacteria, while dopamine production is not yet fully understood (Sperandio *et al.*, 2003). However, it has been demonstrated that certain bacteria, particularly *B. subtilis*, can release noradrenaline and dopamine from the cell which could indicate a role in intercellular communications between microbes and the host. *E. coli K-12* has also been identified as a dopamine and noradrenaline producer *in vitro* as well as being capable of producing the precursor DOPA. Micromolar concentrations of DOPA and nanomolar concentrations of noradrenaline and dopamine were measured in *E. coli K-12* cultures by (Shishov *et al.*, 2009). Furthermore, dopamine production in culture by certain lactic acid bacteria, including *Lactococcus lactis* subsp. *cremoris* (MG 1363), *Lactococcus lactis* subsp. *lactis* (IL1403), *Lactobacillus plantarum* (FI8595) and *Streptococcus thermophilus* (NCFB2392) has been demonstrated by Ozogul *et al.* (2012).

The modulation of noradrenaline and dopamine by the human microbiota *in vivo* has not been confirmed, however evidence suggests that it may play a role in host biosynthesis/catabolism. Asano *et al.* (2012) demonstrated that bacteria which are commonly found in the mouse microbiome are capable of the *in vivo* production of significant quantities of noradrenaline. GF mice were found to have substantially reduced noradrenaline levels in the caecal lumen (3.8 ± 1.3 ng/g compared to 35 ± 5 ng/g) and in the tissue (5.0 ± 0.5 ng/g vs. 115 ± 14 ng/g) compared to SPF mice. Furthermore, colonisation of the GF mice or transfer of a mixture of 46 species restored noradrenaline levels in the murine GI tract. Therefore, this study strongly indicates that the microbiota play a role in influencing noradrenaline levels in the lumen; however whether the bacteria were directly producing noradrenaline or modulating host production was not determined (Asano *et al.*, 2012). Beyond the gut, an increased turnover rate of noradrenaline and dopamine has also been observed in the brain of GF mice (Diaz Heijtz *et al.*, 2011), which could lead to reduced availability in systemic circulation independent of microbial production.

This ability of the microbiota to influence the catecholamine system could have important consequences, as antibiotic depletion of microbiota in mice increased sensitivity to the behavioural effects of cocaine, which was associated with elevated activity of the D1 dopamine receptor Drd1 and the GluR2 AMPA receptor Gria2 in the nucleus accumbens (Kiraly *et al.*, 2016). Interestingly, SCFA supplementation normalised this response to cocaine in antibiotic treated animals, suggesting an indirect path for the microbiota to influence reward behaviour.

2.3.4 Acetylcholine

The neurotransmitter acetylcholine plays a critical role in cognitive function, particularly in memory and learning. It acts in both the central and peripheral nervous systems and has also been identified in non-neuronal tissues such as gastrointestinal, respiratory and urogenital epithelial cells (Wessler, Kirkpatrick and Racke, 1999). It is synthesised by choline acetyltransferase in the CNS and by choline acetyltransferase and carnitine acetyltransferase in the peripheral nervous system (Tuček, 1982; Tuček, 1988). Studies have shown that acetylcholine is both a component of bacterial strains such as *Lactobacillus plantarum* and *Bacillus subtilis,* and a microbial metabolite (Girvin and Stevenson, 1954; Rowatt, 1948). *Lactobacillus plantarum* was identified as an acetylcholine producer and cell free enzymes of acetylcholine synthesis were also detected in this strain (Girvin and Stevenson, 1954). *Escherichia coli* JCM 5491, *Staphylococcus aureus* JCM 2151 and *Bacillus subtilis* PCI 219 were examined for acetylcholine production by Horiuchi *et al.* (2003) who detected a substantial concentration of acetylcholine in *B. subtilis* (55.7 pmol/10^{10} colony forming units) and smaller amounts in *E. coli* (2.22 pmol) and *S. aureus* (0.39 pmol). The authors hypothesised that an acyltransferase other than choline acetyltransferase and carnitine acetyltransferase was responsible for acetylcholine synthesis in these bacteria due to the low levels of acetylcholine synthesis observed.

2.3.5 Histamine

Histamine is a modulatory neurotransmitter in the mammalian brain which, in addition to vasoactive mediation, plays an important role in the maintenance of wakefulness, and dysfunction in the histaminergic system has been linked to narcolepsy (Panula and Nuutinen, 2013). Studies suggest that the histaminergic system is also important for cognitive function (Alvarez, 2009), with decreased levels of histamine observed in the hippocampus, temporal cortex and hypothalamus of patients with Alzheimer's disease, indicating that histaminergic neurons may undergo degeneration and contribute to cognitive decline in this disorder (Alraksinen *et al.*, 1991). Studies have shown that specific ablation of histaminergic neurons results in repetitive movements (Rapanelli *et al.*, 2017) resembling those observed in Tourette syndrome (Baldan *et al.*, 2014). Furthermore, a nonsense mutation at the human histidine decarboxylase *(HDC)* gene (W317X) has been identified in a family of patients with this syndrome (Ercan-Sencicek *et al.*, 2010) and mice completely deficient in *HDC* gene transcription develop a Tourette-like syndrome (Xu *et al.*, 2015; Huang *et al.*, 2018). Histamine is also synthesised in enterochromaffin-like cells (ECL) in the stomach and is released upon gastrin and acetylcholine stimulation, resulting in stomach acid secretion from parietal cells (Tanaka *et al.*, 2002; Hersey, 1995). Mice deficient in the *HDC* gene are unable to fully acidify gastric contents, which can lead to indigestion, diarrhoea, or constipation (Saltzman *et al.*, 1994).

Histamine is produced from histidine, an essential amino acid for humans which is present in several dietary foods (Wu, 2009). This reaction is catalysed by histidine decarboxylase (HDC) which removes the carboxyl group from histidine. Histamine synthesis primarily occurs in mast cells, basophils, histaminergic neurons in the basal ganglia of the brain, and ECL in the stomach (Huang *et al.*, 2018). The *HDC* gene is also reported to be present in certain fermentative bacteria, such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Enterococcus* strains which are capable of histamine production (Landete *et al.*, 2008; Coton *et al.*, 1998). However, production of histamine by certain bacterial strains is an unwanted marker of food spoilage and is regarded as a health risk, as headaches, vomiting

and hypertension have been linked to the ingestion of food containing high concentrations of histamine (Shalaby, 1996). Conversely, histamine production by the probiotic strain *Lactobacillus reuteri* (ATCC PTA 6475) resulted in the suppression of human proinflammatory tumour necrosis factor (TNF) production (Thomas *et al.*, 2012). Moreover, *HDC* gene expression and histamine production by *L. reuteri* was increased with histidine supplementation.

2.3.6 Short-Chain Fatty Acids

SCFAs are the major end products of the bacterial fermentation of carbohydrates and proteins in the gut (Kovatcheva-Datchary and Arora, 2013; Macfarlane and Macfarlane, 2012), and their absorption and metabolism allows the host to salvage energy from resistant starch and fibres that are not digested in the upper GIT. Consequently, they have a variety of important functions such as providing a source of energy for muscles, kidneys, the heart and brain, and affecting epithelial cell growth and differentiation, epithelial cell transport and metabolism, and hepatic control of lipid and carbohydrates (Cummings and Englyst, 1995). SCFAs are also involved in promoting serotonin production and expression of TPH1, the serotonin biosynthetic enzyme, in enterochromaffin cells (Reigstad *et al.*, 2015). Receptors and transporters for SCFAs are expressed in the GIT, where they may be relevant to GI function (Karaki *et al.*, 2008; Tazoe *et al.*, 2008; Tazoe *et al.*, 2009; Milligan, Stoddart and Smith, 2009; Ganapathy *et al.*, 2013; Dass *et al.*, 2007), for example, SCFAs may modulate both enteroendocrine serotonin secretion and peptide YY release, an important neuropeptide at various levels of the gut-brain axis (Holzer, Reichmann and Farzi, 2012). Furthermore, SCFAs may play a role in cell signalling by modulating intracellular calcium levels in neutrophils, as well as influencing neurotransmitter synthesis and release through regulation of tyrosine hydroxylase gene expression, a key enzyme in catecholamine synthesis (DeCastro *et al.*, 2005).

SCFAs are important hormones of the microbiota and could mediate several functions assigned to the microbiota through endocrine signalling (Naccache *et al.*, 1988; Nakao, Fujii and Niederman, 1992; Nakao *et al.*, 1998). *Bacteroides,*

Bifidobacterium, Propionibacterium, Eubacterium, Lactobacillus, Clostridium, Roseburia, and *Prevotella* are examples of bacteria that produce SCFAs (Macfarlane and Macfarlane, 2012). Studies have shown that sodium butyrate elicits an antidepressant effect in the mouse brain (Schroeder *et al.*, 2007). Administration of SCFAs to mice subjected to psychosocial stress alleviated anhedonia, the heightened stress response, and stress-induced increases in intestinal permeability (van de Wouw *et al.*, 2018). Microglia from new-born GF mice exhibit altered gene expression profiles compared to their conventionally colonised counterparts, suggesting a role for maternal microbiota in early life programming of microglial development (Matcovitch-Natan *et al.*, 2016). Postnatal supplementation with SCFAs normalised these alterations in microglial morphology, abundance and gene expression, suggesting that SCFA-producing bacterial species may restore abnormalities in microglia of GF or antibiotic-treated mice (Erny *et al.*, 2015). These effects on microglia could be indirectly influenced by the role of SCFAs in a variety of functions including influencing gastrointestinal physiology, peripheral immunity, liver metabolism and blood brain barrier integrity (Fung, Olson and Hsiao, 2017).

2.3.7 Conjugated Linoleic Acid

CLA is a group of positional and geometric isomers of linoleic acid which are naturally produced in ruminant milk and tissue fat from the action of ruminal microbiota on dietary linoleic acid (Yang *et al.*, 2017; Kepler *et al.*, 1966). It is associated with several health benefits including anticarcinogenic, immune-modulatory, anti-obesity and anti-atherosclerotic activities (Gaullier *et al.*, 2004; Ip, Masso-Welch and Ip, 2003; Bassaganya-Riera *et al.*, 2012; Kritchevsky *et al.*, 2000). Furthermore, CLA also interacts with the CNS due to its ability to cross the blood brain barrier before being incorporated and metabolised in the brain (Fa *et al.*, 2005) where it has been demonstrated to exert antiangiogenic effects (Sikorski, Hebert and Swain, 2008). Additionally, consumption of CLA as a dietary supplement resulted in the protection of cortical neurons from excitotoxicity (Hunt *et al.*, 2010).

The gut microbiota plays an important role in the endogenous production of CLA, which was first reported by Chin *et al.* (1994) who demonstrated that the CLA tissue content in conventional rats was increased in response to increasing dietary linoleic acid, an effect which was not observed in GF rats. Furthermore, commensal lactobacilli and bifidobacteria from the human GIT, particularly *B. breve* strains and *L. plantarum* strains, have been identified as CLA producers, predominantly the *cis*-9, *trans*-11 (c9, t11) isomer from free linoleic acid (Barrett *et al.*, 2007; Rosberg-Cody *et al.*, 2004; Hughes and Sperandio, 2008; Kishino *et al.*, 2002). For example, *B. breve* NCIMB702258 converts linoleic acid to CLA in the murine gut, leading to significantly elevated c9, t11 CLA in the liver (Wall *et al.*, 2009). While *B. breve* is one of the most efficient CLA producers (with a reported conversion rate of 90.1% for *B. breve* CCFM683 (Gao *et al.*, 2020)) other *Bifidobacterium* strains have also demonstrated good conversion rates *in vitro* such as *Bifidobacterium bifidum* and *Bifidobacterium dentium* (Coakley *et al.*, 2003; Rosberg-Cody *et al.*, 2004). Dairy products are the principal food sources of CLA in the human daily diet (Chin *et al.*, 1992; Shantha, Crum and Decker, 1994), however, the amount of CLA in these foods is insufficient to meet the daily requirement (Schmid *et al.*, 2006). Fermented dairy products contain more CLA than unfermented products, therefore using a CLA producer as a starter culture could be a useful technique to increase the CLA content. *Bifidobacterium* strains in particular have high potential due to their probiotic characteristics and their ability to colonise the GI tract of both adults and infants (McCartney, Wenzhi and Tannock, 1996). To this end, Gao *et al.* (2020) assayed 152 bifidobacteria belonging to seven species and found that only *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium pseudocatenulatum* and *Bifidobacterium dentium* generated CLA.

The enzyme linoleic acid isomerase is responsible for the conversion of linoleic acid to CLA by the gut microbiota (Lin, Lin and Wang, 2002), in a manner similar to ruminant bacteria, and has been sequenced in various *Lactobacillus* and *Bifidobacterium* strains (Macouzet, Robert and Lee, 2010; Macouzet, Lee and Robert, 2010). Microbial CLA production has been demonstrated to have a positive impact on colitis, which was improved in mice with colitis following administration

of the probiotic mixture VSL#3. This resulted in high colonic concentrations of c9, t11 CLA which improved colitis by activating peroxisome proliferator-activated receptor gamma (PPAR γ) in macrophages (Bassaganya-Riera *et al.*, 2012). The *in vivo* production of the CLA isomer, *trans*-10, *cis*-12 (t10, c12) CLA by two strains of human origin, *Lactobacillus rhamnosus* PL60 and *Lactobacillus plantarum* PL62 has also been reported. Furthermore, administration of these two strains to mice resulted in increased t10, c12 CLA concentrations in sera with subsequent reductions in adipose tissue and body weight without impacting on food intake (Lee *et al.*, 2006; Lee *et al.*, 2007). Prebiotic supplementation has also been shown to increase CLA content in caecal tissue by increasing substrate availability and by modulating gut microbiota composition (Druart *et al.*, 2013).

2.3.8 Vitamins

Vitamins are essential micronutrients which are often precursors of various enzymes involved in vital biochemical reactions. Humans are incapable of synthesising most vitamins so they must be obtained exogenously, for example in food. However, deficiencies can occur due to insufficient food intake or poor eating habits. The gut microbiota is believed to be a rich source of vitamins to the host as they synthesise vitamins for their own requirements in excess, which is then available to the host through absorption. The importance of the gut microbiota as a vitamin source is evident by the observation that GF animals require vitamin supplementation while normalised animals do not (Hill, 1997). Therefore, the ability of food-related lactic acid bacteria and gut commensals such as bifidobacteria to synthesise and supply vitamins is important for human health and may represent a more natural alternative to supplementation using chemically synthesised pseudo-vitamins. Furthermore, microbial vitamin synthesis would enable the production of fermented foods containing elevated concentrations of vitamins that are less likely to cause undesirable side effects (LeBlanc *et al.*, 2013). In humans, it has been demonstrated that gut microbiota are capable of synthesising the fat-soluble vitamin K as well as several water-soluble B vitamins including biotic, cobalamin,

folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin and thiamine (Hill, 1997).

Folate is a B-group vitamin which is involved in many essential functions such as DNA replication, repair, and methylation as well as synthesis of nucleotides, vitamins, and certain amino acids. Bifidobacteria are confirmed to have folate biosynthetic properties, and certain strains such as *Bifidobacterium bifidum* and *Bifidobacterium longum subsp. infantis* are high level folate producers while *Bifidobacterium breve, Bifidobacterium longum subsp. longum* and *Bifidobacterium adolescentis* are low level producers (Pompei *et al.*, 2007a). These observations have been confirmed *in vivo* as administration of high-producing folate strains increased faecal levels of folate in both rats and humans (Pompei *et al.*, 2007b; Strozzi and Mogna, 2008). Riboflavin, also known as vitamin B2, is essential for cellular metabolism. While most yoghurt starter cultures decrease riboflavin concentrations, others can increase levels up to 160% of the initial concentration present in unfermented milk, indicating microbial synthesis (Kneifel *et al.*, 1992). Increased levels of other B group vitamins such as niacin and pyridoxine have been observed for certain lactic acid bacteria used in yoghurt, cheese and fermentations (Alm, 1982; Shahani and Chandan, 1979). For example, soy fermentation with *Streptococcus thermophilus, Lactobacillus helveticus* and *Bifidobacterium longum* has been shown to increase thiamine and pyridoxine concentrations (Champagne *et al.*, 2010). Finally, vitamin B12 is a cobalamine which is essential for normal DNA synthesis, particularly in bone marrow, and deficiency leads to pernicious anaemia (Hill, 1997). This is the only vitamin that is exclusively produced by microorganisms, particularly by anaerobes, as animals, plants and fungi are incapable of its production (Martens *et al.*, 2002; Roth, Lawrence and Bobik, 1996; Smith *et al.*, 2007). *Propionibacteria freudenreichii* was one of the first model organisms used to study B12 biosynthesis and is now used in its commercial production. The genetic pathway responsible for vitamin B12 production by *Lactobacillus reuteri* has also been described for two *L. reuteri* strains (Saulnier *et al.*, 2011). Gut bacteria are also a source of vitamin K (Fernandez and Collins, 1987), which is an essential component of the blood clotting cascade, with deficiency leading to haemorrhaging

and failure of the clotting system. Vitamin K is also essential to electron transport in bacteria. Faecal concentration estimates are 1-20 µmol/L per day in comparison to the daily requirement of 0.1 µmol/L per day, half of which is estimated to come from the gut microbiota (Passmore and Eastwood, 1986). Vitamin K deficiency occurs in patients who have undergone total colonic sterilisation, which is a further indication of the importance of the gut microbiota as a source of vitamin K in humans. However, GF animals can still produce vitamin K which suggests that menaquinone synthesis is not fully dependent on the gut microbiota (Davidson *et al.*, 1998).

2.4 Screening Techniques: Challenges and Limitations

Our understanding of the potential functions and metabolites of the gut microbiota has been limited by the fact that many gut bacteria are not readily culturable, potentially because they are adapted to a community environment and not pure cultures, so therefore there is potential bias towards specific organisms. In fact, early metagenomic studies targeting the 16S rRNA gene revealed that 80% of the identified bacteria had not yet been cultured (Lagier *et al.*, 2015). Therefore, it is vital to use techniques other than culture-dependent methods to screen for metabolite production to progress this field. An increased understanding of the key microbial genes involved in its various functions will be essential to fully understand and exploit the endocrine role of the gut microbiota. To this end, significant progress has been made in understanding the potential of the gut microbiota due to the exponential advances in culture-independent techniques, particularly genomic approaches. One such method involves targeting the 16S rRNA gene, which is present in all prokaryotes and contains variable domains that allow different taxa to be identified. Although these compositional analyses provide a large volume of data, they fail to generate information regarding the viability and functionality of the microbiota present (Patterson *et al.*, 2014). It is possible to go beyond the 16S gene using metagenomic analysis which targets small fragments of metagenomic DNA to characterise the full genetic content and functional potential

of the microbial community (Qin *et al.*, 2010). However, a significant challenge for functional interpretation of metagenomes in microbiota-gut-brain research is the lack of a dedicated reference database of gut microbial neuroactive metabolic potential (Valles-Colomer *et al.*, 2019). To address this, Valles-Colomer *et al.* (2019) developed a module-based analytical framework which described the microbial pathways that metabolise molecules with the potential to interact with the human nervous system, creating the first catalogue of gut microbiota neuroactive potential. This research enabled targeted profiling and interpretation of metagenomic data in the context of microbiota-gut-brain communication and studied the microbiota-gut-brain interaction beyond taxonomic measures. They curated and annotated 56 gut-brain modules (GBMs), each corresponding to a single neuroactive compound production or degradation process. They identified 14 rarely observed GBMs related to dopamine, acetylcholine, kynurenine, histamine, and serotonin II synthesis. The strongest observed association was histamine synthesis to *Fusobacterium*. In order to identify potential host-microbe interaction mechanisms, the GBM prevalence in genomes of gut-associated versus free-living taxa were compared and it was found that GABA synthesis pathways were significantly more prevalent in gut microorganisms. Furthermore, they assessed whether neuroactive compound metabolism is associated with quality of life and depression and found three GBMs linked to quality of life scores: synthesis of 3,4-dihydroxyphenylacetic acid (DOPAC), which was positively correlated; isovaleric acid synthesis potential II, also positively correlated; and histamine synthesis potential, which had a negative correlation to quality of life scores. DOPAC synthesis was strongly associated with *Coproccus*. Interestingly, decreased DOPAC levels in cerebrospinal fluid have been suggested as a biomarker for PD (Goldstein *et al.*, 2018), and the end product of this module has anti-proliferative activity on colon cancer cells (Gao *et al.*, 2006). Further insights into microbial activity and host-microbe interactions have been provided by methods which can analyse gene expression (metatranscriptomics), protein products (metaproteomics) and metabolic profiles (metabolomics) of the gut microbiota (Guinane and Cotter, 2013). Large-scale metagenomic studies such as National Institutes of Health-funded Human Microbiome Project (http://commonfund.nih.gov/hmp), the

European-funded Metagenomics of the Human Intestinal Tract (http://www.metahit.eu) and the International Human Microbiome Consortium (http://www.human-microbiome.org) best exemplify this. For example, the Human Microbiome Project sampled 18 body sites, which provided an in-depth definition of the human microbiome (Weinstock, 2012).

Although substantial progress has been made in culturing many members of the gut microbiota, a possible limitation in the culturing of certain species is likely the lack of key growth factors, such as quinones, in artificial media that are normally provided by neighbouring bacteria *in situ*. To investigate this, Strandwitz *et al*. (2018) utilised a co-culture method to isolate KLE1738, which is present on the National Institutes of Health's "Most Wanted" list, indicating that it has not been previously cultured. This isolate required the presence of *B. fragilis* ATCC 25852 to grow, supporting the theory proposed above. It was found that GABA was the growth factor produced by *B. fragilis* which enabled KLE1738 to grow by acting as a carbon and energy source. This finding led them to isolate a variety of GABA-producing bacteria by using the growth of KLE1738 as an indicator, thereby providing a novel screening assay for microbial GABA production. By utilising this assay, they were able to determine that *Bacteroides* spp. produced large quantities of GABA, while members of the *Parabacteroides*, *Eubacterium* and *Bifidobacterium* genera were also identified as GABA producers. Strandwitz *et al.* (2018) then profiled the GABA-modulating potential of the human gut microbiome *in silico* and found that 97 organisms had the capability to produce GABA, mostly via Gad, over 25% of which belonged to either *Bacteroides* or *Parabacteroides* genera. An additional method for screening for microbial GABA production has been described by (Barrett *et al.*, 2012). They screened 91 intestinally derived bacteria using a pH-controlled anaerobic faecal fermentation supplemented with 30 mg/ml of MSG. Determination of conversion of MSG to GABA was achieved by analysing the free amino acid content using a Jeol JLC-500/V amino acid analyser fitted with a Jeol Na+ high-performance cation exchange column (McDermott *et al.*, 2016). This method has been utilised in several subsequent studies (Patterson *et al.*, 2019; Linares *et al.*, 2016).

As mentioned previously, certain bacteria are capable of producing serotonin *in vitro* and this has been analysed using HPLC (Lyte, 2011; Jiménez *et al.*, 2013; Shishov *et al.*, 2009). However, it is hypothesised that <10% of peripheral serotonin is contributed directly by microbial synthesis, as demonstrated in studies involving TPH1 KO mice, which lack >90% of intestinal and blood serotonin levels (Savelieva *et al.*, 2008). Yano *et al.* (2015) have described a mechanism in which the gut microbiota regulate serotonin biosynthesis from colonic enterochromaffin cells in a postnatally inducible and reversible manner. Indigenous spore-forming bacteria from the mouse and human microbiota stimulate serotonin biosynthesis from colonic enterochromaffin cells, which supply serotonin to the lumen, mucosa and circulating platelets. Faecal metabolites that were increased by spore formers and that elevated serotonin in chromaffin cell cultures were identified as part of this study, indicating direct metabolic signalling of gut microbes to enterochromaffin cells. Research from Sudo (2019) indicated that gut microbes not only stimulate the release of free serotonin from EC stores but are also involved in the de-conjugation of conjugated serotonin. Furthermore, colonic and blood serotonin levels were increased in GF mice when luminal concentrations of particular microbial metabolites were elevated. The serotonin-related metabolites mentioned in this study are involved in various metabolic pathways which include, but are not limited to, amino acid metabolism, xenobiotics metabolism, peptide metabolism, lipid metabolism, nucleotide metabolism, and metabolism of cofactors and vitamins. Therefore, this study highlights a key role for host-microbiota interactions in regulating serotonin-related biological processes and demonstrates that spore forming bacteria are important modulators of host serotonin (Yano *et al.*, 2015).

Although promising results have come from preclinical studies, it is important to consider the obstacles that might prevent successful translation to the clinic, as illustrated by the diverging results of two different candidate psychobiotics (Allen *et al.*, 2016; Kelly *et al.*, 2017). Mice have become the model of choice in microbiota-gut-brain axis research and have been vital in understanding signalling pathways and the consequences of disturbed gut microbiota, however, it is important that differences between murine and human systems are considered in translational

studies (Mayer, Tillisch and Gupta, 2015). There are differences between the human and murine digestive tract, and human gut microbiota studies typically use faecal samples, while caecal samples are more frequently studied in the mouse microbiome. The ability of mice to reflect all relevant influences on the human gut microbiota is limited because several factors, including mode of delivery, diet and genetics contribute to shaping the human microbiome (Wiley *et al.*, 2017). Large-scale metagenomics studies could aid translation to clinical studies, however their interpretation is limited by a lack of reference databases and tools to study gut microbial neuroactive potential (Valles-Colomer *et al.*, 2019). Furthermore, many microbes are not yet characterised because of the difficulty of comparing data between studies due to the variability of sample processing and analysis, and the inability to culture all members of the microbiome. To address this inability to culture certain anaerobic bacteria, development of new methods for collection and transport of stool samples as well as the use of antioxidant agents when culturing will be important to allow testing for health-promoting metabolite production (Lagier *et al.*, 2015). Our knowledge is also limited by the difficulty of obtaining samples from different regions of the GIT. Most clinical studies have been performed in non-psychiatric patients; therefore, it will be important to translate findings to large scale longitudinal studies in well-phenotyped psychiatric populations. Potential confounding variables such as medication use, diet and supplements will need to be considered, and standardised methods will have to be employed. Interventional studies in psychiatric patients with probiotics and monitoring changes in brain response after these interventions could provide additional information on mechanisms of action of probiotics.

2.5 Implications for Neuropsychiatric Disorders

As discussed previously, health-beneficial bioactive metabolite-production and modulation have significant potential for therapeutic targeting of the gut microbiota in both stress-related disorders and metabolic diseases. Preclinical studies indicate the presence of melancholic microbes which influence psychiatric

disorders such as depression and anxiety (Dinan and Cryan, 2013; Park *et al.*, 2013). Therefore, Strandwitz *et al.* (2018) sought to investigate whether *Bacteroides*, which they postulate to be the major bacterial producers of GABA in the human gut, were associated with clinically diagnosed Major Depressive Disorder (MDD). They found that the relative abundance of faecal *Bacteroides* was inversely associated with functional connectivity between an expansive region of the Default Mode Network (DMN) and the left dorsolateral prefrontal cortex (DLPC). This region overlapped extensively with both the left medial Pre Frontal Cortex (PFC) and left frontopolar cortex, regions highly interconnected to the limbic system which are salient for reward processing and emotional reappraisal (Koechlin and Hyafil, 2007; Wager *et al.*, 2008). A study of 40 healthy women demonstrated that levels of *Bacteroides* were associated with increased grey matter in the cerebellum, hippocampus, and frontal brain regions. Furthermore, these women experienced reduced levels of anxiety, distress and irritability after looking at photographs to evoke an emotional response (Tillisch *et al.*, 2017). A high fat diet has been shown to reduce GABA levels in the rat PFC by ~40%, which was associated with reduced levels of *Bacteroides* and depressive-like behaviour (Hassan *et al.*, 2019). There is a distinct difference in microbiota composition of humans with MDD compared to healthy controls. Furthermore, faecal transfer of MDD microbiota to GF mice results in enhanced immobility time during the forced swim test and reduces centre distance travelled in the open field test (Zheng *et al.*, 2016). A recent study by Valles-Colomer *et al.* (2019) linked microbiota neuroactive capacity with quality of life and depression by analysing the Belgian Flemish Gut Flora Project (FGFP), a large faecal microbiome population study, with validation in various public and newly sequenced data sets. Reference genome mining allowed them to associate the relative abundances of microbial genera with quality of life scores and to catalogue the neuroactive potential of gut isolates. They observed that butyrate-producing *Faecalibacterium* and *Coproccus* bacteria were associated with higher quality of life indicators and their relative abundance correlated with stool butyrate concentrations. *Dialister* and *Coproccus* spp. were also depleted in depression, after correcting for confounding effects of antidepressant treatment, while *Butyricicoccus* were linked to antidepressant treatment. Consequently, Valles-

Colomer and colleagues concluded that *Dialister* and *Coproccus* should be regarded as potential targets for follow-up psychobiotic research. Conversely, *Flavonifractor* was negatively associated with physical functioning and was reportedly increased in major depression disorder patients. The *Bacteroides* enterotype 2, which has a reduced microbial load, was also associated with depression and lower quality of life in the FGFP cohort. Microbial synthesis potential of the dopamine metabolite 3,4-dihydroxyphenylacetic acid correlated positively with mental quality of life, while a potential role of microbial GABA production in depression was indicated, according to gut-brain module analysis employed in this study. Caspani *et al.* (2019) proposed that gut microbial metabolites such as neurotransmitters, SCFAs, indoles, bile acids, choline metabolites, lactate and vitamins affect depressive behaviour through both direct and indirect mechanisms including direct stimulation of central receptors; peripheral stimulation of neural, endocrine, and immune mediators; and epigenetic regulation of histone acetylation and DNA methylation.

Bravo *et al.* (2011) reported that administration of the probiotic bacterium *Lactobacillus rhamnosus* JB-1 reduced stress and depression-like behaviour in a vagus nerve-dependent manner in mice. Changes in the expression of GABA receptors in several areas of the brain, including the amygdala and hippocampus were also observed. Several *L. rhamnosus* strains have been shown to produce GABA at pH 3.6-5.2 (Lin, 2013). Furthermore, it has been shown that mice exhibit elevated levels of GABA in the brain after four weeks of supplementation with JB-1 (Janik *et al.*, 2016). Some *Bifidobacterium* strains have also been shown to produce GABA (Barrett *et al.*, 2012), and supplementation with a GABA-producing *Bifidobacterium* strain ameliorated visceral sensitivity in a rat model of pain (Pokusaeva *et al.*, 2017). In addition to preclinical studies, there is also support from clinical studies to indicate the potential for psychobiotics to improve depression. The translational potential of *B. longum* 1714 was demonstrated in healthy volunteers where it modulated stress, electrophysiology and neurocognition (Allen *et al.*, 2016). In a double-blind, placebo-controlled, randomised clinical trial where healthy subjects were given a mixture of probiotics containing *Lactobacilllus helveticus* R0052 and *B. longum* R0175 or placebo, the treatment group displayed

significantly less psychological distress than controls, combined with decreased urinary free cortisol output (Messaoudi *et al.*, 2011). In a similar study, subjects were fed a probiotic-containing milk drink or placebo for three weeks before mood and cognition were assessed. Significant improvements were found in subjects who initially scored in the lowest third for depressed mood (Benton, Williams and Brown, 2007). Administration of a fermented milk product containing a probiotic to healthy human volunteers altered brain activity in regions relevant to cognitive performance (Tillisch *et al.*, 2013). A randomised, double-blind, placebo-controlled trial administered a probiotic capsule consisting of *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium bifidum* to patients with MDD. Following eight weeks of intervention, patients who received the probiotic had significantly decreased Beck Depression Inventory scores compared with the placebo group (Akkasheh *et al.*, 2016). Importantly, results from a human study by Kootte *et al.* (2017) suggest that GABA levels can be adjusted by manipulating the microbiome. They found that transplantation of a faecal microbiome from lean to obese individuals resulted in increased levels of GABA in the plasma. Therefore, data from Strandwitz *et al.* (2018), combined with these reports, suggest that microbial-derived GABA may influence the host, and may have the potential for improving GABA-related disorders in humans.

Data suggest that the microbiota also influence the development of behavioural disorders such autism through modulation of the host metabolome and neuropeptide production (Fung, Olson and Hsiao, 2017). In the maternal immune activation model (MIA) of ASD, increased intestinal permeability and alterations in gut microbiota composition is observed in the offspring. Supplementation with *B. fragilis* to MIA offspring during weaning restored microbiota composition and significantly improved behavioural abnormalities in tests such as marble burying, open field exploration and ultrasonic vocalisation (Hsiao *et al.*, 2013). It alleviated autistic-like behavioural abnormalities in communication, social behaviour and restricted/repetitive behaviour compared to untreated control mice which were symptomatic of this disorder. Personality traits have also been shown to be influenced by the microbiota, whereby the diversity and composition of the human

gut microbiota were correlated with different personality traits. For example, high neuroticism and low conscientiousness groups were associated with high abundance of *Gammaproteobacteria* and *Proteobacteria*, respectively. Furthermore, an increased abundance of butyrate-producing bacteria such as Lachnospiraceae were observed in high conscientiousness groups (Kim *et al.*, 2018).

Profound alterations in gut microbiota composition and reduced α-diversity are evident in patients with schizophrenia compared to healthy controls. Schizophrenia severity has also been linked to specific taxa such as Veillonellaceae and Lachnospiraceae, while decreased abundance of Lachnospiraceae and Ruminococcaceae were also evident in patients with schizophrenia. Furthermore, colonisation of GF mice with microbiota from human schizophrenia patients resulted in behavioural changes similar to those observed in glutamatergic mouse models of schizophrenia, coupled with lower glutamate and higher glutamine and GABA in the hippocampus. This decrease in brain glutamate and disrupted glutamate-glutamine-GABA cycle, along with altered amino acid and lipid metabolism, are suggested potential mechanisms underlying this altered behaviour (Zheng *et al.*, 2019). An additional study by Zhu *et al.* (2019) provided further evidence of the role of gut microbiota in the pathophysiology of schizophrenia. They demonstrated that transplantation of faecal microbiota from drug-free schizophrenia patients to SPF mice caused schizophrenia-like behavioural abnormalities such as psychomotor hyperactivity, impaired learning and memory, as well as elevated activity of the kynurenine-kynurenic acid pathway of tryptophan degradation in the periphery and the brain. There were significant differences in 60 species of donor-derived bacteria between mice colonised with patient versus control microbiota.

The gut microbiota has also been linked to neurodegenerative diseases such as AD and PD. Pathogenic microbes may contribute to neurodegeneration, as bacterial (Hammond *et al.*, 2010; Huang *et al.*, 2014), viral (Karim *et al.*, 2014), fungal (Pisa *et al.*, 2015), and parasitic (Prandota, 2014) infections targeting the CNS increase the risk of AD, likely by promoting inflammatory responses in the CNS which could contribute to synaptic degradation and amyloidosis (Glass *et al.*, 2010).

Furthermore, memory and learning can be altered through microbiota manipulation by antibiotics or probiotic treatment (Fröhlich *et al.*, 2016; Wang *et al.*, 2015). Alterations in microbial composition have also been observed in PD, a neurodegenerative disorder that is often associated with impaired gastric motility (Fasano *et al.*, 2015) and elevated levels of α-synuclein in the intestine (Shannon *et al.*, 2012) which potentially indicates a role for the intestinal environment in regulating the development of this disease. Patients with PD have been found to harbour a reduced abundance of Prevotellaceae compared to healthy controls. Furthermore, Enterobacteriaceae may play a role in regulating motor function as this was elevated in a subset of patients with severe postural instability and gait difficulty (Scheperjans *et al.*, 2015). PD patients also experience increased colonic inflammation and intestinal permeability (Devos *et al.*, 2013; Forsyth *et al.*, 2011). An additional study found increased *Blautia, Coprococcus, Roseburia* and *Proteobacteria* in PD patients, while *Faecalibacterium* and Prevotellaceae were decreased (Keshavarzian *et al.*, 2015). Several studies have demonstrated that bacterial species which contain sequences similar to tyrosine decarboxylase (tyrDC), such as *Enterococcus faecalis*, can metabolise Levodopa (L-dopa), suggesting that the gut microbiome could reduce peripheral availability of L-dopa (Jameson and Hsiao, 2019; Maini Rekdal *et al.*, 2019; van Kessel *et al.*, 2019). Furthermore, over 50 *Enterococcus* strains, predominantly *E. faecium* and *E. faecalis*, as well as *Lactobacillus* and *Staphylococcus* species also express genes encoding for tyrosine decarboxylase and convert L-dopa to dopamine (Morales-Briceño and Fung, 2019).

2.6 Conclusion

The importance of the commensal bacteria that colonise the gut for major aspects of physiology, particularly functions related to behaviour, brain structure and function is being increasingly recognised (Shanahan, 2013; Lyte, 2014). Research in this area has expanded rapidly in recent years, becoming one of the most studied areas of microbiology with significant potential to benefit clinical practice (Weinstock, 2012). Therefore, microbiome manipulation using probiotics or

prebiotics to strengthen host-microbe symbiosis is likely to play a role in the future prevention and treatment of immune and psychiatric disorders. A microbiota which is capable of producing health-beneficial metabolites including neurotransmitters, vitamins and SCFAs will be particularly important, as these play a role in regulating distal organs including the brain. However, there are still several challenges that need to be addressed. Research needs to progress beyond characterising the microbiota composition in various disease states and investigate potential mechanisms by which altered microbiota composition contributes to pathophysiology of disease, and by which potential probiotic bacteria exert beneficial effects on host health, potentially by metabolite production (Kelly *et al.*, 2015). Additionally, animal studies which combine microbiome intervention with neurotransmitter antagonists will provide further insight into mechanisms of communication between gut microbiota and the host (Strandwitz, 2018). Lagier *et al.* (2015) proposed that sequencing the genomes of all microorganisms cultured for which no sequences are available, which he termed microbiogenomics, will represent a significant advance in clinical microbiology. Nevertheless, further research is necessary to ascertain whether manipulation of human neurotransmission by gut microbiota is associated with additional physiological implications and how it may be employed therapeutically.

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3. Investigating Neuroactive Metabolite Production and Probiotic Characteristics of Gut-Derived Lactobacilli

Niamh C. Wiley[1,2,3], Amy B. Murphy[1,2], Gerard Clarke[2,3], Francisca J. Villalobos[2,3], John F. Cryan[2,4], Timothy G. Dinan[2,3], R. Paul Ross [2,5], Catherine Stanton[1,2]

1 Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland

2 APC Microbiome Ireland, University College Cork, Cork, Ireland

3 Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

4 Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

5 College of Science, Engineering and Food Science, University College Cork, Cork, Ireland

3.1 Abstract

The gut microbiome plays a vital role in functions related to metabolism, the immune system, behaviour, brain structure and function. Recent research indicates that therapeutic targeting of the gut microbiota has the potential to be useful in the treatment of both stress-related disorders and metabolic diseases. An important method by which the gut microbiome can influence the gut-brain axis is through microbial production of psychoactive metabolites. Several bacteria have been shown to produce metabolites which can impact host health such as short chain fatty acids, conjugated linoleic acid, antimicrobials, exopolysaccharides, and vitamins as well as neurotransmitters such as serotonin, gamma-aminobutyric acid (GABA), catecholamines and acetylcholine. Therefore, this study aims to screen gut-derived microbiota isolated from healthy adult males for psychoactive metabolite production which is of relevance to stress-related disorders, specifically GABA and serotonin synthesis. A total of 34 gut-derived strains as well as 46 fermented food isolates were screened for serotonin production, GABA production and other probiotic characteristics such as antimicrobial activity. One *Lactobacillus plantarum* GABA producer, which was not as efficient as the positive control *Lactobacillus brevis* DPC6108, and two *Lactobacillus paracasei* potential serotonin synthesisers were identified. Whole genome sequencing was carried out to investigate mechanisms of action underlying psychobiotic potential, locating the *tryptophan synthase* operon regulating serotonin synthesis as well as genes promoting antimicrobial activity, acid tolerance and adhesion capability in the strains of interest.

3.2 Introduction

The term “psychobiotics” was coined by Dinan *et al.* (2013) to describe probiotics which have a positive mental health benefit due to their ability to produce various metabolites, including peptides and mediators which are normally associated with neurotransmission (Dinan, Stanton and Cryan, 2013). Accumulating preclinical evidence points towards the possibilities of their use in disorders such as

depression and anxiety and may possibly have the potential as a novel therapeutic strategy in the prevention and/or treatment of certain neurological and neuropsychiatric conditions. Furthermore, numerous studies have suggested that several molecules with neuroactive functions, including serotonin, GABA, acetylcholine and catecholamines are microbially derived, and many of these have been isolated from bacteria within the human gut (Sudo, 2019) (Table 1). It is also possible to modulate plasma concentrations of tryptophan by manipulating microbiota composition (Clarke *et al.*, 2014). This is relevant because tryptophan is an essential amino acid and precursor to serotonin which plays a vital role in both the enteric and central nervous systems. The gut microbiota also exerts control over the hypothalamic-pituitary-adrenal (HPA) axis, although the mechanisms are indirect and unknown. Therefore, this ability to synthesise neuroactive compounds allows probiotics to function as pharmacological agents and act as drug delivery vehicles (Lyte, 2011). The delivery of neurochemicals by probiotics may either be in the amount already contained in the bacterium at time of ingestion or what is actively produced by the bacterium once ingested and viable in the gastrointestinal tract (Wall *et al.*, 2014). The main genera used as probiotics are *Lactobacillus* and *Bifidobacterium* because many of them can survive gastrointestinal transit, have the ability to adhere to intestinal epithelial cells and are considered safe for human consumption (Marques *et al.*, 2014).

Table 1: Serotonin and GABA Production by Gut Bacteria

	Bacterial Strain	**Reference**
Serotonin	Streptococcus Thermophiles	(Özoğul *et al.*, 2012)
	E. coli K-12	(Shishov *et al.*, 2009)
	Lactococcus lactis	(Özoğul *et al.*, 2012)
	Lactococcus cremoris	(Özoğul *et al.*, 2012)
	Lactobacillus plantarum	(Özoğul *et al.*, 2012)
	Morganella morganii	(Özoğul, 2004)
	Klebsiella pneumonia	(Özoğul, 2004)

	Hafnia alvei	(Özoğul, 2004)
GABA	Lactobacillus brevis DPC6108	(Barrett *et al.*, 2012)
	Lactobacillus rhamnosus	(Lin, 2013)
	Bifidobacterium adolescentis	(Lin, 2013)
	Bifidobacterium dentium	(Lin, 2013)
	Bifidobacterium infantis	(Lin, 2013)
	Streptococcus Thermophilus	(Linares *et al.*, 2016)

3.2.1 GABA

GABA is the major inhibitory neurotransmitter in the CNS and therefore plays an important role in functions such as neurotransmission, diuretic effects, pain sensation, anxiolytic effects (Hayakawa *et al.*, 2004; Linares *et al.*, 2016), the regulation of cardiovascular functions such as blood pressure and heart rate (Mody *et al.*, 1994), and an altered GABAergic profile has been linked to CNS disorders such as anxiety and depression (Cryan and Kaupmann, 2005). It has been reported that GABA levels are significantly decreased in both the stool and blood of germ free mice (Matsumoto *et al.*, 2017), and certain strains of *Lactobacillus* and *Bifidobacterium* are capable of GABA production via the same biosynthetic pathway as in neuronal tissue, indicating that the microbiota may contribute to circulating levels of GABA (Komatsuzaki *et al.*, 2008). A previous study by Barrett *et al.* (2012) screened 91 strains of human-derived bifidobacteria and lactobacilli and found five strains capable of GABA production, with *Lactobacillus brevis* DPC6108 and *Bifidobacterium dentium* being the most efficient producers. *Lactobacillus brevis* DPC6108 is a highly efficient producer and has been shown to convert up to 90% of glutamate to GABA, while also retaining the capability to produce GABA in the presence of other gut-derived bacteria.

Harnessing the production capability of the intestinal microbiota or consuming probiotic bacteria that can utilise dietary monosodium glutamate to synthesise GABA is therefore an exciting means to increase GABA levels (Barrett *et al.*, 2012)

which could have the potential to improve both neuropsychiatric and metabolic disorders. For example, intervention with *L. brevis* strains has been shown to attenuate several abnormalities associated with metabolic dysfunction, causing a reduction in the accumulation of mesenteric adipose tissue, increased insulin secretion following glucose challenge, improved plasma cholesterol clearance and reduced despair-like behaviour and basal corticosterone production during the forced swim test (Patterson *et al.*, 2019). At gene expression level, ingestion of *L. rhamnosus* JB-1, one of the most cited GABA producers, altered the mRNA expression of both $GABA_A$ and $GABA_B$ receptors, which have been implicated in anxiety and depression and are expressed in regions responsible for maintaining normal mood and fear responses (Bravo *et al.*, 2011). Therefore, this study aims to screen gut-derived lactobacilli for GABA synthesis, while utilising *Lactobacillus brevis* DPC6108 as a positive control, as such GABA producing strains have great potential as novel psychobiotics.

3.2.2 Serotonin

Serotonin is a monoamine neurotransmitter which is biochemically derived from tryptophan and is primarily found in the gastrointestinal tract, blood platelets, and CNS of humans. It plays a key role in the microbiota-gut-brain axis at both the ENS (Mawe and Hoffman, 2013) and CNS (Ruddick *et al.*, 2006) levels. In addition to its role as a neurotransmitter, serotonin is an important regulatory factor in other organ systems such as the gastrointestinal tract where over 90% of the body's serotonin is synthesised. Gut-derived serotonin regulates diverse functions, including enteric motor and secretory reflexes (Gershon and Tack, 2007), platelet aggregation (Mercado *et al.*, 2013), blood pressure regulation (Watts *et al.*, 2012), immune responses (Baganz and Blakely, 2012), bone development (Chabbi-Achengli *et al.*, 2012; Yadav *et al.*, 2008), and cardiac function (Côté *et al.*, 2003). Furthermore, disruption of peripheral serotonin is evident in several disease states, including irritable bowel syndrome (Stasi *et al.*, 2014), cardiovascular disease (Ramage and Villalón, 2008), and osteoporosis (Ducy and Karsenty, 2010).

Microbiota play a role in regulating serotonin levels, as studies have shown decreased serum, plasma, colonic and faecal serotonin concentrations in germ free compared to specific pathogen free mice (Sjögren *et al.*, 2012; Wikoff *et al.*, 2009). Studies in germ free mice indicate that the gut microbiota regulate the peripheral availability of circulating tryptophan, which is critical for serotonin synthesis (Clarke *et al.*, 2013). The mechanism underlying microbial regulation of circulating tryptophan concentrations is unknown but is hypothesised to involve control over tryptophan degradation along an alternative metabolic pathway, the kynurenine pathway (Schwarcz *et al.*, 2012; Stone, Stoy and Darlington, 2013). Additionally, strains such as *Clostridium sporogenes* and *Ruminococcus gnavus* are capable of tryptamine synthesis, which plays a role in signalling and inducing the release of serotonin from enterochromaffin cells, by the decarboxylation of tryptophan (Williams *et al.*, 2014). Therefore, the ability of the microbiota to regulate influential precursors such as tryptophan, tryptamine and kynurenine likely contributes significantly to its ability to influence CNS and ENS neurotransmission (Gheorghe *et al.*, 2019). Additionally, it is hypothesised that specific subsets of gut microbes are responsible for influencing host serotonin pathways. Certain bacteria, such as the probiotic *Bifidobacterium infantis* (Desbonnet et al., 2008), are capable of tryptophan biosynthesis via enzymes which include tryptophan synthase (Raboni, Bettati and Mozzarelli, 2009; Yanofsky, 2007), while some strains also have the ability to produce serotonin from tryptophan (Lyte, 2011; Jiménez *et al.*, 2013; Shishov *et al.*, 2009). Therefore, considering the role of gut microbiota in serotonin production and the regulation of serotonin metabolism pathways, this study aims to investigate the serotonin production capabilities of gut-derived *Lactobacillus* strains with the aim of identifying novel psychobiotic strains.

3.2.3 Tryptophan Metabolism Pathways

Tryptophan is an essential amino acid which cannot be synthesised by human cells and must therefore be supplied through diet (Dehhaghi, Kazemi Shariat Panahi and Guillemin, 2019), predominantly as a constituent of protein and the most common

tryptophan sources include fish, bananas, milk, oats, chicken and chocolate (Richard *et al.*, 2009). Interestingly, breast milk also contains a more immediately accessible non-protein portion for infants which is hypothesised to be important for postnatal development (Csapo and Salamon, 2009). Tryptophan plays an important role in multiple psychological and physiological processes, including contributing to controlling emotional states, the cellular ageing process and energy production through its conversion into serotonin or NAD+, which are the end products of the serotonin pathway and kynurenine pathway respectively (Dehhaghi, Kazemi Shariat Panahi and Guillemin, 2019). The first step of the serotonin pathway is the conversion of tryptophan to 5-hydroxytryptophan by the rate-limiting enzyme tryptophan hydroxylase. This short-lived intermediate product is then converted to serotonin via aromatic amino acid decarboxylase. However, approximately 90% of tryptophan is metabolised along the kynurenine pathway (O'Mahony *et al.*, 2015), and the rate of metabolism is dependent on the action of the largely hepatic-based enzyme tryptophan-2,3-dioxygenase (TDO) and the ubiquitous indoleamine-2,3-dioxygenase (IDO). The bacterial genes encoding the enzymes for kynurenine production have been identified as *kynA* and *kynB*, encoding tryptophan-2,3-dioxygenase and kynurenine formamidase respectively. Kynurenine can also be further converted to quinolinate via the enzymes kynurenine 3-monooxygenase, kynureninase, and 3-hydroxyanthranilate-3,4-dioxygenase, encoded by *KMO*, *KYN* and *HAD* respectively (Kaur, Bose and Mande, 2019). Kynurenine is further metabolised along two separate arms of the pathway, resulting in the production of the neuroprotective kynurenic acid or the neurotoxic quinolinic acid. Furthermore, while kynurenic acid can be neuroprotective against quinolinic acid-induced toxicity, in high concentrations it can induce cognitive impairment (O'Mahony *et al.*, 2015). It is also a ligand of the aryl hydrocarbon receptor (AHR), which plays an important role in neuroinflammatory processes, has immunoregulatory and anti-inflammatory properties in the GI tract, and has been shown to inhibit proliferation of colon cancer cells *in vitro* (Walczak, Turski and Rajtar, 2014). Kynurenine pathway metabolites are associated with inflammatory responses, neurological disorders and immune system regulation (Dehhaghi, Kazemi Shariat Panahi and Guillemin, 2019; Gao *et al.*, 2020). Other metabolites include picolinic acid which is

neuroprotective with antifungal, antiviral, antitumoral and cell growth regulatory activities (Dehhaghi, Kazemi Shariat Panahi and Guillemin, 2019), and quinolinic acid which is neuroprotective in low concentrations but neurotoxic at high concentrations, contributing to neurodegenerative diseases such as Alzheimer's disease, AIDS dementia complex, Huntington disease and Multiple Sclerosis (Chen and Guillemin, 2009; Dehhaghi *et al.*, 2019). Therefore, increased kynurenine pathway activity not only limits the tryptophan availability for central serotonin synthesis, it also results in increased production of neuroactive metabolites which can have a significant impact in both the CNS and ENS (O'Mahony *et al.*, 2015).

Tryptophan metabolism and the gut microbiota are tightly linked as demonstrated by germ free mice which show reduced tryptophan metabolism along the kynurenine pathway, higher tryptophan availability, a decreased ratio of kynurenine: tryptophan which is an indicator of IDO/TDO activity, and lower kynurenine levels in blood circulation, all of which can be normalised following colonisation immediately post weaning (O'Mahony *et al.*, 2015; Desbonnet *et al.*, 2015). Similarly, antibiotic treatment also results in reduced metabolism along the kynurenine pathway, increased circulating tryptophan availability and reduced colonic serotonin levels, accompanied by a down-regulation of the key synthase tryptophan hydroxylase 1 in the colon (Desbonnet *et al.*, 2015; Ge *et al.*, 2017; Jiang *et al.*, 2017). While the majority of tryptophan provided by ingested protein is absorbed in the small intestine, tryptophan which reaches the large intestine is degraded by the microbiota. Tryptophan metabolism pathways were found to be enriched in five gut-associated phyla, namely Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria, as well as five genera, namely *Clostridium, Burkolderia, Streptomyces, Pseudomonas,* and *Bacillus* (Kaur, Bose and Mande, 2019).

Indole is the main metabolite which is produced by gut microbiota in a single-step reaction through the enzyme tryptophanase (Gao *et al.*, 2020) which can be found in genera such as *E. coli, Clostridium* and *Bacteroides*. Indole is an important intercellular signal (Lee, Wood and Lee, 2015) and also interacts with the intestinal epithelium by promoting tight junction resistance and expressing anti-inflammatory

cytokines (Whitfield-Cargile *et al.*, 2016). Indole also influences brain function and behaviour once absorbed into the blood circulation, as rats overproducing indole display higher helplessness in the tail suspension test as well as enhanced anxiety-like behaviour in the novelty, elevated plus maze and open field tests (Jaglin *et al.*, 2018). Tryptamine is a monoamine which is structurally similar to serotonin and is another product of tryptophan metabolism by gut bacteria. It can be generated by decarboxylation of tryptophan through the action of tryptophan decarboxylases and is lacking in germ free mice. It has been demonstrated that at least 10% of the human population harbours at least one bacterium encoding a tryptophan decarboxylase, for example, members of *Clostridium, Ruminococcus, Blautia*, and *Lactobacillus* have been identified as producers of tryptamine from tryptophan in a tryptophan decarboxylase- dependent manner (Williams *et al.*, 2014). Tryptophan can also be metabolised by microbiota into indolic compounds such as indole-3-aldehyde, indole-3-acetic acid and indole-3-propionic acid (Agus, Planchais and Sokol, 2018). Indole-3-acetic acid is synthesised from indole-acetamide, which is converted from tryptophan by the enzyme tryptophan monooxygenase present in bacteria such as *Clostridium, Bacteroides,* and *Bifidobacterium* (Smith and Macfarlane, 1996; Roager and Licht, 2018). Additionally, tryptophan can be converted to the intermediate indole-3-lactic acid by some members of *Lactobacillus* and *Bifidobacterium* by the aromatic amino acid aminotransferase and indoleactic acid dehydrogenase-dependent pathway (Cervantes-Barragan *et al.*, 2017; Aragozzini *et al.*, 1979). Furthermore, *Clostridium* and *Peptostreptococcus* have been reported to convert indole-3-lactic acid to indole-3-propionic acid in a reaction catalysed by phenyllactate dehydratase (Williams *et al.*, 2014; Wlodarska *et al.*, 2017). These metabolites affect intestinal permeability and host immunity (Jennis *et al.*, 2018) as demonstrated by a recent study which showed that the microbial indole metabolites of tryptophan including indole, indole-3-acetic acid, and indole-3-propionic acid can activate aryl hydrocarbon signalling in astrocytes, thereby suppressing CNS inflammation (Rothhammer *et al.*, 2016).

As mentioned previously, certain commensal microbiota, especially spore-forming bacteria promote serotonin biosynthesis in colonic enterochromaffin cells (Yano *et*

al., 2015). Furthermore, commensal microbiota have been demonstrated to directly utilise luminal tryptophan for serotonin synthesis as *Lactococcus, Lactobacillus, Streptococcus, E. coli,* and *Klebsiella* have been reported to synthesise serotonin by expressing tryptophan synthase (O'Mahony *et al.*, 2015; Gao *et al.*, 2020). Bacteria can also synthesise tryptophan via the enzyme tryptophan synthase (Raboni, Bettati and Mozzarelli, 2009; Yanofsky, 2007). Interestingly, certain strains can also influence serotonin synthesis indirectly. For example, the probiotic *Lactobacillus casei* 327 has been shown to promote colonic serotonin synthesis by increasing TPH1 expression, thereby affecting the peripheral serotonin pool (Hara *et al.*, 2018). Oral treatment with *Lactobacillus johnsonii* cell-free supernatant in rats resulted in increased serum serotonin levels accompanied by decreased serum kynurenine levels and reduced intestinal indoleamine-2,3-deoxygenase activity (Valladares *et al.*, 2013). Taken together, these studies indicate that certain probiotic *Lactobacillus* species have the potential to shift the host tryptophan metabolism by suppressing the kynurenine pathway, thereby indirectly promoting serotonin biosynthesis. Furthermore, other *Lactobacillus* species are reported to be capable of tryptophan degradation into indolic compounds including indole-3-aldehyde, indole-3-lactic acid, and indole-3-acetic acid which, as mentioned previously are important for brain function, behaviour, and the inflammatory response (Cervantes-Barragan *et al.*, 2017; Zelante *et al.*, 2013). Therefore, targeting the gut microbiota could be a promising approach for modulating tryptophan metabolism and promoting serotonin biosynthesis (O'Mahony *et al.*, 2015).

3.2.4 Study Aims

MyNewGut is a global research consortium which aims to identify the effects of environmental factors on the gut microbiome as well as investigate the influence of the gut microbiome on brain, immune, and metabolic programming, development, and function. As part of the MyNewGut project, stool samples from 37 male C-section delivered and 39 healthy male control participants have been collected during a period of naturalistic stress and processed using both culture dependent

and independent approaches, isolating putative *Lactobacillus* and *Bifidobacterium* species for characterisation as future probiotics. This study aims to screen these strains for bacteria with psychobiotic potential, the ability to produce neurotransmitters and neuroactive compounds which could influence the microbiota-gut-brain-axis, with the rationale that probiotic bacteria may have the potential as a novel therapeutic strategy in the prevention and/or treatment of certain neurological and neuropsychiatric conditions. Therefore, the development of a biobank of psychobiotics may improve the standard of living for large numbers of people by improving mood and stress. As part of this study, gut-derived *Lactobacillus* strains from the biobank were assessed for psychobiotic potential with a particular focus on serotonin and GABA.

Serotonin is of particular interest because it plays an important role in the regulation of a number of bodily functions, including gastrointestinal tract motility, secretion, sensation and mood. Furthermore, there is significant overlap between behaviours regulated by the serotonergic system and those that are influenced by the microbiota. Several *Lactobacillus* species from the biobank were compared to serotonin producers identified from the literature including *Lactococcus lactis* subsp. *cremoris* MG1363, *Lactococcus lactis* subps. *cremoris* 1614, *Lactococcus lactis* subsp. *cremoris* 712, *Streptococcus thermophilus* 5655, *Streptococcus thermophilus* 5656, *Lactobacillus plantarum* 891, and *Lactobacillus plantarum* 6805 (Özoğul *et al.*, 2012) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit and high performance liquid chromatography (HPLC) analysis. Both methods were compared to determine whether there is any difference in detection capability, and which screening method may be more suitable for any future work.

Strains from the biobank were also assessed for GABA production. As discussed previously, sufficient evidence exists to suggest that microbially-produced GABA can impact brain function and behaviour. Strains were grown in media supplemented with monosodium glutamate to stimulate GABA production and GABA was then quantified using the previously validated method of amino acid analysis (Patterson *et al.*, 2019). Using *Lactobacillus brevis* DPC6108 as a positive control, 45 samples were prepared for amino acid analysis comprising of 17 isolates

from vaginally-delivered MyNewGut samples, 17 isolates from Caesarean section-delivered MyNewGut samples, *Lactobacillus brevis* DPC6108, as well as blank broths and samples grown in de Man, Rogosa and Sharpe (MRS) without monosodium glutamate as negative controls. A second screen was also carried out consisting of isolates from fermented food products such as saurkraut, kimchi, salted turnip, goats milk kefir and buffalo cheese.

Any isolates of interest which demonstrated GABA or serotonin production were identified using Sanger sequencing and further characterised by whole genome sequencing to investigate potential mechanisms of action underlying neurotransmitter production. Whole genome sequencing will be particularly important for characterising the serotonin producers as it will provide an additional means of comparing the ELISA and HPLC assays, as well as provide an insight into the metabolic pathways involved in the underlying mechanisms of action. This data was also utilised to identify genes promoting probiotic characteristics such as acid and bile tolerance, antimicrobial activity, and adhesion capability, as well as confirming that genes promoting toxin production and antibiotic resistance are not present within the genomes of the strains of interest. As an additional test for probiotic characteristics, bacteriocin screening was carried out on a subset of strains from the biobank using deferred antagonism assays, well diffusion assays, as well as Proteinase K activity and heat stability assays.

3.3 Materials and Methods

3.3.1 Creation of Biobank of Potential Probiotics

A biobank containing approximately 300 putative lactobacilli was created with the aim of screening for psychobiotic potential. The biobank was created from isolates from faecal samples obtained as part of the MyNewGut project. The participants in this project were healthy adult males, born *via* Caesarean section but not prone to stress. 0.1 g of each faecal sample was aliquoted into tubes and tenfold serial dilutions were performed in MRD before 100 µl was spread-plated on lactobacillus

selection (LBS) agar plates to select for lactobacilli. The plates were then incubated anaerobically at 37°C for five days. After incubation, approximately 10 single colonies of different morphologies and colours from each sample were picked with sterile inoculation loops and triple streaked on fresh LBS plates for purity. After 48 hours, single colonies were picked and inoculated in 10ml of MRS broth for 24-48 hours. When the cultures had sufficient growth, they were checked under the microscope and any cultures which consisted of cocci morphology were discounted. Cultures which appeared pure and had rod morphology were stocked in glycerol and stored at −80°C for further downstream characterisation.

3.3.2 *Selection of Positive Controls and Media Types for Serotonin Production*

Lactococcus lactis subsp. *cremoris* MG 1363 was selected as a positive control for serotonin production following a literature search. Ozogul *et al.* (2012) demonstrated that it was capable of producing 0.71 mg/L serotonin in arginine decarboxylase broth as measured using HPLC. *Lactococcus lactis* subsp. *cremoris* 1614 and *Lactococcus lactis* subsp. *cremoris* NCDO 712, were also selected as potential positive controls. To assess growth conditions, all three strains were streaked on Lactose Indicator Agar. Briefly, this media was prepared by dissolving 20 g tryptone, 5 g yeast extract, 10 g lactose, 4 g sodium chloride, 1.5 g sodium acetate, 0.5 g ascorbic acid, 2.5 g gelatin, and 1% agar in 1L of water. The pH was adjusted to 6.8 before being autoclaved at 121°C for 15 minutes. 200 µl of 4% bromocresol purple was added to a 200 ml bottle prior to pouring the media. These strains were then triple streaked on the plates to assess whether they use glucose or lactose as an energy source, depending on a colour change of the media – from purple to yellow for glucose utilisation, and from purple to white for lactose utilisation. Glucose M17 or Lactose M17 (Sigma-Aldrich) was then prepared for the strains in order to create stocks. *Streptococcus thermophilus* 5655 and *Streptococcus thermophilus* 5656 were also selected, along with *Lactobacillus plantarum* 891 and *Lactobacillus plantarum* 6805 based on literature searches (Clarke *et al.*, 2014).

Ozogul *et al.* (2012) used arginine decarboxylase broth to screen for serotonin producers, therefore this was prepared using 2 g peptone, 1 g Lab-Lemco powder (Oxoid, England), 5 g NaCl (Merck, Germany), 8.02 g L-arginine (Sigma Aldrich, Germany) and 5 mg pyridoxal HCl (Sigma, Germany) in 1 L distilled water. The pH was adjusted using 2.5M HCl and 3M NaOH until the pH reached 6. Arginine decarboxylase broth was decanted into 250 ml bottles and then autoclaved at 121°C for 15 minutes prior to use. However, it was found when tested that most strains had a poor ability to grow in this growth medium. Fresh arginine decarboxylase broth was then prepared as above but with the addition of 5 g of D-Glucose.

Test broth containing tryptophan, the precursor to serotonin, was prepared for each media type. 2x concentrated bottles of broth were prepared for each media type according to manufacturer's instructions. A tryptophan stock was then prepared separately to the media types because tryptophan cannot be autoclaved. 1.5 g of tryptophan was found to be the maximum amount of tryptophan that was dissolvable in 100 ml of water, without the addition of acid. Neutralising the pH resulted in the tryptophan coming back out of solution; consequently, it was necessary to avoid the addition of acid. A stock solution of tryptophan was prepared by dissolving 1.5 g of tryptophan in 100 ml of water before being filter sterilised into 100 ml of each media type, to give a final volume of 200 ml broth and a final tryptophan concentration of 0.75%.

3.3.3 Serotonin Screening using ELISA

The potential positive controls, along with a selection of putative lactobacilli from the MyNewGut biobank, were selected for screening using the Serotonin High Sensitive ELISA kit from Eagle Biosciences (10 Columbia Dr, Amherst, NH 03031, United States), with a sensitivity of 0.39 pg/sample (Serotonin Ultrasensitive ELISA Assay Kit SEU39-K01). Each strain was triple streaked on the relevant media type and grown anaerobically for 48 hours before single colonies were selected and inoculated into 10 ml of standard broth (GM17, LM17, MRS, or arginine

decarboxylase broth as appropriate). After 24 hours, 100 µl from each culture was inoculated into 10 ml fresh standard broth as above in order to stabilise the growth of the strains. This was carried out in technical duplicates. After a further 24 hours, 100 µl from each culture was added to 10 ml of test broth to be used in the ELISA and allowed to grow for 24 hours before the ELISA was carried out. The test broths consisted of both standard broths, for use as a baseline measurement, as well as tryptophan broths, to stimulate the production of serotonin. All broths were supplemented with 0.1% ascorbic acid to protect serotonin in the cultures from oxidation in the ELISA, as recommended by the manufacturer.

Prior to carrying out the ELISA, wash buffer was prepared according to the manufacturer's instructions and kept at 4°C where it is stable for up to four weeks. Briefly, reagents and samples were first allowed to reach room temperature before the standards and controls were diluted according to the manufacturer's instructions. The samples were prepared by centrifuging 2 ml of each culture for five minutes and filtering the supernatant to remove any remaining cells. The acylation agent was then dissolved in 2.5 ml of solvent and placed on an orbital shaker for five minutes. 25 µl of acylation buffer was pipetted into each well, before 20 µl of the diluted controls, the standards, and the filtered samples were pipetted into the respective wells. The plate was mixed for 10 seconds on the orbital shaker, which was placed beside a Bunsen burner in order to ensure sterility. 10 µl of acylation agent was pipetted into all of the wells, resulting in a colour change. The plate was then incubated at room temperature on an orbital shaker as described above for one hour. 25 µl of deactivator was pipetted into all wells, and the plate was incubated at room temperature on the orbital shaker for three hours. 50 µl from each well of the acylation plate was then transferred to the ELISA plate. This was then covered and incubated at 2-8°C overnight. Following this incubation period, the contents of the ELISA plate were discarded before the wells were washed four times by the addition 300 µl of wash buffer, ensuring to remove all residual liquid. 100 µl of enzyme conjugate was then added to all wells, before the plate was placed on the orbital shaker for one hour. The wells were again washed four times, as described above, and 100 µl of substrate was pipetted into all wells.

The plate was incubated for 20-30 minutes at room temperature on an orbital shaker, before 100 µl of stop solution was pipetted into all wells. The optical density was then read at 450 nm on a Synergy HT plate reader (Mason Technology). A second ELISA was later performed with further putative lactobacilli from the biobank as described above but with the following modifications: tryptophan broth was prepared to a concentration of 0.1% instead of 0.75%, as this was the tested cross-reactivity of the ELISA kit. Furthermore, the standards and controls were prepared with broth instead of with the dilution buffer supplied in the kit in order to account for background readings.

3.3.4 Screening for Serotonin using HPLC

Preliminary HPLC analysis was carried out to determine whether serotonin can be detected in the cell culture matrix using this method. Bacterial cell cultures were spiked with a known concentration of serotonin (50 µM, Sigma Aldrich) and the supernatants were measured using HPLC. Two different centrifugation methods were selected based on a literature search: 2 ml was centrifuged for two minutes at 11,500 rpm at 4°C, the supernatant was then removed and frozen for HPLC analysis; and 2 ml was centrifuged for two minutes at 11,500 rpm at 4°C, the supernatant was removed, 2 ml of water was added and centrifuged again at the same settings. This supernatant was removed and frozen until HPLC analysis could be carried out as follows (Park *et al.*, 2011):

I. Sample Preparation:

20 µl of 4M Perchloric acid was added to 200 µl of sample which were then centrifuged at 14000 rpm for 20 minutes. 150 µl of supernatant was added to 150 µl of homogenisation buffer (HPLC mobile phase spiked with 4 ng/20 µl internal standard) and the samples were centrifuged again at 14000 rpm. The supernatant (20 µl) was injected onto the HPLC system.

II. HPLC Method

The mobile phase contained 0.1 M citric acid, 5.6 mM octane-1-sulphonic acid (Sigma), 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Cork) and 11.1% (v/v) methanol (Alkem/Reagecon) and was adjusted to pH 2.8 using 4 N NaOH (Alkem/Reagecon). The HPLC system consisted of a CBM-20A system controller, a EC3000 Recipe amperometric detector, a LC-20AD XR pump, a CTO-20A column oven at 30°C, a SIL-20AC XR autosampler, and a Prominence DGU-20A3 degasser. A reverse-phase column (Kinetex 2.6u C18 100A 100 mm X 4.6 mm, Phenomenex) was employed in the separation using a flow rate of 0.9 mL/min. Each neurotransmitter and metabolite (serotonin and 5-hydroxyindoleacetic acid) were identified through their characteristic retention times and their concentration was determined using the ratios of peak heights of analyte versus internal standard provided by the LabSolutions software (Shimadzu). Results were expressed as μg of neurotransmitter (or metabolite) per mL of supernatant.

3.3.5 Amino Acid Analysis for Tryptophan Detection

As an adjunct method of screening for serotonin production using the ELISA kit, tryptophan analysis was also carried out using amino acid analysis in order to determine whether strains in the cultures were using the supplied tryptophan to synthesise serotonin. It was hypothesised that a corresponding tryptophan reduction would be detected accompanied by an increase in serotonin. The cultures from the first ELISA were also prepared for amino acid analysis during the incubation step of the ELISA protocol. Samples were deproteinised by mixing equal volumes of 24% (w/v) trichloroacetic acid and culture and allowed to stand for 10 minutes before undergoing centrifugation at 14,000 rpm for 10 minutes. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125 nm/ml. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column. The analyser uses an ion exchange column with post-

column on-line reactor derivatisation with ninhydrin (McDermott *et al.*, 2016). In the JLC-500/V amino acid analyser, specified amounts of sample, set in vials, were introduced into a column packed with cation exchange resin and by flowing buffers at different pHs through the column, each amino acid component of the sample was separated and eluted from the column. The ninhydrin reagent was then added to the eluent, and when heated, it reacted with amino acids creating pigments, which are measured by the flow colorimetric method. The signals detected here were sent to the data processing unit to analyse the amino acids qualitatively and quantitatively.

3.3.6 GABA Screening using Amino Acid Analysis

As an additional screen for psychobiotic potential, putative lactobacilli from the MyNewGut biobank were also screened for GABA production using the previously described method of amino acid analysis (Patterson *et al.*, 2019). *Lactobacillus brevis* DPC6108, which is a highly efficient GABA producer capable of converting up to 90% of monosodium glutamate to GABA (Barrett *et al.*, 2012), was selected as a positive control. This strain, along with putative lactobacilli from the MyNewGut biobank, was streaked on MRS plates and grown anaerobically for 48 hours. Single colonies were then selected and inoculated in 10 ml of MRS broth for 24 hours. 100 µl from each culture was inoculated into 10 ml fresh standard broth as above in order to stabilise the growth of the strains. The cultures were also checked under the microscope for purity at this time. After a further 24 hours, 100 µl from each culture was inoculated in MRS containing 3% (w/v) monosodium glutamate, the substrate for GABA, at 37°C for 24 hours. The cultures were centrifuged at 4,000 rpm for 20 minutes and prepared for amino acid analysis as described above. GABA was then quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column. The analyser used an ion exchange column with post-column on-line reactor derivatisation with ninhydrin (McDermott *et al.*, 2016). MRS and MRS

containing 3% MSG broths which did not contain any strain were used as blanks for the analysis.

3.3.7 Antimicrobial Activity

There are many techniques for estimating antimicrobial activity, however two separate tests were used in this case. Putative lactobacilli were tested for antimicrobial activity initially using the deferred antagonism test, a method which assesses the ability of one bacterium to inhibit the growth of another through the production of antimicrobial compounds or through competition for nutrients. The extent of inhibition can be measured quantitatively, through the size of the zone of clearing around the inhibitor-producing strain, and qualitatively, by assessing the clarity of the inhibition zone (Moran *et al.*, 2016). Antimicrobial activity was further assessed with the cell-free supernatant from the potential bacteriocin-producing isolates using the agar well diffusion method, to determine whether an antimicrobial substance was secreted into the growth medium. *Lactobacillus bulgaricus* was used as the sensitive indicator strain for both tests. Briefly, the isolates to be tested and the indicator strain were streaked on MRS and incubated anaerobically at 37°C for 48 hours. Single colonies were then inoculated into MRS broth and allowed to grow anaerobically at 37°C for 24 hours. Spot bioassays were then carried out on the isolates by spotting 3 µl of each culture onto MRS plates; the plates were then incubated for 24-48 hours anaerobically at 37°C before being overlaid with 10 ml of sloppy (0.75%) MRS agar seeded with a 1% inoculum of the overnight indicator culture. The plates were incubated anaerobically overnight and then checked for clear zones around the spotted colonies; any zones which appeared hazy could possibly be the result of acid production.

Strains which produced zones in the deferred antagonism assay were then further characterised using the well diffusion assay. Briefly, cell-free supernatants were prepared from 10 ml overnight cultures of the putative antimicrobial producers by centrifugation (Sorvall Legend RT). 20 ml of 1% MRS agar seeded with a 1% inoculum of the indicator culture was rapidly dispensed into sterile petri dishes and

allowed to set. Once set, a sterile glass Pasteur pipette was used to bore wells in the agar, leaving enough space between holes to view any zones which may be produced. 50 µl of each supernatant was added to the wells. The plates were carefully placed in the anaerobic hood, ensuring not to spill any supernatant from the wells, and incubated anaerobically for 24 hours before checking for zones of clearing (O'Sullivan *et al.*, 2018).

Modifications were also made to the well diffusion assay to establish the heat stability of bacteriocins produced. Cell-free supernatants were heat-treated for 20 minutes at 80°C and at 100°C before performing a well diffusion assay as previously described. A further well diffusion assay was carried out with the addition of Proteinase K (Sigma) to investigate whether the antimicrobials produced were proteinaceous in nature. The assay was carried out as described above, with the addition of 3 µl of Proteinase K to the edge of each well. This test was performed to rule out acid production being the cause of any observed zones of clearing.

3.3.8 Identifying Isolates of Interest

In order to identify the isolates of interest, 16S rRNA sequencing was carried out with Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom). Genomic DNA was extracted from the strains (grown overnight in MRS broth at 37°C) using the GenElute Bacterial Genomic DNA Kit according to the manufacturer's instructions (Sigma-Aldrich Ireland Limited, Vale Road, Arklow, Co. Wicklow, Ireland). Briefly, lysozyme was first prepared by adding 3 ml of water to 135 mg of lysozyme. Proteinase K supplied in the kit was also reconstituted with 500 µl of water. 1.5 ml of an overnight bacterial broth culture was pelleted by centrifugation for two minutes at 12000-16000 rpm. The culture medium was removed; the pellet was then thoroughly resuspended in 200 µl of Lysozyme Solution and then incubated at 37°C for 45 minutes. 20 µl of RNase A solution was added before a further incubation step for two minutes at room temperature. To lyse the cells, 20 µl of Proteinase K solution was added, followed by 200 µl of Lysis Solution C. The solution was mixed by vortex before incubating at 55°C for 10 minutes. 200 µl of

ethanol (95-100%) was added to the lysate and vortexed for 5-10 seconds before being added to the binding columns, which were previously prepared with Column Preparation Solution. The tubes were centrifuged at 10000 rpm for one minute and the binding column was placed in a new collection tube. 500 µl of Wash Solution 1 was added to the columns and centrifuged at 10000 rpm for one minute. The collection tube was discarded and 500 µl of Wash Solution was added to the column before centrifugation for three minutes at maximum speed. 200 µl of Elution Solution was pipetted directly onto the centre of the column, incubated for five minutes at room temperature and centrifuged at 10000 rpm for one minute to elute the DNA.

For 16S rRNA sequencing, universal primers (Merck) 27F 5-GAGAGTTTGATCCTGGGTCAG-3 and 1492R 5-TACCTTGTTACGACTT-3 were used for PCR reactions to initially identify the strains of interest. The PCR reaction consisted of 2 µl DNA, 1 µl 27F primer, 1 µl 1492R primer, 25 µl Biomix and 21 µl of water to give a final reaction volume of 50 µl. PCR amplification was then carried out as follows: run conditions: initial denaturation: 94°C × 2 min, cycling conditions: 35 cycles of 94°C × 20 s, 52°C × 30 s, 72°C × 1 min, final extension: 72°C × 10 min. Quantification of the extracted DNA and PCR products was conducted using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). The PCR products were then cleaned up using the GenElute PCR Clean-Up Kit according to manufacturer's instructions (Sigma-Aldrich Ireland Limited, Vale Road, Arklow, Co. Wicklow, Ireland). Briefly, Wash Solution was prepared with the addition of 48 ml of 100% ethanol. The columns were inserted into collection tubes, followed by the addition of 500 µl of Column Preparation Solution. The columns were then centrifuged at 12000 rpm for 30-60 seconds. Five volumes of Binding Solution (250 µl) were added to one volume of the PCR reaction (50 µl), mixed, and transferred to the binding column before undergoing centrifugation at maximum speed for one minute. The eluate was discarded and 500 µl of diluted Wash Solution was added to the column before a further centrifugation step was carried out. 50 µl of Eluate Solution was added to the center of each column and incubated at room temperature for one minute. The DNA was eluted by centrifuging at maximum speed for one minute. Once

sequencing was completed by Genewiz, Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) was used to compare the sequencing data of the isolates to existing genomic data.

3.3.9 Whole Genome Sequencing

The three strains of interest were streaked out on MRS plates before a single colony was taken from each plate and mixed in 200 µl sterile buffer (for example 1xPBS). 100 µl of this buffer was inoculated in 25-50 mL of sterile MRS + cysteine broth while the remaining 100 µl was streaked on MRS agar plates to assess the purity of the culture. The cultures were incubated until the upper exponential phase (for ~24 hours) before being pelleted in a centrifuge (10 minutes at 500 x g). The liquid was then discarded, taking care not to disturb the pellet. The cell pellet was weighed to ensure that a wet weight of 300-600 mg was achieved. The agar plate prepared at the beginning of the protocol was checked once the colonies had grown for contamination. Finally, the pelleted cells were fully resuspended in 500 µL of the cryopreservant liquid from the barcoded bead tube supplied by MicrobesNG before being transferred into the barcoded bead tube which was then sealed and shipped to Microbes NG for enhanced genome analysis.

Lactobacillus paracasei 231 was sequenced using standard Illumina whole genome sequencing as it was not possible for MicrobesNG to extract DNA from the pelleted cells. Plated cultures of each isolate were inoculated into a cryoperservative (Microbank™, Pro-Lab Diagnostics UK, United Kingdom). 10-20 µl of the suspension were lysed with 120 µL of TE buffer containing lysozyme (final concentration 0.1 mg/mL) and RNase A (ITW Reagents, Barcelona, Spain) (final concentration 0.1 mg/mL), incubated for 25 minutes at 37°C. Proteinase K (VWR Chemicals, Ohio, USA) (final concentration 0.1mg/mL) and SDS (Sigma-Aldrich, Missouri, USA) (final concentration 0.5% v/v) were added and incubated for five minutes at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer (Qiagen, Germany). DNA was quantified with the Quant-iT dsDNA HS kit

(ThermoFisher Scientific) assay in an Eppendorf AF2200 plate reader (Eppendof UK Ltd, United Kingdom). Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: 2 ng of DNA were used as input, and PCR elongation time was increased to one minute from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced with the Illumina HiSeq using a 250bp paired end protocol.

Lactobacillus paracasei 110 underwent enhanced whole genome sequencing as DNA extraction from the pelleted cells was successful. Broth cultures of each isolate were pelleted out and the pellet was resuspended in the cryoperservative of a Microbank™ (Pro-Lab Diagnostics UK, United Kingdom) tube and stored in the tube. Approximately $2x10^9$ cells were used for high molecular weight DNA extraction using Nanobind CCB Big DNA Kit (Circulomics, Maryland, USA). DNA was quantified with the Qubit dsDNA HS assay in a Qubit 3.0 (Invitrogen) Eppendof UK Ltd, United Kingdom). Long read genomic DNA libraries were prepared with Oxford Nanopore SQK-RBK004 kit and/or SQK-LSK109 kit with Native Barcoding EXP-NBD104/114 (ONT, United Kingdom) using 400-500 ng of HMW DNA. 12-24 barcoded samples were pooled together into a single sequencing library and loaded in a FLO-MIN106 (R.9.4 or R.9.4.1) flow cell in a GridION (ONT, United Kingdom).

Illumina reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger, Lohse and Usadel, 2014). Genome assembly was performed using Unicycler v0.4.0 (Wick *et al.*, 2017). The contigs were annotated using Prokka 1.11 (Seemann, 2014). Metagenomic analysis of the whole genome sequencing results was carried out using Artemis (Release 18.1.0), a genome browser and annotation tool, to visualise the genome and search for the relevant operons (Carver *et al.*, 2012). Antimicrobial activity was assessed using the online server BAGEL4 (de Jong *et al.*, 2006), a web-based bacteriocin genome mining tool, to determine whether bacteriocin genes were present within the

sequenced strains. AntiSMASH (version 5.0) was also used to search for secondary metabolites as it allows the rapid genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial genomes (Blin *et al.*, 2019).

3.4 Results

3.4.1 Serotonin ELISA Screening

A number of methods were tested to determine an effective means of screening for microbial serotonin production, including using a commercially available ELISA kit from Eagle Biosciences. First, a number of strains from the literature were selected as positive controls including *Lactococcus lactis* subsp. *cremoris* 1363, *Lactococcus lactis* subsp. *cremoris* 1614, *Lactococcus lactis* subsp. *cremoris* 712, *Streptococcus thermophilus* 5655, *Streptococcus thermophilus* 5656, *Lactobacillus plantarum* 891, *Lactobacillus plantarum* 6805 (Özoğul *et al.*, 2012). These strains were tested initially, along with human gut-derived strains, to determine whether ELISA was a suitable method for serotonin screening. Strains were grown in standard broth as well as tryptophan supplemented broth to stimulate serotonin production. High background levels of serotonin were detected in all samples, including the blanks, highlighting that caution is required when interpreting results from this assay (Figure 1). Out of the strains tested, *Lactococcus lactis* subsp. *cremoris* MG1363 and *Lactococcus lactis* subsp. *cremoris* 1614 were the only positive controls tested which had higher levels of serotonin than their respective blanks, indicating that they could be serotonin producers. An increase in detected serotonin of 45.84% in standard broth as well as 45.88% in tryptophan supplemented broth was calculated for *Lactococcus lactis* subsp. *cremoris* MG1363, while *Lactococcus lactis* subsp. *cremoris* 1614 had increases of 3.75% and 56.53% for standard versus tryptophan supplemented broth respectively. Of the MyNewGut strains tested, two strains had increased levels of serotonin compared to the blank with an increase of 8.96% in standard broth and 2.57% in tryptophan supplemented broth for putative *lactobacillus* 110, and an increase of 24.7% in tryptophan supplemented broth for

putative *lactobacillus* 231 indicating that these strains may be potential serotonin producers. These strains were therefore carried forward for further analysis and characterisation.

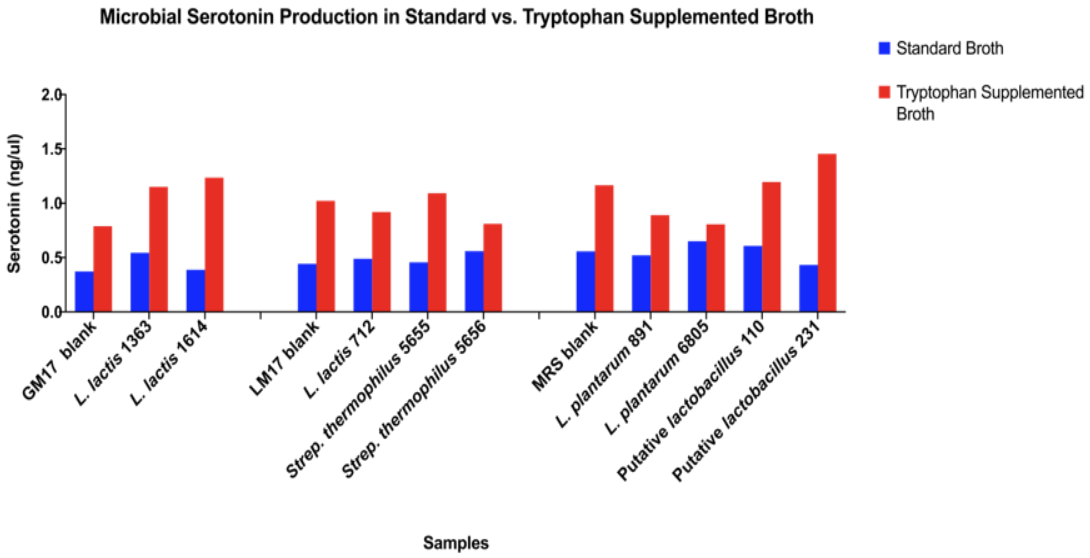


Figure 1: ELISA results for positive controls and for MyNewGut biobank isolates tested in both standard (blue) and tryptophan supplemented (red) broth. Serotonin concentrations are measured in ng/ml.

An additional ELISA was carried out with the aim of mitigating the high levels of serotonin detected in the blank broths in order to improve the accuracy of this assay. For the second ELISA, *Lactococcus lactis* subsp. *cremoris* MG1363 and 1614 strains were carried forward as positive controls and additional strains from the MyNewGut biobank were tested in duplicate. A number of alterations were implemented for this ELISA, including using a lower concentration of tryptophan broth (0.1% instead of 0.75%) to reduce the background levels. Furthermore, the standards and controls were prepared with broth instead of the dilution buffer from the kit, and water was also tested as a negative control to test for kit contamination. The results from this ELISA were determined to be inaccurate because several of the samples had readings above the 4 parameter logistic

standard curve so it was impossible to determine the percentage increase. Table 2 outlines the percentage increases for all strains tested in both ELISA assays.

Table 2: Percentage increases of serotonin detected in both standard and tryptophan supplemented broth for all strains tested by the ELISA method.

Identity	Serotonin Production (ng/ul)	% Increase in Standard Broth	% Increase in Tryptophan-Supplemented Broth
***Lactobacillus paracasei* 110**	1.196	8.96	2.57
Lactobacillus plantarum	0.194	NA	NA
***Lactobacillus paracasei* 231**	1.454	-22.4	24.7
Lactobacillus rhamnosus	2.12	-92.6	-64.23
Lactobacillus mucosae	3.59	NA (>curve)	NA (>curve)
Lactobacillus rhamnosus	1.73	-99.7	-70.8
***L. lactis* 1363**	1.151	45.84	45.88
***L. lactis* 1614**	1.235	3.75	56.53
***L. lactis* 712**	0.919	10.38	-10.17
***Strep. thermophilus* 5655**	1.092	2.7	6.74
***Strep. thermophilus* 5656**	0.811	26.19	-20.72
***L. plantarum* 891**	0.891	-6.6	-23.58
***L. plantarum* 6805**	0.806	16.66	-30.87

3.4.2 Serotonin HPLC Analysis

HPLC analysis was also utilised as an extra validation method to compare with the results obtained from the ELISA assay. First, blank broths were spiked with the serotonin standard in order to confirm that serotonin was detectable in broth using

this method. The centrifugation method which involved freezing the supernatant immediately for HPLC analysis was found to result in increased serotonin detection compared to the centrifugation method where the pellet was resuspended in water before centrifugation and freezing for subsequent HPLC analysis. The positive controls *Lactococcus lactis* subsp. *cremoris* MG1363 and *Lactococcus lactis* subsp. *cremoris* 1614 were tested along with the MyNewGut strains of interest identified from the ELISA assay. Blank broths were also used as negative controls. As well as measuring serotonin levels, this HPLC method also detected 5-hydroxyindoleacetic acid, which is the main metabolite of serotonin and is used to determine serotonin levels in the body through chemical analysis of urine samples. Therefore, measuring this metabolite could provide further insight into the serotonin metabolism capabilities of the strains tested here compared to the ELISA method.

Similarly to the ELISA method, HPLC analysis revealed a strong effect of tryptophan supplementation in the media as it was not possible to get a reliable reading of serotonin concentration for samples grown in tryptophan-supplemented broth, possibly due to interfering peaks which made it impossible to integrate a serotonin peak (Table 3). Furthermore, high background levels of serotonin or 5-hydroxyindoleacetic acid were detected in all samples, including the blanks, highlighting that caution is required when interpreting results from this HPLC screen. Nevertheless, it was possible to integrate a serotonin peak for the putative positive controls, and a serotonin concentration of 3.4 µg/ml and 12.7 µg/ml were detected for the positive controls *Lactococcus lactis* subsp*. cremoris* 1614 and *Lactococcus lactis* subsp*. cremoris* MG1363 respectively, when grown in GM17 broth. Therefore, this HPLC assay provided similar overall findings to the serotonin ELISA, although the individual concentrations detected using this method were different. Similarly, 5-hydroxyindoleacetic acid was also detected in all samples, including the blanks, albeit at lower levels than in the strains tested. Interestingly, tryptophan supplementation consistently resulted in lower serotonin, when detectable, and higher 5-hydroxyindoleacetic acid in the inoculated samples compared to the blanks. This effect is possibly a result of tryptophan supplementation shifting bacterial metabolism away from serotonin and towards

other pathways, leading to increased bacterial indole production which the HPLC method was unable to separate from 5-hydroxyindoleacetic acid. The 5-hydroxyindoleacetic acid/serotonin ratio presented in Table 3 is a measure of serotonin turnover and can be interpreted here as indicating increased breakdown of serotonin following tryptophan supplementation.

Table 3: Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentration (μg/ml) detected in potential serotonin producers identified from the literature and lactobacilli from the MyNewGut biobank compared to blank tryptophan-supplemented broth as measured using HPLC.

Sample	5-HIAA (μg/ml)	5-HT (μg/ml)	5-HIAA/5-HT (μg/ml)
110 MRS	4.057	NA	NA
110 Tryptophan	8.914	NA	NA
231 MRS	2.723	NA	NA
231 Tryptophan	9.977	NA	NA
1614 GM17	5.509	3.380	3.585
1614 Tryptophan	39.611	0.148	588.152
1363 GM17	9.762	12.748	1.685
1363 Tryptophan	83.589	NA	NA
106 MRS	4.591	NA	NA
106 Tryptophan	61.915	NA	NA
148 MRS	5.718	NA	NA
148 Tryptophan	60.080	NA	NA
MRS blank	3.199	NA	NA
MRS + tryptophan blank	15.392	NA	NA
GM17 blank	6.416	6.788	2.079
GM17 + tryptophan blank	40.197	0.307	287.622

3.4.3 Tryptophan Amino Acid Analysis

As an adjunct method of screening for serotonin production using the ELISA kit, tryptophan analysis was also carried out using amino acid analysis to determine whether strains in the cultures were using the supplied tryptophan to synthesise serotonin. The cultures from the first ELISA screen, consisting of potential positive producers from the literature as well as putative lactobacilli selected from the MyNewGut biobank, were also prepared for amino acid analysis during the ELISA incubation step. Increased concentrations of tryptophan were detected in all inoculated samples compared to the tryptophan-supplemented blanks (Table 4). This could indicate tryptophan synthesis in the strains or potentially other metabolites derived from tryptophan metabolism, such as indole or tryptamine, which the amino acid analysis method was unable to distinguish from tryptophan.

Table 4: Tryptophan concentration (µg/ml) detected in potential serotonin producers identified from the literature and lactobacilli from the MyNewGut biobank compared to blank tryptophan-supplemented broth.

Sample information	Concentration µg/ml
GM17 + 0.75% tryptophan	17828.345
Lactococcus lactis subsp*. cremoris* 1363	20770.140
Lactococcus lactis subsp*. cremoris* 1614	18767.6775
LM17 + 0.75% tryptophan	19292.128
Lactococcus lactis 712	19928.4105
Streptococcus thermophilus 5655	19948.5395
Streptococcus thermophilus 5656	20488.369
MRS + 0.75% tryptophan	18429.085
Lactobacillus plantarum 891	20488.369
Lactobacillus plantarum 6805	20867.835
Lactobacillus paracasei (MNG 110)	18992.381
Lactobacillus casei (MNG 231)	18498.997

3.4.4 GABA Amino Acid Analysis

In this study, a series of amino acid analysis assays were carried out to screen for microbial GABA production. In the first screen, 34 human-derived *Lactobacillus* strains obtained from the MyNewGut project were assessed for the ability to generate GABA from monosodium glutamate. As several putative lactobacilli were isolated from each MyNewGut participant, care was taken to select strains from different participants at different time points to minimise repetition and 17 strains were selected from individuals born by Caesarean section while 17 were isolated from those born vaginally. *Lactobacillus brevis* DPC6108, which is a highly efficient GABA producer, was used as a positive control, and blank MRS broth, blank monosodium glutamate-supplemented MRS broth as well as both *Lactobacillus brevis* DPC6108 and a test strain grown in MRS broth without monosodium glutamate were also included as negative controls. From this initial screen, *Lactobacillus brevis* DPC6108 had the highest GABA production while a *Lactobacillus plantarum* strain from the MyNewGut biobank also had high GABA levels detected and no GABA production was observed in the negative controls, as expected. This first screen was later duplicated, and similar results were observed, with *Lactobacillus brevis* DPC6108 again having the highest production and the *Lactobacillus plantarum* strain also identified as a potential GABA producer. A third screen was carried out to again test *Lactobacillus plantarum* and *Lactobacillus brevis* DPC6108. Fermented food isolates which were previously isolated from sources such as kimchi, goats milk kefir and buffalo cheese, were also tested in this assay, although minimal GABA concentrations were detected from these strains. Table 5 outlines the percentage GABA conversion rates observed as an average over the three GABA screens. In conclusion, one human-derived *Lactobacillus plantarum* strain was identified as a GABA producer with an average conversion rate of 38.4% and was therefore carried forward for further downstream analysis and identification. However, *Lactobacillus brevis* DPC6108 was the most efficient of the strains tested with an average conversion rate of 45.35%.

Table 5: GABA amino acid analysis results presented as percentage monosodium glutamate to GABA conversion rates calculated as an average over all available screens.

Strain	Source	% Conversion
***Lactobacillus brevis* DPC 6108**	Positive Control	45.35%
Lactobacillus plantarum	MNG - vaginal delivery	38.40%
Lactobacillus paracasei	MNG - vaginal delivery	2.58%
Putative *lactobacillus*	MNG - vaginal delivery	3.07%
Putative *lactobacillus*	MNG - vaginal delivery	3.70%
Putative *lactobacillus*	MNG - vaginal delivery	3.22%
Lactobacillus rhamnosus	MNG - vaginal delivery	2.91%
Putative *lactobacillus*	MNG - vaginal delivery	1.96%
Putative *lactobacillus*	MNG - vaginal delivery	3.25%
Putative *lactobacillus*	MNG - vaginal delivery	3.14%
Lactobacillus mucosae	MNG - vaginal delivery	2.47%
Lactobacillus rhamnosus	MNG - vaginal delivery	9.17%
Putative *lactobacillus*	MNG - vaginal delivery	3.58%
Putative *lactobacillus*	MNG - vaginal delivery	3.76%
Putative *lactobacillus*	MNG - vaginal delivery	3.45%
Putative *lactobacillus*	MNG - vaginal delivery	3.01%
Putative *lactobacillus*	MNG - vaginal delivery	2.15%
Putative *lactobacillus*	MNG - vaginal delivery	3.19%
Lactobacillus plantarum	MNG - C section delivery	6.47%
Lactobacillus paracasei	MNG - C section delivery	2.40%
Putative *lactobacillus*	MNG - C section delivery	4.77%
Putative *lactobacillus*	MNG - C section delivery	3.20%
Putative *lactobacillus*	MNG - C section delivery	2.39%
Putative *lactobacillus*	MNG - C section delivery	2.66%
Putative *lactobacillus*	MNG - C section delivery	1.91%

Putative *lactobacillus*	MNG - C section delivery	2.98%
Putative *lactobacillus*	MNG - C section delivery	2.29%
Putative *lactobacillus*	MNG - C section delivery	2.41%
Putative *lactobacillus*	MNG - C section delivery	2.92%
Putative *lactobacillus*	MNG - C section delivery	2.42%
Putative *lactobacillus*	MNG - C section delivery	2.33%
Putative *lactobacillus*	MNG - C section delivery	2.51%
Putative *lactobacillus*	MNG - C section delivery	2.10%
Putative *lactobacillus*	MNG - C section delivery	2.85%
Putative *lactobacillus*	MNG - C section delivery	2.58%
Lactobacillus paracasei	Fermented food isolate	4.16%
Lactobacillus paracasei	Fermented food isolate	4.66%
Lactobacillus paracasei	Fermented food isolate	4.44%
Lactobacillus sunkii	Fermented food isolate	5.16%
Lactobacillus sunkii	Fermented food isolate	2.43%

3.4.5 Screening for Antimicrobial Activity

A series of assays for antimicrobial activity were carried out on both the MyNewGut isolates (n=26) and the fermented food isolates (n=46) using the deferred antagonism assay. Any isolates which produced a zone in this assay were carried forward and antimicrobial activity was further assessed using the agar well diffusion method. All MyNewGut isolates tested produced zones in the deferred antagonism assay, however the zones were cloudy which indicated acid production (Figure 2A). All but two fermented food isolates also produced zones in this assay (Figure 2B) and were consequently further investigated using well diffusion. None of the fermented food isolates produced a zone in the well diffusion assay, while a zone was visible for one MyNewGut isolate using this assay, namely the *Lactobacillus paracasei* strain which showed potential serotonin production (Figure 2C). Although the observed zone was small, further well diffusion assays were carried out on this

strain including heat treating the supernatant at 99°C and 80°C. as well as using Proteinase K. To investigate whether the antimicrobials produced were proteinaceous in nature, the cell-free supernatant of all antimicrobial-producing isolates were incubated with Proteinase K. No zones were observed when the supernatant was heat treated although small zones were visible when the supernatant was at room temperature (Figure 2D). In conclusion, these results indicate weak antimicrobial activity, and the strain was carried forward for identification by Sanger sequencing.

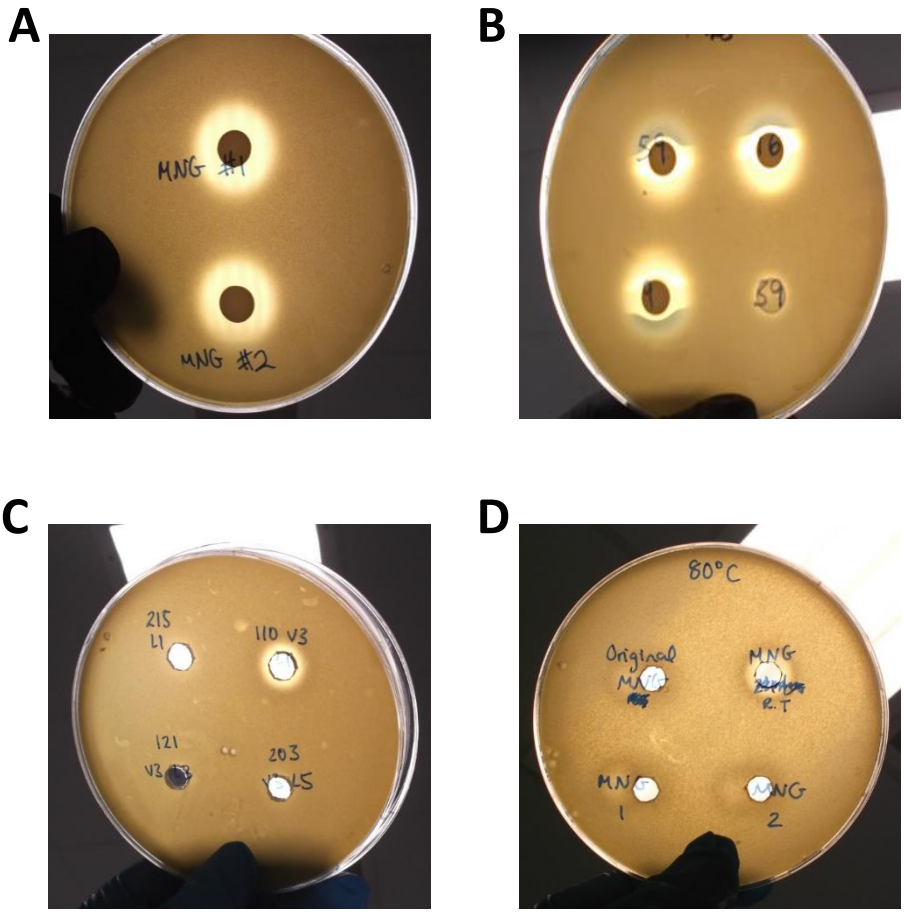


Figure 2: Selection of images from bacteriocin screens showing deferred antagonism assay zones produced by MyNewGut isolates (A) and fermented food isolates (B), well diffusion assay results (C), and follow-up heat treated well diffusion assays with the *Lactobacillus paracasei* strain of interest (D).

3.4.6 Sanger Sequencing

All strains of interest identified from the GABA (n = 5), serotonin (n = 3) and bacteriocin (n = 1) screens were further characterised by Sanger sequencing to determine their identity. Seven MyNewGut isolates and two fermented food isolates were identified as *Lactobacillus paracasei* (n = 3), *Lactobacillus sunkii* (n = 1), *Lactobacillus plantarum* (n = 2), *Lactobacillus rhamnosus* (n = 2), and *Lactobacillus mucosae* (n = 1). This analysis identified the strains and confirmed that they were all lactobacilli and not potentially pathogenic strains.

3.4.7 Whole Genome Sequencing

Two potential serotonin producing strains were carried forward for whole genome sequencing to further investigate their potential as serotonin producers and to interrogate the accuracy of the ELISA and HPLC screening methods. Whole genome sequencing confirmed the most frequent genus to be *Lactobacillus* for both 231 (99.11%) and 110 (97.62%), while the most frequent species was identified as *paracasei* for both 231 (71.29%) and 110 (54.34%). *L. paracasei* 231 had a total of 89 contigs, a total length of 2651999 nucleotides and a GC content of 46.56% while *L. paracasei* 110 contained 126 contigs, had a total length of 2963247 nucleotides and a GC content of 46.23%.

Artemis was employed to search for the *trp* operon in the genomes of both strains to determine whether they contained the genes responsible for tryptophan metabolism and serotonin synthesis. The *Lactococcus lactis* subsp*. cremoris* MG1363 genome (Genbank accession number AM406671) was used as a reference strain and was found to contain the entire *trp* operon, consisting of the following genes: *trpE*, encoding anthranilate synthase component 1; *trpG*, encoding anthranilate synthase component 2, both of which can be involved in the first step of tryptophan synthesis; *trpD*, encoding anthranilate phosphoribosyltransferase which is involved in step two of tryptophan synthesis; *trpF*, encoding phosphoribosylanthranilate isomerase involved in the third step of tryptophan

synthesis; *trpC*, encoding indole-3-glycerol phosphate synthase involved in step four of tryptophan synthesis; *trpA*, encoding tryptophan synthase alpha chain involved in step five of tryptophan synthesis; and *trpB*, encoding tryptophan synthase beta chain which catalyses the final step of tryptophan synthesis. In comparison, the genome of *L. paracasei* 110 was found to contain the following genes of the tryptophan pathway: *trpA*, which encodes the tryptophan synthase alpha chain involved in the final step of tryptophan biosynthesis; *trpB*, which encodes the tryptophan synthase beta chain which also catalyses the final step in tryptophan biosynthesis; *trpC* which encodes indole-3-glycerol phosphate synthase, involved in the fourth step of tryptophan biosynthesis; *trpD* which encodes anthranilate phosphoribosyltransferase which catalyses the second step of tryptophan biosynthesis; *trpF* which encodes N-(5'-phosphoribosyl) anthranilate isomerase involved in step 3 of tryptophan biosynthesis; and *trpS2*, tryptophan-tRNA ligase 2 (Figure 3). In contrast, Artemis revealed that *L. paracasei* 231 only contained *trpA* at the nucleotide position 2651049-2651327 and *trpS2* at the position 905720-906736. Taken together, these results indicate that *L. paracasei* 110 is capable of tryptophan synthesis because it contains the *trp* operon and of serotonin synthesis because it contains the gene which encodes for tryptophan synthase (O'Mahony *et al.*, 2015; Gao *et al.*, 2020). While *L. paracasei* 231 does contain the gene *trpA* it does not contain the genes to encode the remaining enzymes involved in tryptophan synthesis. Therefore, it is likely that this strain is capable of synthesising indole as this gene contributes to tryptophan-independent indole biosynthesis and that tryptophan supplementation is leading to increased bacterial indole production which the HPLC method is unable to separate from 5-hydroxyindoleacetic acid.

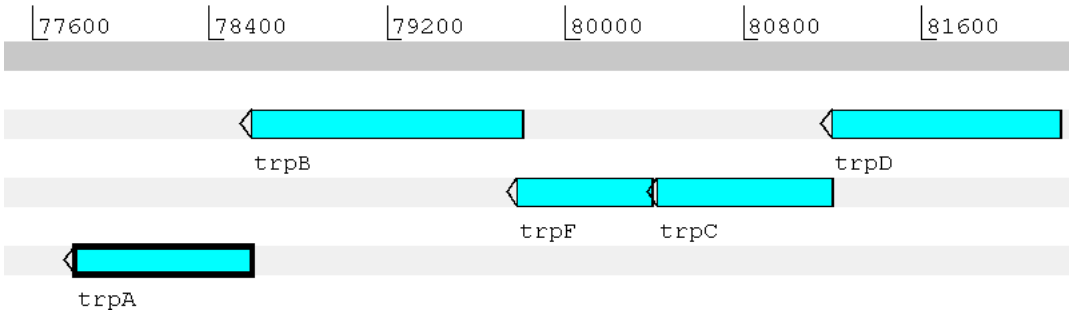


Figure 3: The *trp* operon in *L. paracasei* 110 as visualised using Artemis. The *trp* operon contains the genes *trpA, trpB, trpF, trpC and trpD*.

As well as searching for the *trp* operon within the genomes of the strains of interest, Artemis was also utilised to determine whether the strains contained genes which encoded enzymes involved in other pathways of tryptophan metabolism apart from serotonin synthesis, namely the kynurenine pathway, indole production and tryptamine production. The genomes were checked for the presence of genes which encode enzymes involved in the kynurenine pathway through the production of kynurenine and quinolinate. *KynA* and *KynB*, which encode the enzymes tryptophan 2,3-dioxygenase and kynurenine formamidase respectively, were not found to be present in either *L. paracasei* 110 or *L. paracasei* 231, indicating that these strains are not involved in kynurenine production. The genes *KMO* and *Kyn*, which encode the enzymes kynurenine 3-monooxygenase and kynurinase for quinolinate production were also not found in either of the genomes of interest, indicating that these strains do not play a role in quinolinate production and are not involved in the kynurenine pathway. The role of these strains in tryptophan metabolism was also assessed by searching for genes identified in playing a role in tryptophan degradation to indole and indole derivatives such as indole-3-acetic acid, indole-3-propionic acid, and tryptamine (Kaur, Bose and Mande, 2019). The gene *tnaA*, which encodes the enzyme tryptophanase which is responsible for indole synthesis from tryptophan, was not found to be present in either of the genomes of interest (Kaur, Bose and Mande, 2019; Gao *et al.*, 2020). However, the gene *trpA* which contributes to tryptophan-independent indole

biosynthesis is present in both strains so indole production through this gene cannot be ruled out, particularly for *L. paracasei* 231 which does not contain *trpB*, the enzyme which catalyses the final step in tryptophan biosynthesis. Similarly, the genes involved in indole-3-acetic acid synthesis, *iaaM* and *ipdC*, which encode tryptophan-2-monooxygenase and indolepyruvate decarboxylase respectively, were not found in the genomes of these strains. The gene *fldABC*, which encodes the enzyme phenyllactate dehydratase for indole-3-propionic acid production, was not present in the genomes (Kaur, Bose and Mande, 2019). Finally, the ability of the strains to synthesise tryptamine was assessed by searching for the genes *TDC*, *CLOSPO-0283*, and *RUMGNA-01526* which encode tryptophan decarboxylase. Neither *L. paracasei* 110 nor *L. paracasei* 231 were found to possess the genes known to be involved in tryptamine synthesis, therefore it is unlikely that either of the strains of interest are capable of tryptamine production. Taken together, these results indicate that the strains described here do not play a role in the kynurenine pathway or are involved in the degradation of tryptophan to form the metabolites IAA, IPA or tryptamine. Therefore, it is unlikely that any of these metabolites were mistaken for serotonin or 5-hydroxyindoleacetic acid in the ELISA and HPLC analysis as mentioned previously. However, the gene *trpA* which contributes to tryptophan-independent indole biosynthesis is present in both of the strains so indole production through this gene cannot be ruled out.

Genomic analysis was also carried out on the strains to identify genes for probiotic characteristics such as antimicrobial activity, tolerance to stressors such as acid, bile, temperature stress and oxidative stress. BAGEL was utilised to identify genes with potential for antimicrobial activity. Two areas of interest were found within *L. paracasei* 110 and were identified as Enterolysin A, with a match of 56.82%, and LSEI 2386, with a match of 100%. Similarly, three areas of interest were uncovered in *L. paracasei* 231 and were subsequently identified as Carnocin CP52, LSEI 2163, and Enterolysin A. AntiSMASH analysis also revealed a bacteriocin gene in region 1.1 from nucleotides 2429536 to 2455162, with a total length of 25627 nucleotides. No matches were found for similar known gene clusters. Three bacteriocin genes were identified in the genome of *L. paracasei* 231 using antiSMASH. These were

located in region 20.1 with a total length of 5836 nucleotides; in region 38.1 with a total length of 7475 nucleotides; and in region 43.1 with a total length of 6326 nucleotides. Similarly, no matches were found to these bacteriocins (Figure 4).

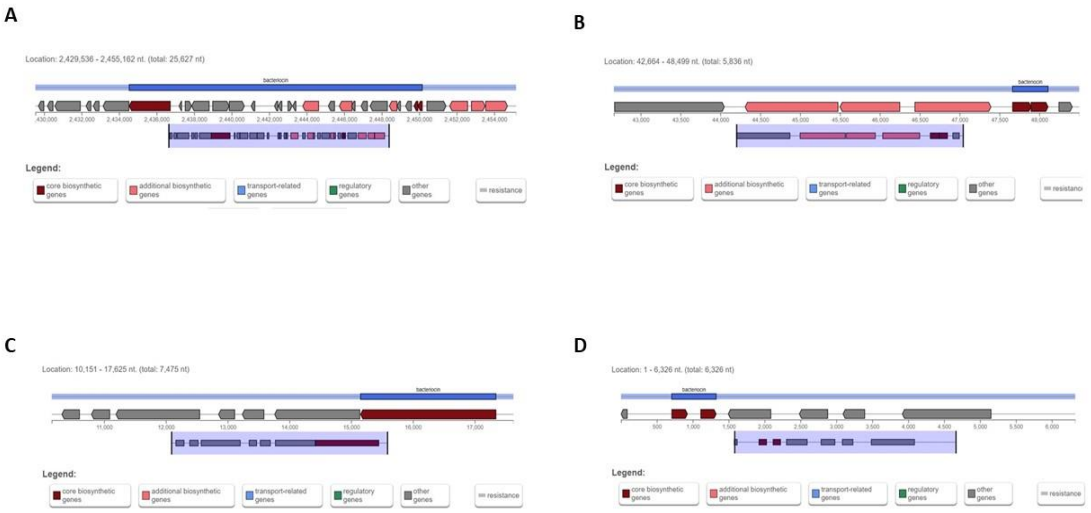


Figure 4: Bacteriocin operons from *L. paracasei* 110 (A) and from *L. paracasei* 231 (B-D) as identified using AntiSMASH.

Genes promoting probiotic characteristics were also searched for within the genomes of the strains of interest. These included *dltA* and *dltD* which confer tolerance to acid, an important feature for a candidate probiotic to possess in order for them to survive gastrointestinal transit and colonise the intestinal mucosa. Interestingly, both *L. paracasei* 110 and 231 possessed the genes *dltA* and *dltD* which indicates that they are capable of withstanding low pH. However, neither strain contained *gadB* which also confers acid tolerance. Similarly, the genes *clpL*, promoting acid and bile tolerance, and *bsh*, promoting tolerance to bile were not present. Adhesion ability of the strains was also investigated and both strains were found to contain the gene *fbp* which promotes adhesion ability, an additional characteristic which is important for candidate probiotics to possess.

3.5 Discussion

The importance of the commensal bacteria that colonise the gut for major aspects of physiology, particularly functions related to behaviour, brain structure and function is being increasingly recognised (Shanahan, 2013; Lyte, 2014). Research in this area has expanded rapidly in recent years, becoming one of the most studied areas of microbiology with significant potential to benefit clinical practice (Weinstock, 2012). Therefore, microbiome manipulation using probiotics or prebiotics to strengthen host-microbe symbiosis is likely to play a role in the future prevention and treatment of immune and psychiatric disorders. A probiotic which is capable of producing health-beneficial metabolites including neurotransmitters, vitamins and SCFAs will be particularly important, as these play a role in regulating distal organs including the brain. Furthermore, the gut microbiota plays a crucial role in modulating tryptophan metabolism, which impacts the balance between serotonin synthesis and tryptophan degradation, as well as producing neuroactive metabolites which influence both CNS and ENS functions (Gao *et al.*, 2020). Gut-derived serotonin plays a role in regulating enteric motor and secretory reflexes (Gershon and Tack, 2007), platelet aggregation (Mercado *et al.*, 2013), blood pressure regulation (Watts *et al.*, 2012), immune responses (Baganz and Blakely, 2012), bone development (Chabbi-Achengli *et al.*, 2012; Yadav *et al.*, 2008), and cardiac function (Côté *et al.*, 2003), and disruption of peripheral serotonin is evident in several disease states, including irritable bowel syndrome (Stasi *et al.*, 2014), cardiovascular disease (Ramage and Villalón, 2008), and osteoporosis (Ducy and Karsenty, 2010). Similarly, GABA is also important for multiple diverse functions in its role as the major inhibitory neurotransmitter including neurotransmission, diuretic effects, anxiolytic effects (Hayakawa *et al.*, 2004; Linares *et al.*, 2016), the regulation of cardiovascular functions, and pain sensation (Mody *et al.*, 1994). Furthermore, an altered GABAergic profile has been linked to CNS disorders such as anxiety and depression (Cryan and Kaupmann, 2005), indicating that a psychobiotic with GABA producing capability has the potential to treat such disorders. Therefore, the development of a biobank of psychobiotics may improve the standard of living for large numbers of people by improving mood and stress. This study aimed to

screen gut derived microbiota isolated from healthy adult males for psychoactive metabolite production which is of relevance to stress-related disorders, specifically GABA and serotonin.

Screening gut-derived bacteria for serotonin synthesis is a relatively novel approach; therefore, a number of methods were tested to determine an effective means of screening for microbial serotonin production, including using a commercially available ELISA kit. In order to test whether this method would be effective and to identify growth requirements, a number of strains from the literature were selected as positive controls including *Lactococcus lactis* subsp. *cremoris* MG1363, *Lactococcus lactis* subsp. *cremoris* 1614, *Lactococcus lactis* subsp. *cremoris* 712, *Streptococcus thermophilus* 5655, *Streptococcus thermophilus* 5656, *Lactobacillus plantarum* 891, and *Lactobacillus plantarum* 6805 (Özoğul *et al.*, 2012). Strains were grown in standard broth as well as tryptophan supplemented broth to stimulate serotonin production. High background levels of serotonin were detected in all samples, including the blanks, highlighting that caution is required when interpreting this assay. Out of the strains tested, *Lactococcus lactis* subsp. *cremoris* 1363 and *Lactococcus lactis* subsp. *cremoris* 1614 were the only positive controls tested which had higher levels of serotonin than their respective blanks, indicating that they could be serotonin producers. A serotonin increase of 45.84% in standard broth as well as 45.88% in tryptophan supplemented broth was calculated for *Lactococcus lactis* subsp. *cremoris* MG1363, while *Lactococcus lactis* subsp. *cremoris* 1614 had increases of 3.75% and 56.53% for standard versus tryptophan supplemented broth respectively. Of the MyNewGut strains tested, two strains had increased levels of serotonin compared to the blank with an increase of 8.96% in standard broth and 2.57% in tryptophan supplemented broth for *Lactobacillus paracasei* 110, and an increase of 24.7% in tryptophan supplemented broth for *Lactobacillus paracasei* 231 indicating that these strains may be potential serotonin producers. Tryptophan supplementation increased serotonin detection but variability masked improved measurement so should be used with caution.

An additional ELISA was carried out to attempt to optimise this method by reducing the high background readings in the blank broths. A number of alterations were

implemented for this ELISA, including using a lower concentration of tryptophan broth (0.1% instead of 0.75%) to try to reduce background levels. Furthermore, the standards and controls were prepared with broth instead of with the dilution buffer supplied in the kit, and water was also employed as a negative control to test for kit contamination. However, this ELISA was unsuccessful because the samples had readings above the standard curve, so it was impossible to determine the percentage increase, possibly due to preparing the standards and controls with broth instead of reagents from the kit. Although the results from the ELISA assay were promising in that they seemed to confirm the serotonin production capability of the positive controls from the literature as well as identifying two potential producers from the MyNewGut biobank, the results from this assay should be viewed as preliminary. Further work will be necessary to optimise this method, potentially by using minimal growth media to reduce the background readings in the broth. It is also necessary to include uninoculated growth media samples as blanks to monitor the background levels detected and provide a more accurate description of serotonin detection in the analysed samples.

The strains of interest from the ELISA screen were further analysed using HPLC as an adjunct method to assess the reliability of the ELISA assay. An additional advantage of using this HPLC method was the ability to detect 5-hydroxyindoleacetic acid, which is the main metabolite of serotonin and is used to determine serotonin levels in the body through chemical analysis of urine samples. Therefore, measuring this metabolite could provide further insight into the serotonin metabolism capabilities of the strains tested here. Similarly to the ELISA method, HPLC analysis revealed a strong effect of tryptophan supplementation in the media as it was not possible to get a reliable reading of serotonin concentration for samples grown in tryptophan-supplemented broth, possibly due to interfering peaks which made it impossible to integrate a serotonin peak. Furthermore, high background levels of serotonin or 5-hydroxyindoleacetic acid were detected in all samples, including the blanks, highlighting that caution is required when interpreting results from this HPLC screen. An important observation from both methods is the necessity of using blank broths to control for high background

interference. Nevertheless, it was possible to integrate a serotonin peak for the putative positive controls, and a serotonin concentration of 3.4 µg/ml and 12.7 µg/ml were detected for the positive controls *Lactococcus lactis* subsp*. cremoris* 1614 and *Lactococcus lactis* subsp*. cremoris* MG1363 respectively, when grown in GM17 broth. Therefore, this HPLC assay provided similar overall findings to the serotonin ELISA, although the concentrations detected using this method differed. Similarly, 5-hydroxyindoleacetic acid was also detected in all samples, including the blanks, albeit at lower levels than in the strains tested. Interestingly, tryptophan supplementation consistently resulted in lower serotonin, when detectable, and higher 5-hydroxyindoleacetic acid in the inoculated samples compared to the blanks. This effect is possibly a result of tryptophan supplementation shifting bacterial metabolism away from serotonin and towards other pathways, leading to increased bacterial indole production which the HPLC method was unable to separate from 5-hydroxyindoleacetic acid. Taken together, the results from this HPLC screen indicate that *Lactococcus lactis* subsp*. cremoris* 1614 and *Lactococcus lactis* subsp*. cremoris* MG1363 are likely to be serotonin producers which is in agreement with the literature (Özoğul *et al.*, 2012). Further investigation will be required in order to determine whether the HPLC method was detecting 5-hydroxyindoleacetic acid or indole.

In order to gain an insight into this, and to provide further information about the potential serotonin production capabilities of these strains, *L. paracasei* 110 and *L. paracasei* 231 underwent whole genome sequencing to search for genes involved in tryptophan metabolism using *Lactococcus lactis* subsp*. cremoris* MG1363 (Genbank accession number AM406671) as reference. Artemis was used to search for the *trp* operon in the genomes of both strains to determine whether they contain the genes responsible for tryptophan metabolism and serotonin synthesis. The *Lactococcus lactis* subsp*. cremoris* MG1363 genome was found to contain the entire *trp* operon, consisting of the following genes: *trpE*, encoding anthranilate synthase component 1; *trpG*, encoding anthranilate synthase component 2, both of which can be involved in the first step of tryptophan synthesis; *trpD*, encoding anthranilate phosphoribosyltransferase which is involved in step two of tryptophan

synthesis; *trpF*, encoding phosphoribosylanthranilate isomerase involved in the third step of tryptophan synthesis; *trpC*, encoding indole-3-glycerol phosphate synthase involved in step four of tryptophan synthesis; *trpA*, encoding tryptophan synthase alpha chain involved in step five of tryptophan synthesis; and *trpB*, encoding tryptophan synthase beta chain which catalyses the final step of tryptophan synthesis. Therefore, the genomes of the two MyNewGut strains of interest were searched for the presence of these genes. In comparison, the genome of *L. paracasei* 110 was found to contain the following genes of the tryptophan pathway: *trpA, trpB, trpC, trpD, trpF,* and *trpS2*. In contrast, Artemis revealed that *L. paracasei* 231 only contained *trpA* at the nucleotide position 2651049-2651327 and *trpS2* at the position 905720-906736. Taken together, these results indicate that *L. paracasei* 110 is capable of tryptophan synthesis because it contains the *trp* operon and of serotonin synthesis because it contains the gene which encodes for tryptophan synthase (O'Mahony *et al.*, 2015; Gao *et al.*, 2020). While *L. paracasei* 231 does contain the gene *trpA* it does not contain the genes to encode the remaining enzymes involved in tryptophan synthesis. Therefore, it is likely that this strain is capable of synthesising indole as this gene contributes to tryptophan-independent indole biosynthesis and that tryptophan supplementation is leading to increased bacterial indole production which the HPLC method is unable to separate from 5-hydroxyindoleacetic acid.

Whole genome sequencing data was also used to investigate whether the strains of interest contained genes which encoded enzymes involved in other pathways of tryptophan metabolism apart from serotonin synthesis, namely the kynurenine pathway, indole production and tryptamine production. It was important to assess whether the strains contained genes responsible for the synthesis of these metabolites because approximately 90% of tryptophan is metabolised along the kynurenine pathway, indole is the main metabolite produced by gut bacteria from tryptophan, and tryptamine is a monoamine which is structurally similar to serotonin (Gao *et al.*, 2020), therefore these metabolites could be interfering with serotonin detection in the ELISA and HPLC methods. It was important to rule out the possibility that these metabolites were misidentified as serotonin or the

serotonin metabolite 5-hydroxyindoleacetic acid particularly when the HPLC results showed that tryptophan supplementation resulted in lower serotonin and higher 5-hydroxyindoleacetic acid, which could indicate that tryptophan led to increased bacterial indole production. A literature search was first carried out to determine the enzymes involved in the production of these metabolites to determine which genes to search for (Kaur, Bose and Mande, 2019). The genomes were checked for the presence of genes which encode enzymes involved in the kynurenine pathway, through the production of kynurenine and quinolinate. *KynA* and *KynB*, which encode the enzymes tryptophan 2,3-dioxygenase and kynurenine formamidase respectively (Kaur, Bose and Mande, 2019), were not found to be present in either *L. paracasei* 110 or *L. paracasei* 231, indicating that these strains may not be involved in kynurenine production. The genes *KMO* and *Kyn*, which encode the enzymes kynurenine 3-monooxygenase and kynurinase for quinolinate production were also not found in either of the genomes of interest, indicating that these strains may not play a role in quinolinate production and are not involved in the kynurenine pathway (Kaur, Bose and Mande, 2019). The role of these strains in tryptophan metabolism was also assessed by searching for genes identified in playing a role in tryptophan degradation to indole and indole derivatives such as indole-3-acetic acid, indole-3-propionic acid, and tryptamine. The gene *tnaA*, which encodes the enzyme tryptophanase which is responsible for indole synthesis from tryptophan, was not found to be present in either of the genomes of interest (Kaur, Bose and Mande, 2019; Gao *et al.*, 2020). However, the gene *trpA* which contributes to tryptophan-independent indole biosynthesis is present in both strains so indole production through this gene cannot be ruled out, particularly for *L. paracasei* 231 which does not contain *trpB*, the enzyme which catalyses the final step in tryptophan biosynthesis. Similarly, the genes involved in indole-3-acetic acid synthesis, *iaaM* and *ipdC*, which encode tryptophan-2-monooxygenase and indolepyruvate decarboxylase respectively, were not found in the genomes of these strains (Kaur, Bose and Mande, 2019). The gene *fldABC*, which encodes the enzyme phenyllactate dehydratase for indole-3-propionic acid production, was not present in the genomes (Kaur, Bose and Mande, 2019). Finally, the ability of the strains to synthesise tryptamine was assessed by searching for the genes *TDC, CLOSPO-0283,*

and *RUMGNA-01526* which encode tryptophan decarboxylase (Williams *et al.*, 2014). Neither *L. paracasei* 110 nor *L. paracasei* 231 were found to possess the genes known to be involved in tryptamine synthesis, therefore it is unlikely that either of the strains of interest are capable of tryptamine production. Taken together, these results indicate that the strains described here do not play a role in the kynurenine pathway or are involved in the degradation of tryptophan to form the metabolites indole-3-acetic acid, indole-3-propionic acid, or tryptamine. Therefore, it is unlikely that any of these metabolites were mistaken for serotonin or 5-hydroxyindoleacetic acid in the ELISA and HPLC analysis as mentioned previously. Additionally, an important conclusion that can be drawn from these results is the value of using multiple screening approaches in combination, including laboratory techniques such as ELISA and HPLC, as well as metagenomic approaches such as whole genome sequencing to search for genes of interest. This provides a greater insight into the metabolic capabilities of strains of interest by determining whether strains have the genes to participate in the relevant pathways and allows a deeper interrogation of the metabolites detected using screening methods which could be structurally similar to the metabolite of interest. Using whole genome sequencing, it is possible to determine whether a strain of interest contains the genes necessary to encode metabolites at each step of the pathways of interest and thereby rule out metabolites which might be structurally similar and not distinguishable using other screening methods.

As mentioned previously an altered GABAergic profile is present in CNS disorders such as anxiety and depression (Cryan and Kaupmann, 2005), therefore a probiotic which is capable of GABA synthesis has potential for treating such disorders. Amino acid analysis was used to assess the ability of 34 human-derived *Lactobacillus* strains obtained from the MyNewGut project as well as strains isolated from fermented food products to generate GABA from monosodium glutamate compared to the positive control *Lactobacillus brevis* DPC6108, which is a highly efficient GABA producer (Patterson *et al.*, 2019). The assay was carried out in triplicate and identified one putative *Lactobacillus* strain as a GABA producer with an average monosodium glutamate to GABA conversion rate of 38.4%. Sanger

sequencing subsequently identified the strain as *Lactobacillus plantarum*. However, this strain is not as efficient a GABA producer as the positive control *L. brevis* DPC6108, with a conversion rate of up to 90% (Barrett *et al.*, 2012). Future studies could further characterise this strain and work to optimise GABA production by testing different concentrations of monosodium glutamate supplied as a precursor.

Bacteriocins are antimicrobial peptides or proteins that inhibit growth of bacteria closely related to the producing organism (Nilsen, Nes and Holo, 2003). Bacteriocin production is often regarded as a desirable probiotic trait and has been shown to promote probiotic survival within the gastrointestinal tract, contribute to the control of pathogens and influence host gene expression in the gut (Hegarty *et al.*, 2017). Furthermore, the growing threat of antimicrobial resistance has resulted in an increased demand for novel antimicrobial therapies (O'Sullivan *et al.*, 2018). Therefore, a series of assays for antimicrobial activity were performed on both the MyNewGut isolates (n=26) and the fermented food isolates (n=46) using deferred antagonism assay and well diffusion assay approaches. All MyNewGut isolates tested produced zones in the deferred antagonism assay; however the zones were cloudy which indicated acid production. All but two fermented food isolates also produced zones in this assay, however no zones were visible in the subsequent well diffusion assay. Well diffusion assays identified *Lactobacillus paracasei* 110 as a potential bacteriocin producer. Although the observed zone was small, further well diffusion assays were carried out on this strain including heat treating the supernatant at 99°C and 80°C as well as using Proteinase K. These tests were carried out because characteristically bacteriocins are relatively heat-stable and are susceptible to degradation by proteolytic enzymes (Jack, Tagg and Ray, 1995; O'Sullivan *et al.*, 2018). No zones were observed when the supernatant was heat treated although small zones were visible when the supernatant was at room temperature. In conclusion, these results indicate weak antimicrobial activity.

However, in order to gain a greater insight into their antimicrobial potential, the genomes of both *Lactobacillus paracasei* strains were also analysed using BAGEL to identify genes with potential for antimicrobial activity. An important characteristic of a bacteriocin producer is the presence of immunity gene(s) within their genome

which protect producers from the antimicrobial effects of the bacteriocin they produce (Cotter, Hill and Ross, 2005). Indeed, both *L. paracasei* 110 and 231 contained a putative carnobacteriocin B2 immunity protein within their genomes at positions 2446898-2447233 and 2615586-2615921 respectively. Two areas of interest were found using BAGEL within *L. paracasei* 110 and were identified as Enterolysin A, with a match of 56.82%, and LSEI 2386, with a match of 100%. Similarly, three areas of interest were uncovered in *L. paracasei* 231 and were subsequently identified as Carnocin CP52, LSEI 2163, and Enterolysin A. Enterolysin A is a class III heat-labile bacteriocin protein with a broad inhibitory spectrum which breaks down the cell walls of sensitive bacteria (Nilsen, Nes and Holo, 2003). It was first purified from *Enterococcus faecalis* LMG 2333 and found to inhibit the growth of selected enterococci, pediococci, lactococci, and lactobacilli (Nilsen, Nes and Holo, 2003). It has previously been shown to inhibit human pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Zhang *et al*., 2013). Interestingly, Nilsen *et al.* (2003) initially only detected antimicrobial activity on agar plates in overlay assays but was later optimised by growing the bacteria in a fermenter under optimised production conditions. This could explain why large zones of clearing were only detected in the deferred antagonism assay while minimal zones were observed in the cell-free supernatant well diffusion assay in the present study. Other bacteriocins identified using BAGEL included Carnocin CP52, LSEI 2386, and LSEI 2163 all of which have previously been identified in *Lactobacillus rhamnosus, casei and paracasei* (Kim, Chang and Kim, 2020; Surachat *et al.*, 2017). Carnocin has previously demonstrated antimicrobial activity against *Carnobacterium, Enterococcus* and *Listeria* spp. as well as *Lactobacillus delbruekii* spp. Carnocin CP52 is an anti-*Listeria* bacteriocin gene which is similar to Carnobacteriocin B2 (Herbin *et al.*, 1997). LSEI 2163 peptide is a class IId bacteriocin which exhibits antimicrobial activity against some lactobacilli and several *Listeria* species. Similarly, LSEI 2386 is a putative pheromone peptide that also has significant bacteriocin activity against several *Listeria* species. Both peptides have been shown to tolerate 121°C for 30 minutes but not treatment with Proteinase K or trypsin (Kuo *et al.*, 2013).

The main categories that contribute to the optimal functioning of probiotics are not only related to probiotic factors which directly contribute to health-promoting effects such as maintaining microbial balance, epithelial protection and immunoregulation, but also adaptation factors which allow optimal adaptation to their new niches. These include stress resistance such as resistance to low pH, digestive enzymes and bile salts; active metabolism adapted to the host environment such as antimicrobial production; and adherence to the intestinal mucosa and mucus, all of which aid in determining the optimal frequency, dose and mode of administration (Lebeer, Vanderleyden and De Keersmaecker, 2008). Therefore, the strains were also searched for genes promoting these probiotic characteristics. These included *dltA* and *dltD* which confer tolerance to acid, which is an important feature for a candidate probiotic to possess in order for them to survive gastrointestinal transit and colonise the intestinal mucosa (Goel, Halami and Tamang, 2020). Interestingly, both *L. paracasei* 110 and 231 possessed the genes *dltA* and *dltD* which indicates that they are capable of withstanding low pH, while the anti-inflammatory potential gene *dltB*, was not found to be present in either of the strains. However, neither strain contained *gadB* which also confers acid tolerance (Yunes *et al.*, 2016). Similarly, the genes *clpL*, promoting acid and bile tolerance, and *bsh*, supporting tolerance to bile were not present (Bustos *et al.*, 2018). Adhesion ability of the strains was also investigated and both strains were found to contain the gene *fbp* which promotes adhesion ability, an additional characteristic which is important for candidate probiotics to possess (Turpin *et al.*, 2012). Therefore, the presence of bacteriocin genes as well as genes promoting tolerance to acid and adherence to intestinal mucosa may provide a competitive advantage to the strains identified in this study, promoting survival in a complex, competitive environment such as the large intestine, which may represent an advantage of these strains as potential probiotics (Dobson *et al.*, 2012; Jia *et al.*, 2020).

3.6 Conclusion

Microbiome manipulation using probiotics, particularly strains which produce neuroactive metabolites, are likely to play a promising role in the future prevention and treatment of immune and psychiatric disorders. However, there are still several challenges that need to be addressed. Research needs to progress beyond characterising the microbiota composition in various disease states and investigate potential mechanisms by which altered microbiota composition contributes to pathophysiology of disease, and by which potential probiotic bacteria exert beneficial effects on host health. Importantly, this study highlighted the value of combining laboratory screening techniques with metagenomic analysis to gain a further mechanistic insight into the psychobiotic potential of strains of interest. Whole genome sequencing allows identification of enzymes involved in the relevant metabolic pathways within the genomes of potential probiotics, enabling the distinction between the metabolite of interest and other secondary metabolites which are structurally similar. Future studies will be required to optimise the methods described in this study, particularly to reduce background readings from media which were observed in the serotonin ELISA and HPLC screens. Optimisation of the synthesis capabilities of potential probiotics will be necessary by adjusting growth conditions, growth medium and precursor concentration. It will also be important to assess whether positive producers are capable of synthesising neuroactive metabolites in the presence of other microbiota by using techniques such as faecal fermentation. Additionally, animal studies which combine microbiome intervention with neurotransmitter antagonists will provide further insight into mechanisms of communication between gut microbiota and the host (Strandwitz, 2018). Lagier *et al.* (2015) proposed that sequencing the genomes of all microorganisms cultured for which no sequences are available, which he termed microbiogenomics, will represent a significant advance in clinical microbiology. Nevertheless, further research is necessary to ascertain whether manipulation of human neurotransmission by gut microbiota is associated with additional physiological implications and how it may be employed therapeutically.

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Author Contributions

N. Wiley designed the study with assistance from C. Stanton and P. Ross, carried out the laboratory assays, performed the data analysis, drafted the manuscript, and assisted with interpretation; A. Murphy, Teagasc, isolated the *Lactobacillus* strains from the MyNewGut faecal samples and assisted with study design and interpretation; G. Clarke and F. Villalobos performed the HPLC analysis and assisted with interpretation; J.F. Cryan, T.G. Dinan, R.P. Ross and C. Stanton assisted with data interpretation, contributed to the writing of the manuscript, and provided resources and intellectual input that supported the study. C. Stanton and R.P. Ross had primary responsibility for final content.

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4. High-Anxious Versus Normal-Anxious Mice: Effect of Environmental Enrichment on the Gut Microbiota Composition

Nicolas Singewald[1], Anupam Sah[1], Niamh C Wiley[2,3], Anna V Golubeva[2,4], Thomaz Batiaanssen[2,4], Fiona Fouhy[2,3], John F Cryan[2,4], Timothy G Dinan[2,5], R. Paul Ross[2,6] and Catherine Stanton[2,3]

[1]Neuropharmacology Unit, Department of Pharmacology and Toxicology, University of Innsbruck, Austria

[2]APC Microbiome Ireland, University College Cork, Cork, Ireland

[3]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[4]Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[5]Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[6]School of Microbiology, University College Cork, Cork, Ireland

4.1 Abstract

Multiple studies have proposed environmental enrichment as a potential mechanism to provide physiological, neurological, and behavioural benefits. Advantages of enrichment include improving cognition and attenuating responses to anxiety-provoking situations by promoting resilience to diverse stressors, and these effects persist over time. It is now known that the composition of the gut microbiome is strongly influenced by stress and the hypothalamic pituitary adrenal axis. Furthermore, the gut microbiota has been implicated in psychological disorders such as anxiety, depression, and abnormal stress responses. Environmental conditions have a substantial influence on microbiome composition and diversity, indicating a link between environmental enrichment, the microbiome and stress. While numerous studies have explored the impact of environmental enrichment on psychological and physiological measures, there has been limited investigation into its effects on gut microbiome composition and diversity, and whether these changes may play a role in mediating the effects of environmental enrichment. Thus, the aim of this study was to investigate the impact of environmental enrichment in both highly anxious and non-anxious mouse breeds. Furthermore, we aimed to determine whether the gut microbiota profile differed between high anxiety-related behaviour and normal anxiety behaviour mice. Finally, we aimed to investigate whether environmental enrichment can alter the gut microbiota composition in mice, and if these alterations were associated with changes in anxiety and depression. Two different mouse phenotypes were used in this study: male mice with innate enhanced anxiety and comorbid depression-like behaviour; and male mice with a "normal" anxiety/depression behavioural phenotype. Normal-anxious and high-anxious mice were housed in a standard environment for a two-week habituation phase before being housed over four weeks in an enriched environment. Additionally, a separate cohort of animals was housed over four weeks in a standard environment to control for the effect of one month change in animals age in the beginning and at the end of the study. As expected, multiple differences in anxiety-like behaviour were evident between high and normal anxiety phenotypes. Exposure to an enriched environment resulted in

improved anxiety-like behaviour in both the Light Dark Test and Open Field Test in high anxiety mice compared to both non-anxious mice post enrichment and highly anxious mice post standard conditions. Multiple differences in microbiome composition were observed at both phylum and genus level. However, no effect of environmental enrichment was observed on alpha diversity while differences in beta diversity appeared to be based on mouse phenotype and not environment.

4.2 Introduction

Animal models are an important tool for studying psychiatric disorders such as anxiety and depression as well as testing clinical efficacy of novel potential treatments. Mice are especially useful because of their small size and minimal requirement of food and space, gene targeting can be applied to the mouse genome, they have similar gene functions to humans and share a number of neuroanatomical, neurochemical and behavioural similarities with humans. However, translating findings from mouse studies into information that can be applied in the clinic remains challenging, potentially because most studies use animals displaying 'normal' anxiety rather than 'psychopathological' animal models with elevated anxiety, which more closely reflect features of human anxiety disorders, thus narrowing the translational gap. To this end, the high anxiety-related behaviour, low anxiety-related behaviour and normal anxiety-related behaviour models of trait anxiety were created at the Max Planck Institute of Psychiatry in Munich by selective in-breeding of CD-1 mice for an extremely high, an extremely low and a normal level of anxiety-related behaviour, displayed on the Elevated Plus Maze and confirmed in other tests of anxiety including the Light Dark Test, Open Field and Open Arm tests, as well as in different laboratories indicating a very robust phenotype (Salomé *et al.*, 2002) that appears to be largely independent of uterine environment, maternal care, sex and age (Landgraf *et al.*, 2007). Compared to low anxiety-related behaviour mice, high anxiety behaviour mice are more anxious and display increased risk assessment behaviour in several tests such as the Elevated Plus Maze Test, Open Arm Test, Light/Dark Avoidance Test, and

Ultrasound Vocalisation Test as well as a preference for immobility/passive stress-coping strategies in paradigms such as Forced Swim and Tail Suspension tests and clear signs of anhedonia assessed by the Sucrose Preference Test (Sah *et al.*, 2012; Schmuckermair *et al.*, 2013). This model has translational power for Generalised Anxiety Disorder in particular as the high anxiety-related behaviour/normal anxiety-related behaviour mouse model operationalises trait anxiety (Sartori, Landgraf and Singewald, 2011), which is evident in individuals suffering from Generalised Anxiety Disorder (Rapee, 1991; Chambers). To confirm the robustness of the anxiety phenotype in this model, Salome´*et al*. (2002) aimed to test the reliability of the differences in anxiety-related behaviour of these animals in two other laboratories (Villeneuve d'Ascq, France and Innsbruck, Austria). The results from this study were in agreement with the studies performed by Liebsch *et al.* (1998), as the divergence in anxiety-related behaviour of the two mouse lines was highly consistent in all tests performed in Villeneuve d'Ascq and Innsbruck. Furthermore, principal component analysis from this study confirmed that the selection of high anxiety-related behaviour and low anxiety-related behaviour rats is based on anxiety behaviour rather than locomotor activity (Salomé *et al.*, 2002). Reduced hippocampal serotonergic transmission has also been demonstrated in high anxiety-related behaviour rats compared with low anxiety-related behaviour rats at both the presynaptic (serotonin release) and the postsynaptic (Serotonin 1A receptor) level. This is of relevance to anxiety and depression-like behaviour because disturbances in serotonergic neurotransmission have been suggested to be closely interlinked with hyperactivity of the hypothalamic pituitary adrenal system, and are likely to be involved in the pathophysiology of anxiety disorders and major depression (Keck *et al.*, 2005). High anxiety mice also display aberrant sleep patterns and alterations in electroencephalogram activities (Jakubcakova *et al.*, 2012). Dysregulation of circadian rhythms is also an important symptom of mood disorders, such as anxiety and depression and this has also been demonstrated in this mouse model of high anxiety-related behaviour (Griesauer *et al.*, 2014).

Environmental enrichment has long been studied as a potential mechanism to provide multiple beneficial physiological, neurobiological and behavioural effects as

early studies showed that enrichment alters brain structure and promotes superior learning (Renner and Rosenzweig, 1987a). Early studies provided a template for environmental enrichment, which typically consists of housing in large groups of six to twelve animals to provide an opportunity for social interactions in spacious cages, and with ready access to a variety of stimulating objects (Belz *et al.*, 2003; Rosenzweig, Bennett and Diamond, 1972; Rosenzweig, Krech and Bennett, 1961). Environmental enrichment also offers increased opportunities to interact with complex inanimate and social stimuli and to explore the environment, which enables the animal to acquire sensory and cognitive experiences (Rosenzweig *et al.*, 1978; Roy *et al.*, 2001). Overall, environmental enrichment is regarded as a continuous enhancement in cognitive, sensorimotor and/or physical activity (Mattson *et al.*, 2001). There is significant evidence to indicate that enrichment induces morphological, neurobiological, physiological and behavioural changes in several animal species (Greenough and Volkmar, 1973; Larsson, Winblad and Mohammed, 2002; Rosenzweig and Bennett, 1996; Van Praag, Kempermann and Gage, 2000; Will *et al.*, 1977). Environmental enrichment has been shown to alter the expression of several genes whose products are involved in plasticity, neuronal structure and neurotransmission (Rampon *et al.*, 2000). Other advantages of environmental enrichment include improving cognition, reducing anxiety (Benaroya-Milshtein *et al.*, 2004; Chamove, 1989; Nilsson *et al.*, 1999) and facilitating recovery from brain lesions (Will *et al.*, 1977). Increasing cage size alone has been shown to improve general well-being and reduce anxiety levels of animals (Manosevitz and Pryor, 1975). Conversely, removal of enrichment features results in adverse effects. Enriched mice lose more body weight than controls when placed in individual cages for only one night (Van de Weerd *et al.*, 2002). Altogether, environmental enrichment offers benefits for both the psychological and physical well-being of animals (Boinski *et al.*, 1999; Hansen and Berthelsen, 2000; Marashi *et al.*, 2003).

A significant advantage of environmental enrichment is that as well as providing protection from excessive anxiety it is also able to reverse the effects of past stressors (Escorihuela, Tobeña and Fernández-Teruel, 1994). For example,

environmental enrichment alleviated the detrimental effects of early maternal separation, as reflected by reduced plasma corticosterone levels and increased exploratory and feeding behaviours (Francis *et al.*, 2002), and these effects are observed even in adolescence. Similarly, environmental enrichment reversed the negative effects of prenatal stress by increasing social interaction, decreasing corticosterone secretion, and strengthening the immune system (Laviola *et al.*, 2004; Morley-Fletcher *et al.*, 2003). These findings revealed that enrichment has a compensating or reversing effect even against prenatal or early life adversity. Multiple studies have shown that enrichment increases the number of open arm entries and time spent in open arms in the Elevated Plus Maze, which is indicative of reduced anxiety (Chapillon *et al.*, 1999; Fernandez-Teruel *et al.*, 1997; Friske and Gammie, 2005). Environmental enrichment confers long-term anxiolytic affects which are resistant to extensive experimental stimulation even when the animals are exposed to enrichment only in adulthood (Larsson, Winblad and Mohammed, 2002). The effects of environmental enrichment last for at least three months following the removal of enrichment and may be epigenetic (Friske and Gammie, 2005). Interestingly, the protective effects of environmental enrichment are particularly apparent in animals that are highly anxious or when the task is especially challenging for the subject, for example, environmental enrichment reduced freezing behaviour in the highly anxious BALB/c mice (Chapillon *et al.*, 1999). Similarly, environmental enrichment reduced freezing behaviour following predator exposure only in females, which are more emotionally reactive to predator exposure (Klein *et al.*, 1994). Furthermore, a more challenging task highlights the anxiolytic effect of environmental enrichment (Fernández-Teruel *et al.*, 2002), for example, enrichment improved maze exploration in the relatively more complex eight-arm maze but not in the four-arm maze (Janus *et al.*, 1995). These results suggest that environmental enrichment may be therapeutically anxiolytic only in situations of pronounced anxiety (Fox, Merali and Harrison, 2006). It is important to note that environmental enrichment does not completely eliminate the stress response but rather reduces it. Although a faster adaptation to repetitive stress may provide advantages for preparing the organism to cope with environmental demands, the stress response is important to allow the individual to

react appropriately to environmental threats (Klein *et al.*, 1994; Moncek *et al.*, 2004).

The mechanisms by which enrichment promotes stress resilience and affects behaviour, physiology, and brain morphology remain to be elucidated. However, various mechanisms of action have been proposed, ranging from behavioural/cognitive to cellular/molecular processes. Early studies proposed that the interaction with inanimate objects, and not social interaction, induced morphological changes in the brain (Ferchmin, Bennett and Rosenzweig, 1975; Rosenzweig *et al.*, 1978). However, the negative impact of social isolation (Woolverton *et al.*, 1989; Renner and Rosenzweig, 1987b) indicates that both elements contribute to the beneficial effects of environmental enrichment (Van Praag, Kempermann and Gage, 2000). It has also been suggested that environmental enrichment provides the animals with a sense of control over their environment by providing opportunities to structure and organise their environment, which is known to reduce the stress level of the animal (Broom, Johnson and Broom, 1993). Enrichment may also represent a mechanism of stress inoculation whereby the repeated introduction of new objects and the opportunity to explore them is comparable to repeated mild stress exposures, which renders the animals more emotionally stable (Chorpita and Barlow, 1998; Larsson, Winblad and Mohammed, 2002). The enhancing effect of environmental enrichment on cognition has been attributed to its induction of hippocampal neurogenesis (Kempermann, Kuhn and Gage, 1997). Therefore, stress resilience following environmental enrichment may also be related to hippocampal neurogenesis as this structure, along with the amygdala and hypothalamus, has been implicated in fear and anxiety (Huttunen and Myers, 1986; LeDoux, 1992). Furthermore, environmental enrichment has been shown to increase total brain and cortical weight (Henderson, 1970; Rosenzweig and Bennett, 1972) along with an increase in neuronal density (Kempermann, Kuhn and Gage, 1997), number of dendritic branches (Greenough and Volkmar, 1973), synapses (Turner and Greenough, 1985; Ickes *et al.*, 2000; Pham *et al.*, 1999) and neurotrophin levels, which may all mediate the impact of environmental enrichment on stress (Ickes *et al.*, 2000). No

consensus has been agreed upon regarding the effects of environmental enrichment on plasma corticosterone levels, however evidence suggests that enrichment renders the hypothalamic pituitary adrenal axis response more adaptive and efficient (Larsson, Winblad and Mohammed, 2002; Mohammed *et al.*, 1993). Environmental enrichment may decrease emotional reactivity by lowering stress hormone levels such as adrenocorticotropic hormone and corticosterone (Fox, Merali and Harrison, 2006).

The microbiota-gut-brain axis has been found to have a particularly strong influence on stress, defined as a perceived or actual threat to an organism that is accompanied with a sense of unpredictability or lack of control (Sanchez, Ladd and Plotsky, 2001). The detrimental effects of stress are long-term and range from behavioural/cognitive deficits to brain damage such as hippocampal atrophy (McEwen, 1998; Mulholland *et al.*, 2005; Smith, 2005). Prenatal or early life stress can have persisting detrimental effects on behaviour and brain function in adulthood (Roy *et al.*, 2001; Sanchez, Ladd and Plotsky, 2001). It is long known that the composition of the gut microbiome is strongly influenced by stress and the hypothalamic pituitary adrenal axis as microbial diversity and composition disruption is evident after several different types of stressors. The early life stressor of maternal separation results in long-term hypothalamic pituitary adrenal axis changes as well as long-term effects on the microbiome (Bailey and Coe, 1999). Faecal microbiome diversity of adult rats exposed to maternal separation is significantly altered compared to non-separated controls (O'Mahony *et al.*, 2009). Mice exposed to a prolonged restraint stressor have significantly altered microbial community structure compared to non-stressed control mice (Bailey *et al.*, 2010). Sudo *et al.* (2004) described an aberrant stress response in germ free mice where a mild restraint stress induced an exaggerated release of corticosterone and adrenocorticotropic hormone compared with specific pathogen free controls. This can be partially reversed by colonisation with faecal matter from specific pathogen free animals and fully reversed by monoassociation with *B. infantis* in a time-dependent manner. Therefore, the gut microbiome is vital for the development of an appropriate stress response later in life, and to ensure normal development of

the hypothalamic pituitary adrenal axis, colonisation must occur in early life. Environmental conditions play a substantial role in influencing microbiome composition and diversity. In fact, a study by Rothschild *et al.* (2018) which examined multiple cohorts concluded that gut microbiome composition is predominantly shaped by environmental factors rather than host genetics, estimating that the average heritability of gut microbiome taxa is only between 1.9% and 8.1%. To further investigate the role of environment in shaping the microbiome, Rothschild and colleagues examined genetically unrelated individuals who share a household compared to relatives who have no history of household sharing and found that the genetically unrelated individuals possess significant similarities in their microbiome composition, while there were no significant similarities among the relatives who have not shared a house. These results are corroborated by previous studies (Song *et al.*, 2013), for example, a twin study demonstrated that the gut microbiomes of twins became more genetically dissimilar when living apart over time (Xie *et al.*, 2016).

The aim of this study was to assess the impact of environmental enrichment on a number of factors in both highly anxious and normal anxious mouse lines. The potential for environmental enrichment to normalise enhanced anxiety and depression-like behaviour in high anxiety-related behaviour versus normal anxiety-related behaviour mice was investigated. Furthermore, we aimed to determine whether the gut microbiota profile differed between high anxiety-related behaviour and normal anxiety-related behaviour mice. Finally, we aimed to investigate whether environmental enrichment can alter the gut microbiota composition in highly anxious mice, and if these alterations were associated with changes in anxiety and depression. 16S rRNA sequencing was employed to characterise the gut microbiota profiles of both high anxiety-related behaviour and normal anxiety-related behaviour mice and to track potential changes as a result of environmental intervention. In order to assess behavioural measures such as depression and anxiety-like behaviour, a number of standard behavioural tests were used, including Open Field, Light Dark Box and Elevated Plus Maze. While numerous studies have explored the impact of environmental enrichment on psychological and

physiological measures, there has been limited investigation into the effects of environmental enrichment on gut microbiome composition and diversity, and whether these changes may play a role in mediating the effects of environmental enrichment. Therefore, findings from this study will present many benefits for animal husbandry since environmental enrichment is known to provide multiple beneficial physiological, neurobiological, and behavioural effects. This study aims to provide insight into potential mechanisms by which enrichment promotes these effects and whether the microbiome may have a role to play.

4.3 Materials and Methods

4.3.1 Animals and experimental groups

Two different mouse phenotypes were used in this study: male mice with innate enhanced anxiety and comorbid depression-like behaviour; and male mice with a "normal" anxiety/depression behavioural phenotype. This was achieved by selectively breeding for over 20 generations for anxiety-like behaviour from outbred CD-1 mice for both high anxiety-related behaviour and normal anxiety-related behaviour. These mice were selected in breeding based on their performance in the Elevated Plus Maze test, and indeed the high anxiety-related behaviour strain shows higher anxiety. Normal-anxious and high-anxious mice were housed in a standard environment for a two-week habituation phase after which faecal matter was collected for a baseline time point. Normal-anxious and high-anxious mice were then housed over four weeks in an enriched environment, consisting of 2.5-3.0 cm thick bedding; a representative image of the enriched environment can be seen below (Figure 1).

4.3.2 Sampling

Faecal samples for the microbiota composition analysis were collected individually from each animal before and after the enriched housing: normal anxiety-related behaviour at baseline and normal anxiety-related behaviour at endpoint (n=6), high

anxiety-related behaviour at baseline and high anxiety-related behaviour at endpoint (n=12). Additionally, a separate cohort of animals was housed over four weeks in a standard environment to control for the effect of one month change in animals age in the beginning and at the end of the study. Faecal samples were collected before and after the standard housing: normal anxiety-related behaviour at baseline and normal anxiety-related behaviour at endpoint (n=5), high anxiety-related behaviour at baseline and high anxiety-related behaviour at endpoint (n=11).



Figure 1: Representative image of the enriched environment.

4.3.3 Elevated Plus Maze test

The elevated plus maze device consisted of a central area (5 x 5 cm), two opposing open arms (30 x 5 cm) and two opposing closed arms (same size) with 14 cm high, nontransparent walls. The apparatus was elevated 73 cm above the floor and illuminated with 100 lux. At the beginning of each trial, a mouse was placed onto the central area of the maze, facing a closed arm. Mice were tested for a period of five minutes and their movements on the maze were tracked and subsequently analysed by the TSE VideoMot2 system (TSE, Technical and Scientific Equipment GmbH, Germany). The following parameters were assessed: open and closed arm entries, open and closed arm time, latency to enter open arm for the first time, total arm entries and percentage of time spent in open arm. Mice were considered to have entered an open or closed arm when both front paws and front shoulders

were on the arm, but full entries (all four paws) also were counted (Krömer *et al.*, 2005).

4.3.4 Light/Dark Test

The light/dark testing arena (41 × 41 × 41 cm; TruScan, Coulbourn Instruments, USA) was divided into two halves, comprising a white, aversive compartment illuminated with 400 lux at floor level and a dark, safe compartment covered by a black top illuminated with 10 lux. The compartments were connected by a small opening (7 × 7 cm) located in the centre of the partition at floor level. Animals were individually placed into the dark compartment facing away from the opening and allowed to freely explore the apparatus for 10 minutes. During the 10-minute testing period the behaviour displayed by each mouse was automatically registered and the following anxiety-related parameters quantified: the latency to the first entry into the lit compartment, number of entries into the lit compartment, time spent in the lit compartment. and overall distance travelled by the mice (Sartori *et al.*, 2012).

4.3.5 Open Field Test

The open field consisted of a plastic box (41 × 41 × 41 cm) equipped with an automated activity monitoring system (TruScan, Coulbourn Instruments, USA). The area of the open field, illuminated with 150 lux, was divided into a 28 × 28 cm central zone and a surrounding border zone. Mice were placed individually into the periphery of the open field and allowed to explore it for 10 minutes. The following anxiety-related parameters were recorded: latency to enter the central zone, entries into the central zone, time spent in it, and the overall distance travelled (Sartori *et al.*, 2012).

4.3.6 Faecal DNA extraction

DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Germany) coupled with an initial bead-beating step. Briefly, each sample was homogenised at maximum speed in a Mini-Bead Beater (BioSpec Products) in 2 ml screw-cap tubes (Sarstedt, Germany) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.0 mm sterile zirconia beads plus a single 3.5 mm diameter bead (BioSpec Products) with 1 mL of Qiagen InhibitEX® buffer. DNA was quantified using the QubitTM 3.0 Fluorometer (Bio-Sciences Dublin, Ireland) and the Qubit® dsDNA HS Assay Kit (Bio-Sciences Dublin, Ireland). Extracted DNA was kept frozen at -20°C until further analysis.

4.3.7 16S rRNA gene sequence-based microbiota analysis

The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol (http://www.illumina.com/content/dam/illumina support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Briefly, the first PCR was carried out using forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3').
Each 25 µl PCR reaction contained 2.5 µl microbial genomic DNA, 5 µl of each primer (1 µM) and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Roche, Sussex, UK). The PCR conditions were as follows: initial denaturation at 95°C x 3 min; 25 cycles of 95°C x 30 s, 55°C x 30 s, 72°C x 30 s; and 72°C x 5 min for final extension. PCR products were purified with Agencourt AMPure XP beads (Labplan, Dublin, Ireland). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, USA). Each 50 µl PCR reaction contained 5 µl purified DNA, 5 µl index primer 1 (N7xx), 5 µl index primer 2 (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix and 10 µl PCR grade water (Sigma Aldrich). PCR amplification was completed using the previous program but with only eight

amplification cycles. Following this, a second clean-up step with the Agencourt AMPure XP system was carried out. PCR products were quantified, normalised, and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Bio-Sciences Dublin, Ireland). 2 × 300 (bp) paired-end sequencing was performed on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

4.3.8 Bioinformatic sequence analysis

Paired-end sequences were assembled using FLASH (min overlap of 30 bp and min length of 460 bp) and analyzed using QIIME v1.8.0. Sequences were quality-checked and clustered into OTUs using USEARCH (v7.0-64bit). Taxonomic ranks were assigned with a BLAST search against the SILVA SSU database v123. Alpha and beta diversity indices were generated in QIIME.

4.3.9 Biostatistical analysis

Statistical analysis was carried out in SPSS, Calypso and R software environment. Relative abundance of bacterial taxa was expressed as percentage of identified sequences. Between-group differences in alpha diversity indices and in the relative abundance of bacterial genera were analysed with paired-sample Wilcoxon test for the following comparisons: normal anxiety-related behaviour pre versus post enrichment, high anxiety-related behaviour pre versus post enrichment, normal anxiety-related behaviour pre versus post standard, and high anxiety-related behaviour pre versus post standard. Kruskal Wallis test was used to estimate the differences between groups for the following comparisons: high anxiety-related behaviour versus normal anxiety-related behaviour mice pre-enrichment, high anxiety-related behaviour versus normal anxiety-related behaviour mice post enrichment, high anxiety-related behaviour versus normal anxiety-related behaviour pre standard, and high anxiety-related behaviour versus normal anxiety-related behaviour mice post standard. A p value < 0.05 was deemed significant; Benjamini-Hochberg adjustment with Q=0.2 was used to correct p values for

multiple testing. PCoA plots based on Bray-Curtis distance matrices were constructed using R Studio (version 1.0.136), and the “vegan” package. Base images were generated using the “ggplot2” package. For PCoA plots (with vectors, 0.8.7) with recommended parameters, Spearman correlation as correlation metric, Benjamini-Hochberg with Q < 0.1 cut-off value as multiple testing correction and method as clustering method.

4.4 Results

4.4.1 Mouse phenotype had a significant impact on anxiety-related behaviour

At the beginning of the study, mice underwent the Elevated Plus Maze behavioural test to confirm differences in anxiety-like behaviour between mouse phenotypes where high anxiety-related behaviour mice were expected to display innate enhanced anxiety/depression-like behaviour while the normal anxiety are considered to show normal anxiety/depression-like behaviour. In all aspects of the Elevated Plus Maze, there was a clear impact of mouse phenotype identified with respect to the number of open arm entries for the high anxiety mice, compared with the normal anxiety mice for both standard and enriched housing types. As expected, there was a significant difference between normal anxiety-related behaviour and high anxiety-related behaviour phenotypes pre enrichment in number of open arm entries ($p = 0.002$), percentage of open arm entries ($p = 0.002$), time spent in open arms ($p = 0.002$), time spent in closed arms ($p = 0.034$), and % time spent in open arms ($p = 0.003$) where high anxiety-related behaviour mice had fewer open arm entries and spent less time in open arms than normal anxiety-related behaviour mice (Figure 2). Similarly, several significant differences were evident between normal anxiety mice and high anxiety mice pre standard housing. High anxiety mice had fewer open arm entries for both number ($p = 0.006$) and percentage ($p = 0.008$), spent less time in open arms ($p < 0.001$), and spent more time in closed arms ($p = 0.001$) compared to normal anxiety mice (Figure 3). Therefore, mouse phenotype had a significant impact on anxiety-related behaviour as measured by the Elevated Plus Maze test and differential anxiety-like behaviour

between high anxiety-related behaviour and normal anxiety-related behaviour mice was confirmed.

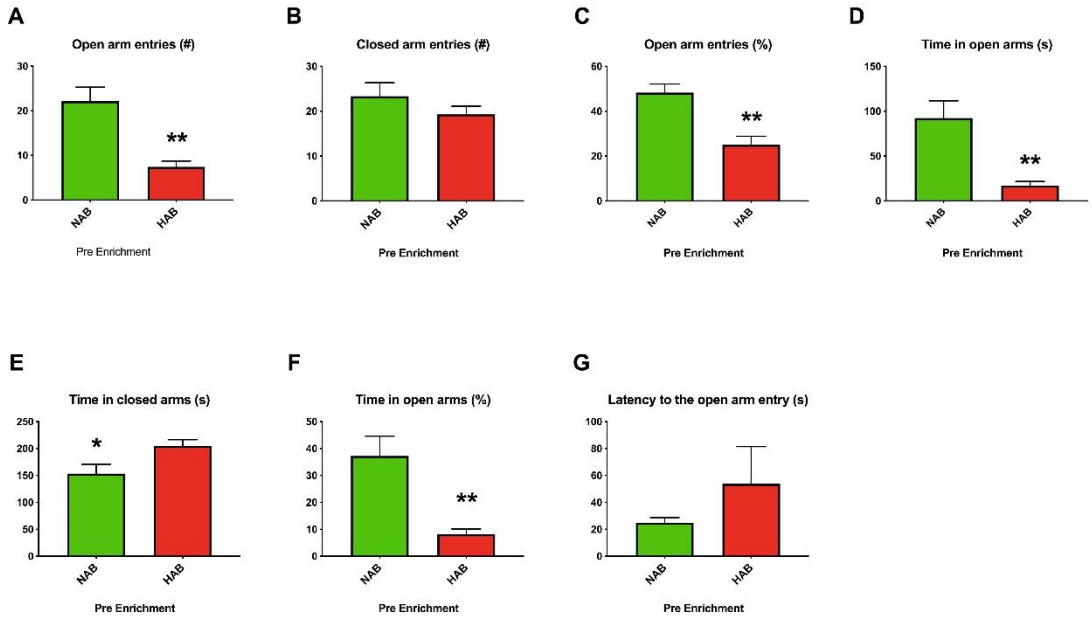


Figure 2: Confirmation of increased anxiety-like behaviour in high anxiety versus normal anxiety mice pre-environmental enrichment as assessed using the Elevated Plus Maze. Anxiety-like behaviour was assessed in both strains through (A) open arm entries (#), (B) closed arm entries (#), (C) open arm entries (%), (D) time in open arms (s), (E) time in closed arms (s), (F) time in open arms (%), (G) latency to the open arm entry (s). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ high anxiety-related behaviour pre-enrichment versus normal anxiety-related behaviour pre-enrichment.

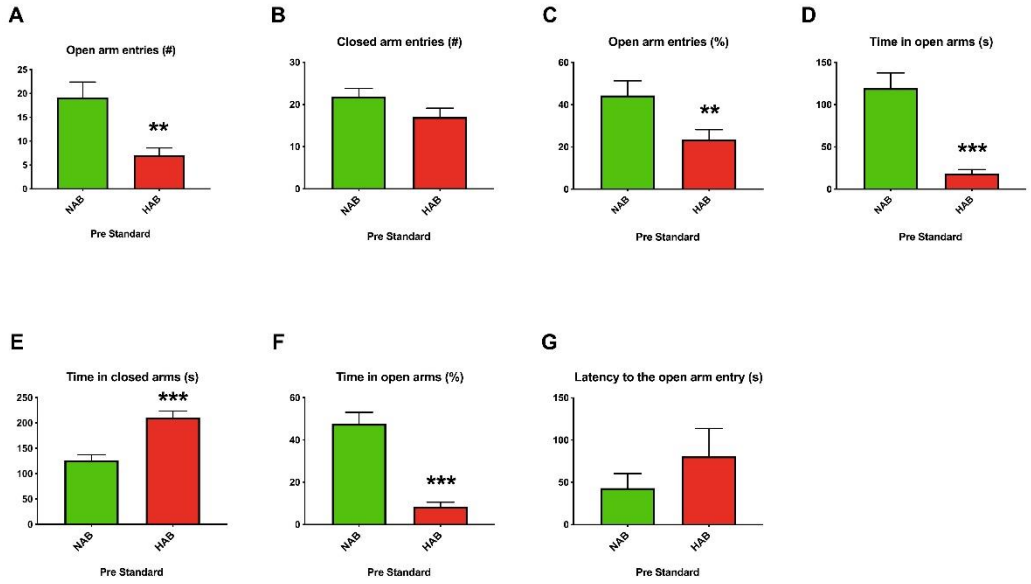


Figure 3: Confirmation of increased anxiety-like behaviour in high anxiety-related behaviour versus normal anxiety-related behaviour mice pre standard housing as assessed using the Elevated Plus Maze. Anxiety-like behaviour was assessed in both strains through (A) open arm entries (#), (B) closed arm entries (#), (C) open arm entries (%), (D) time in open arms (s), (E) time in closed arms (s), (F) time in open arms (%), (G) latency to the open arm entry (s). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ high anxiety-related behaviour pre-enrichment versus normal anxiety-related behaviour mice pre standard housing.

4.4.2 High anxiety mice displayed improved anxiety-like behaviour in the Light Dark and Open Field Tests following enriched but not standard housing

Significant differences were observed in the Light Dark Test between high anxiety-related behaviour and normal anxiety-related behaviour mice post standard conditions. There were significant differences in latency to enter the light ($p = 0.003$), time in the light ($p = 0.01$), number of visits into the light ($p = 0.002$), distance in the light ($p = 0.001$) and total distance in the Light Dark Test ($p = 0.001$) whereby normal anxiety-related behaviour mice had a greater number of visits into the light, travelled a greater distance in the light as well as in the Light Dark Test in

total compared to high anxiety-related behaviour mice (Figure 4). Interestingly, when comparing highly anxious versus non-anxious mice post enrichment, no differences were observed in any of the measurements (Figure 5). This is in stark contrast to the results observed following standard housing and suggests that environmental enrichment attenuated the enhanced anxiety-like behaviour in highly anxious mice, normalising it to levels similar to non-anxious mice.

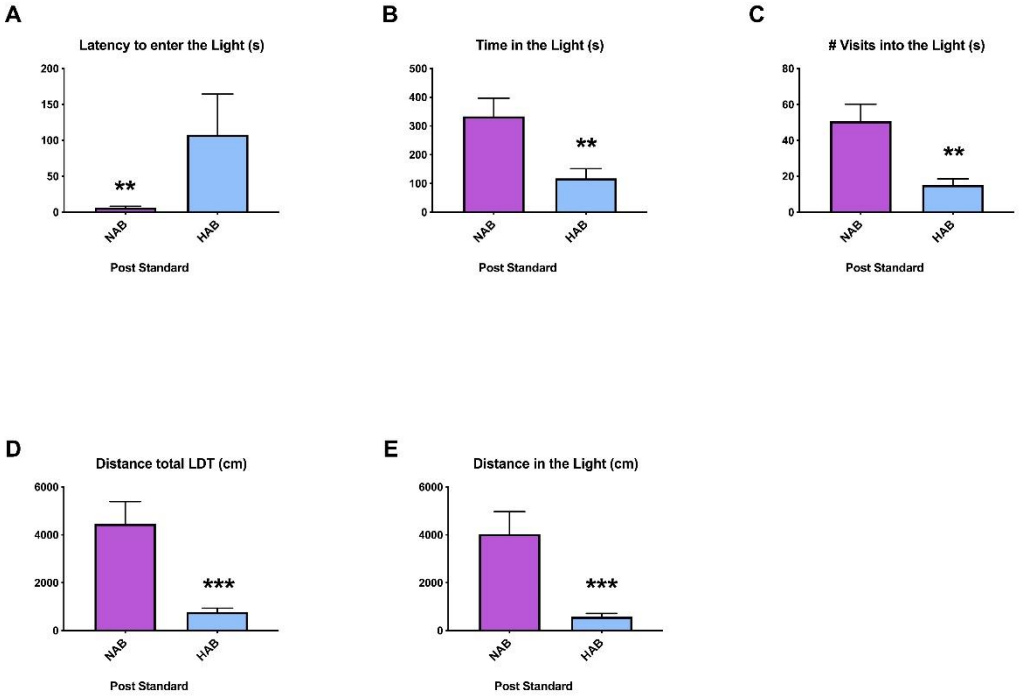


Figure 4: High anxiety-related behaviour mice display increased anxiety-like behaviour in the Light Dark Test compared to normal anxiety-related behaviour mice following housing in a standard environment. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the light (s), (B) time in the light (s), (C) visits into the light (#), (D) distance total Light Dark Test (cm), (E) distance in the light (cm). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ high anxiety-related behaviour post enrichment versus normal anxiety-related behaviour post standard.

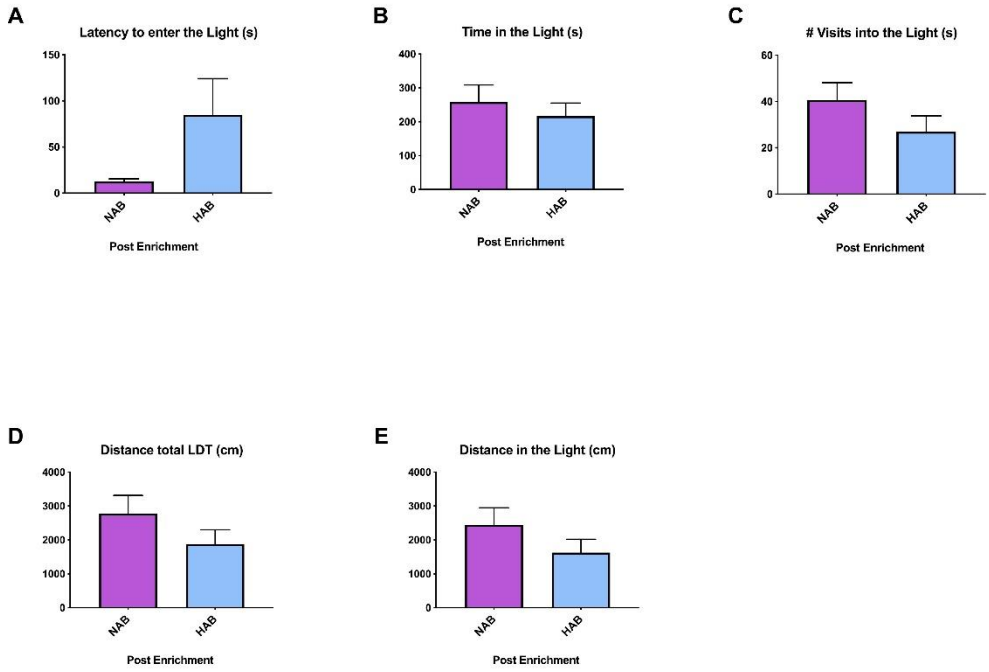


Figure 5: No differences in anxiety-like behaviour were observed in the Light Dark Test between high anxiety-related behaviour and normal anxiety-related behaviour following housing in an enriched environment. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the light (s), (B) time in the light (s), (C) visits into the light (#), (D) distance total Light Dark Test (cm), (E) distance in the light (cm). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ high anxiety-related behaviour post enrichment versus normal anxiety-related behaviour post enrichment.

The Open Field Test was also carried out to further investigate the differences in anxiety-like behaviour between groups. Similarly, highly anxious behaviour mice showed reduced anxiety-like behaviour in the Open Field Test following enriched but not standard housing. When comparing normal anxiety-related behaviour and high anxiety-related behaviour mice post environmental enrichment, significant differences were observed in number of visits into the centre ($p = 0.035$) whereby high anxiety-related behaviour mice visited the centre more than normal anxiety-related behaviour mice following environmental enrichment. They also tended to travel a greater distance in the centre ($p = 0.058$) and spend greater time in the centre ($p = 0.073$) compared to normal anxiety-related behaviour mice (Figure 6).

In contrast, when comparing high anxiety-related behaviour and normal anxiety-related behaviour mice post standard housing, a significant difference in total distance travelled in the Open Field Test was observed (p = 0.009) whereby normal anxiety mice travelled a greater distance than high anxiety mice (Figure 7). Together, the results from these behavioural tests indicate that environmental enrichment reduced anxiety-like behaviour of high anxiety phenotype mice to levels comparable to or even lower than normal anxiety phenotype mice. This is a very interesting finding, and it will be important to assess whether the gut microbiota has a role to play in the underlying mechanisms involved.

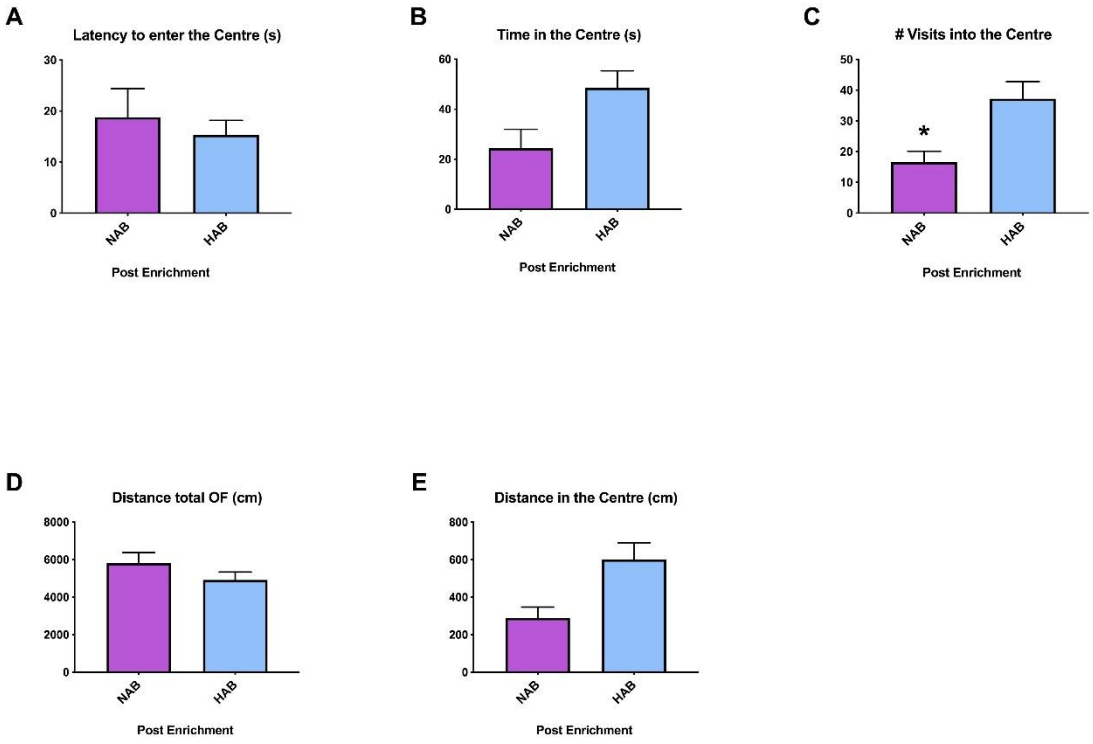


Figure 6: High anxiety-related behaviour mice display reduced anxiety-like behaviour in the Open Field test compared to normal anxiety-related behaviour mice following environmental enrichment. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the centre (s), (B) time in the centre (s), (C) visits into the centre (#), (D) distance total Open Field Test (cm), (E) distance in the centre (cm). * $p < 0.05$ high anxiety-related behaviour post enrichment versus normal anxiety-related behaviour post enrichment.

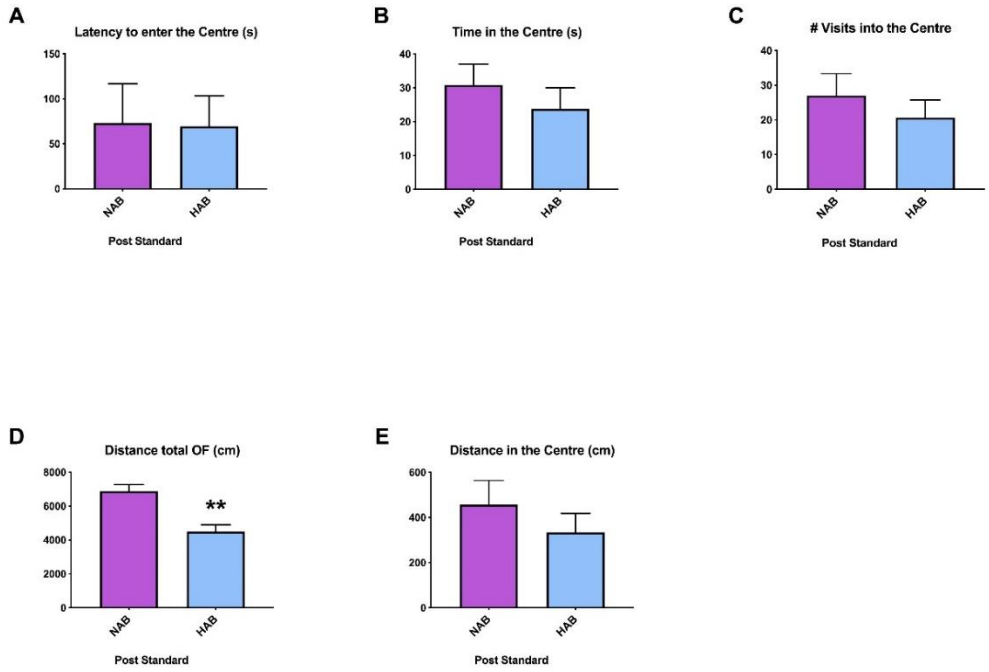


Figure 7: Normal anxiety-related behaviour mice travelled a greater distance during the Open Field Test compared to high anxiety-related behaviour mice following housing in standard conditions. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the centre (s), (B) time in the centre (s), (C) visits into the centre (#), (D) distance total Open Field Test (cm), (E) distance in the centre (cm). ** $p \leq 0.01$ high anxiety-related behaviour post enrichment versus normal anxiety-related behaviour post enrichment.

Table 1: Comparison of anxiety behaviour in highly anxious versus non-anxious mice in both enriched and standard conditions as assessed using the Light Dark Test and the Open Field Test.

Behaviour Test	Measurement	NAB vs HAB Post Enrichment	NAB vs HAB Post Standard
Light Dark Test	Latency to Enter the Light (s)	0.542	0.003 **
	Time in the Light (s)	0.574	0.01 **
	# Visits into the Light	0.083	0.002 **
	Distance in the Light (cm)	0.111	0.001 ***
	Distance Total Light Dark Test (cm)	0.16	0.001 ***
Open Field Test	Latency to Enter the Centre (s)	0.712	0.801
	Time in the Centre (s)	0.073	0.412
	# Visits into the Centre	0.035 *	0.315
	Distance in the Centre (cm)	0.058 *	0.314
	Distance Total Open Field Test (cm)	0.343	0.009 **

4.4.3 Significant differences in beta diversity were observed based on mouse phenotype and based on colonisation effect of differential facility of origin

Principal component analysis was performed to examine changes in the relative abundance of all bacterial genera in high anxiety-related behaviour and normal anxiety-related behaviour mice housed in either enriched or standard environment. To determine if any significant differences in beta diversity occurred based on environmental enrichment, principal component analysis plots were constructed based on Bray Curtis distance matrices. We sought to investigate if samples from the same mouse phenotype or environmental housing would cluster together. The resultant principal component analysis plot clearly showed the separation of the high anxiety-related behaviour and normal anxiety-related behaviour datasets indicating differential effect of housing environment on the high anxiety mice

microbiota structure (Figure 8A). Furthermore, housing in enriched environment caused a larger shift in the microbial community of high anxiety than of normal anxiety mice (p = 0.041, Supplementary Figure 1). There was a significant difference in beta diversity, as measured using Bray Curtis distance matrices, between high anxiety-related behaviour mice pre enrichment and normal anxiety-related behaviour mice pre enrichment (p=0.008), and between high anxiety-related behaviour post enrichment and normal anxiety-related behaviour post enrichment mice (p=0.008), indicating that mouse phenotype may have a greater influence on microbial beta diversity than housing in an enriched environment versus a standard environment. Clustering was also seen on principal component analysis plots comparing all enriched groups where samples clustered based on mouse phenotype (p = 0.001) (Figure 8A). Similar clustering was observed when comparing all groups housed in a standard environment (p = 0.001, Figure 8B).

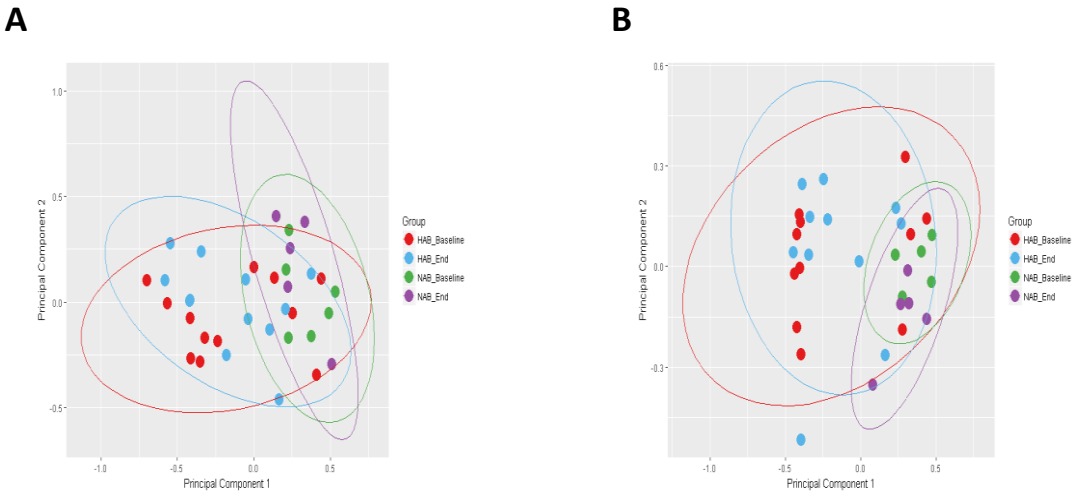


Figure 8: Visualisation of the principal component analysis based on Bray-Curtis distance matrices at genus level to determine if there is separation of samples based on mouse phenotype and time for mice in enriched (A) and standard (B) cohorts. Red represents high anxiety-related behaviour mice at baseline, while blue represents high anxiety-related behaviour mice post environmental intervention. Normal anxiety-related behaviour mice at baseline are presented in green and normal anxiety-related behaviour mice post intervention are shown in purple.

Furthermore, principal component analysis showed that while normal anxiety-related behaviour mice clustered very tightly together, high anxiety-related behaviour mice displayed quite a heterogeneous microbiota profile. Even before the enriched housing was commenced, around 50% of high anxiety-related behaviour mice clearly separated from normal anxiety-related behaviour counterparts ("high anxiety related behaviour-like" cluster), while the remaining high anxiety-related behaviour mice grouped together with normal anxiety mice ("normal anxiety related behaviour-like" cluster). To identify the bacterial species that were "driving" the separation of "high anxiety related behaviour-like" from the "normal anxiety related behaviour-like" microbiota, the relative abundance of all bacterial OTUs was correlated with the two major components of the principal component analysis plot. Differences were observed between the Standard and the Enriched cohort; however, the key taxa associated with the "high anxiety-related behaviour-like" microbiota were identified as species of *Parabacteroides, Prevotellaceae* UCG-001, *Lachnoclostridium*, *Lachnospiraceae UCG-008 and NK4A136* group, *Ruminiclostridium* and *Ruminococcus* 1 (Supplementary Figures 2A and C). The "normal anxiety related behaviour-like" cluster was associated with *Alistipes, Anaerotruncus, Lactococcus, Ruminiclostridium* 5 and 6, among other taxa. Furthermore, standard housing had very little effect on the "high anxiety-related behaviour-like" microbiota cluster, associated with *Enterohabdus*, *Parabacteroides, Prevotellaceae UCG-001, Lachnospiraceae NK4A136* group and *Ruminiclostridium* species. The composition and the abundance of this cluster remained largely intact (Supplementary Figure 2C and D, the left section of the principal component analysis plots). In contrast, enriched housing induced a substantial re-structuring of the high anxiety-related behaviour microbiota, creating a unique high anxiety-related behaviour cluster dominated almost exclusively by different members of *Lachnospiraceae* family (Supplementary Figure 2A and B). When investigating the associations, patterns of responders and non-responders were evident and it was determined that this may be due to a colonisation effect from where the animals were reared, therefore, this was investigated further.

4.4.4 Behaviour analysis and MiSeq analysis of faecal microbiota of high anxiety-related behaviour and normal anxiety-related behaviour mice housed in different environmental conditions excluding high anxiety-related behaviour mice born in CCB facility

When analysing the PCoA results, it was discovered that there was an underlying facility factor at play which was affecting the 16S rRNA sequencing data. A subsection of the high anxiety-related behaviour mice cohort was born in a different facility (CCB) to the remainder of the high anxiety-related behaviour mice and the entire cohort of normal anxiety-related behaviour mice (PM1), which was consequently skewing the results. High anxiety-related behaviour mice born in the CCB facility maintained a very different microbiota profile despite being raised in the PM1 facility and clustered separately from the remainder of the high anxiety-related behaviour mice on principal component analysis plots. Therefore, it was decided to re-analyse the results with the high anxiety-related behaviour CCB mice omitted. This resulted in an n number lower than six which is insufficient for statistical analysis as the power is too low, therefore these results are regarded as preliminary. The final n numbers for each group were as follows: high anxiety-related behaviour pre-enrichment n = 5; high anxiety-related behaviour post enrichment n = 5; normal anxiety-related behaviour pre-enrichment n = 6; normal anxiety-related behaviour post enrichment n = 5; high anxiety-related behaviour pre standard n = 4; high anxiety-related behaviour post standard n = 4; normal anxiety-related behaviour pre standard n = 5; normal anxiety-related behaviour post standard n = 5.

4.4.5 Elevated Plus Maze confirmed differential anxiety-like behaviour between normal anxiety-related behaviour and high anxiety-related behaviour phenotypes

At the beginning of the study, mice underwent the Elevated Plus Maze behavioural test to confirm differences in anxiety-like behaviour between mouse phenotypes where highly anxious mice were expected to display innate enhanced anxiety/depression-like behaviour while the normal anxiety phenotype are

considered to show normal anxiety/depression-like behaviour. In all aspects of the Elevated Plus Maze test, there was a clear impact of mouse phenotype identified with respect to the number of open arm entries for the high anxiety-related behaviour mice, compared with the normal anxiety-related behaviour mice. As expected, there was a significant difference between normal anxiety-related behaviour and high anxiety-related behaviour phenotypes pre-enrichment in number of open arm entries ($p = 0.036$), percentage of open arm entries ($p = 0.039$) and time spent in open arms ($p = 0.039$) where high anxiety-related behaviour mice had fewer open arm entries and spent less time in open arms than normal anxiety-related behaviour mice (Figure 9). Similarly, several significant differences were evident between normal anxiety-related behaviour and high anxiety-related behaviour mice pre standard housing. High anxiety mice had fewer open arm entries for both number ($p = 0.029$) and percentage ($p = 0.038$), fewer closed arm entries ($p = 0.046$), spent less time in open arms ($p = 0.008$), spent more time in closed arms ($p = 0.008$) compared to normal anxiety mice (Figure 10). Therefore, mouse phenotype had a significant impact on anxiety-related behaviour as measured by the Elevated Plus Maze test and differential anxiety-like behaviour between high anxiety and normal anxiety mice was confirmed.

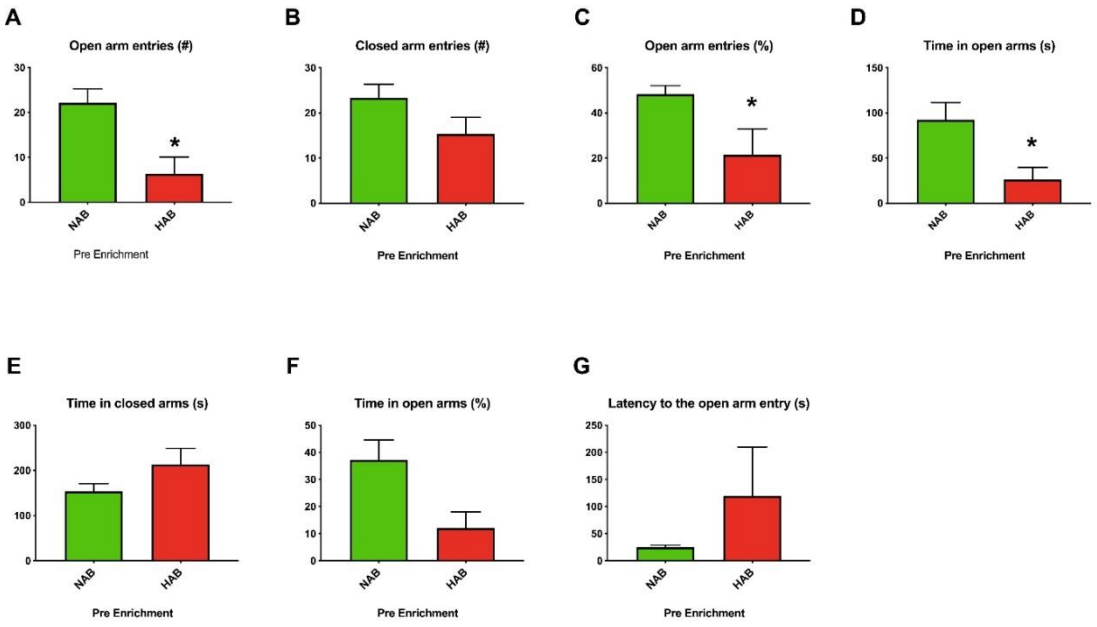


Figure 9: Confirmation of increased anxiety-like behaviour in high anxiety-related behaviour versus normal anxiety-related behaviour mice pre-environmental enrichment as assessed using the Elevated Plus Maze. Anxiety-like behaviour was assessed in both strains through (A) open arm entries (#), (B) closed arm entries (#), (C) open arm entries (%), (D) time in open arms (s), (E) time in closed arms (s), (F) time in open arms (%), (G) latency to the open arm entry (s). * $p < 0.05$ high anxiety-related behaviour pre-enrichment versus normal anxiety-related behaviour pre-enrichment.

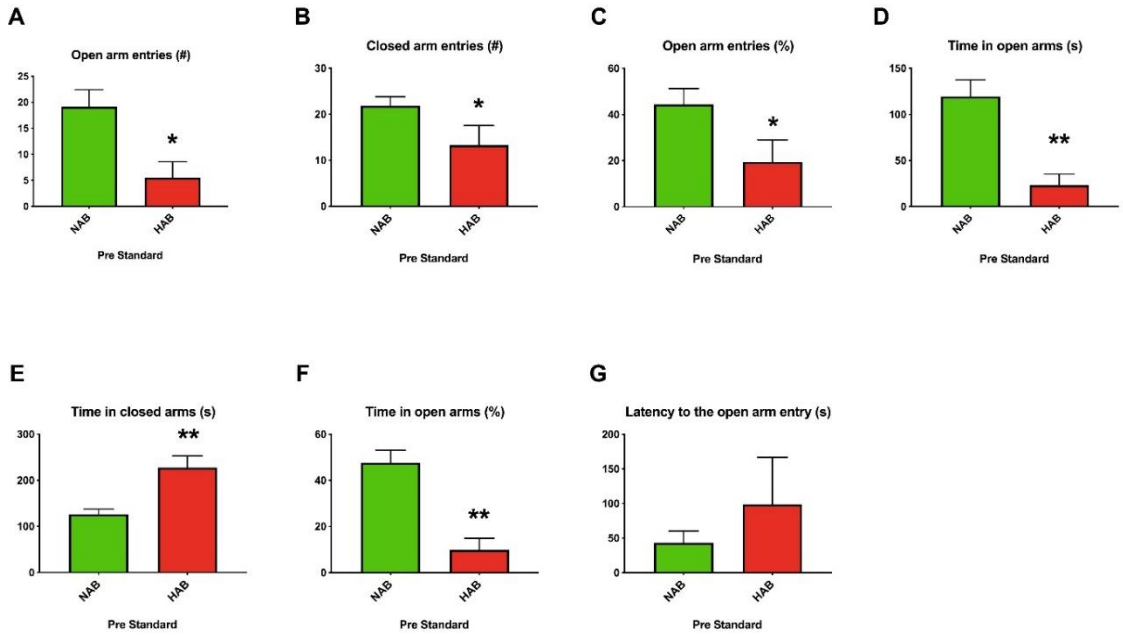


Figure 10: Confirmation of increased anxiety-like behaviour in high anxiety-related behaviour versus normal anxiety-related behaviour mice pre standard conditions as assessed using the Elevated Plus Maze. Anxiety-like behaviour was assessed in both strains through (A) open arm entries (#), (B) closed arm entries (#), (C) open arm entries (%), (D) time in open arms (s), (E) time in closed arms (s), (F) time in open arms (%), (G) latency to the open arm entry (s). * $p < 0.05$ high anxiety-related behaviour pre standard versus normal anxiety-related behaviour pre standard, ** $p < 0.01$ high anxiety-related behaviour pre standard versus normal anxiety-related behaviour pre standard.

4.4.6 Mouse phenotype had a significant impact on microbiota composition and beta diversity but not alpha diversity

At phylum level, high anxiety-related behaviour and normal anxiety-related behaviour mice shared common phyla pre-enriched and standard housing. The most prevalent phyla in all baseline groups were Bacteroidetes, Firmicutes and Proteobacteria, with average abundances of 50%, 35% and 11% respectively. Actinobacteria, Deferribacteres, Tenericutes, Verrucomicrobia, Chlamydiae, Cyanobacteria and Saccharibacteria contributed smaller proportions of the sequencing reads. Many of the significant differences were lost after removal of the CCB mice, however there was a significant difference in Chlamydiae between high anxiety-related behaviour and normal anxiety-related behaviour mice pre-enrichment ($p = 0.037$) and between high anxiety-related behaviour and normal anxiety-related behaviour mice pre standard housing ($p = 0.007$), with a higher abundance present in highly anxious mice.

At genus level, high anxiety-related behaviour and normal anxiety-related behaviour mice shared common genera pre-enriched and standard housing (Figure 11). The most prevalent genera were *Bacteroides* (5.6%), *Bacteroidales S24-7* group uncultured (13%), *Lachnospiraceae NK4A136* group (11%), *Alistipes* (10%), *Helicobacter* (8.6%), and *Prevotellaceae UCG001* (8%). When comparing high anxiety-related behaviour and normal anxiety-related behaviour phenotypes pre-enrichment, 21 significant differences at genus level were observed including *Staphylococcus* ($p = 0.032$), *Lactococcus* ($p = 0.017$) and *Tyzzerella* ($p = 0.044$). When comparing high anxiety-related behaviour and normal anxiety-related behaviour mice housed in standard environmental conditions, 25 significant differences were observed such as *Enterococcus* ($p = 0.014$), *Roseburia* ($p = 0.027$) and *Parabacteroides* ($p = 0.014$, Table 2).

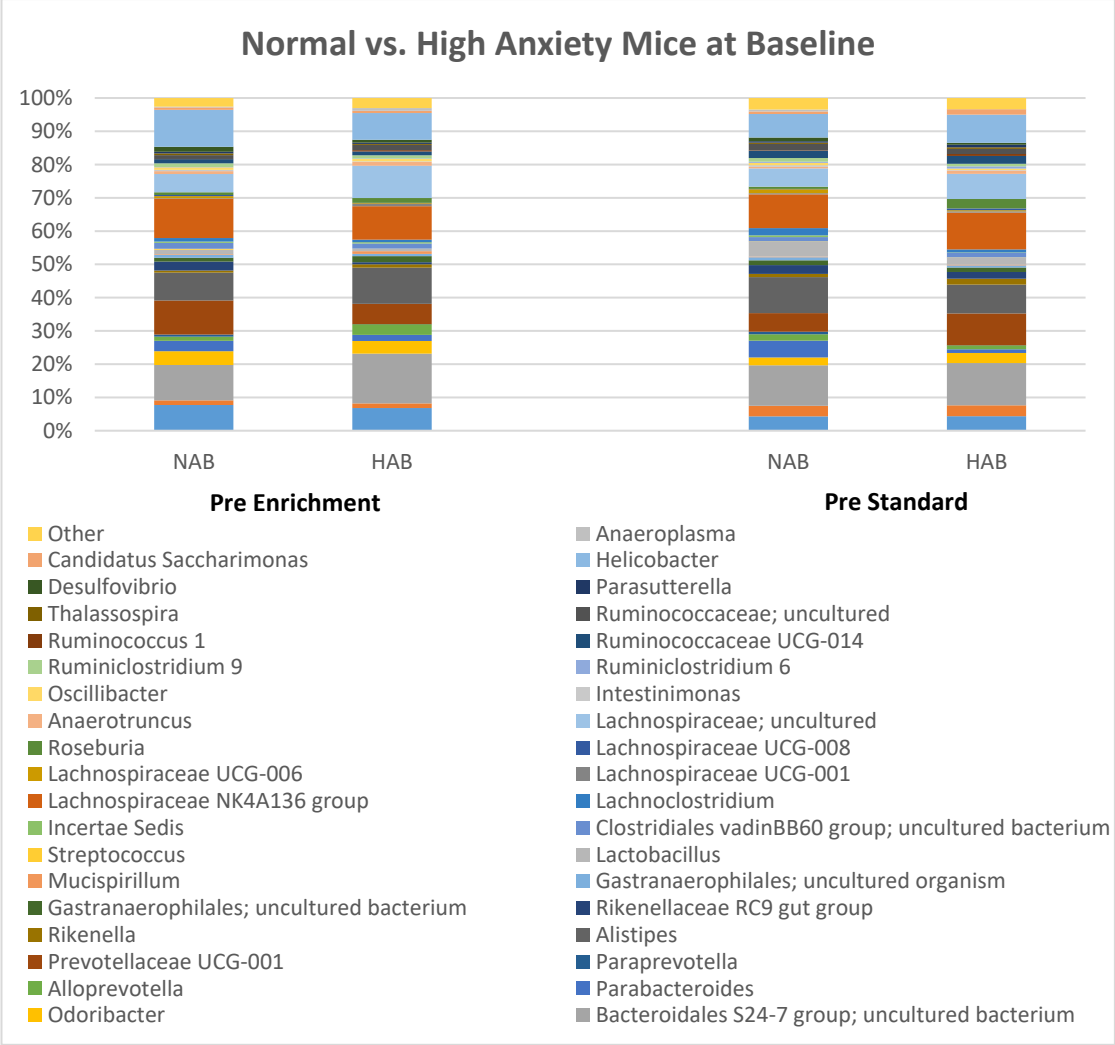


Figure 11: Relative abundances (%) distribution of bacterial genera in high anxiety-related behaviour and normal anxiety-related behaviour mice samples pre-environmental enrichment versus pre standard housing. Represented only taxa >1% in at least one group. The Other category contains all other genera present at <1% of assignable reads at genus level.

Table 2: Significant differences at genus level between high anxiety and normal anxiety mice pre-enrichment and high anxiety and normal anxiety mice pre standard housing ($p<0.05$) as calculated using the Kruskal Wallis test for non-paired groups.

Genus	FDR-corrected p value	Comparison
Coriobacteriaceae UCG 002	0.022	HAB vs. NAB Pre-Enrichment
Bacteroidetes VC2.1 Bac22 uncultured bacterium	0.004	HAB vs. NAB Pre-Enrichment
Bacteroidales S24.7 group unidentified	0.018	HAB vs. NAB Pre-Enrichment
Paraprevotella	0.005	HAB vs. NAB Pre-Enrichment
Rikenellaceae RC9 gut group	0.006	HAB vs. NAB Pre-Enrichment
Chlamydia	0.037	HAB vs. NAB Pre-Enrichment
Gemella	0.034	HAB vs. NAB Pre-Enrichment
Staphylococcus	0.032	HAB vs. NAB Pre-Enrichment
Lactococcus	0.017	HAB vs. NAB Pre-Enrichment
Christensenellaceae uncultured	0.009	HAB vs. NAB Pre-Enrichment
Defluviitaleaceae UCG 011	0.037	HAB vs. NAB Pre-Enrichment
Anaerovorax	0.045	HAB vs. NAB Pre-Enrichment
Acetitomaculum	0.022	HAB vs. NAB Pre-Enrichment
Coprococcus 2	0.028	HAB vs. NAB Pre-Enrichment
Lachnospiraceae UCG 005	0.035	HAB vs. NAB Pre-Enrichment
Lachnospiraceae UCG 006	0.018	HAB vs. NAB Pre-Enrichment
Tyzzerella	0.044	HAB vs. NAB Pre-Enrichment
Tyzzerella 3	0.01	HAB vs. NAB Pre-Enrichment
Lachnospiraceae Eubacterium ventriosum group	0.035	HAB vs. NAB Pre-Enrichment
Intestinimonas	0.045	HAB vs. NAB Pre-Enrichment
Ruminococcaceae UCG 013	0.045	HAB vs. NAB Pre-Enrichment
Bacteroidetes VC2.1 Bac22 uncultured bacterium	0.013	HAB vs. NAB Pre-Standard
Parabacteroides	0.014	HAB vs. NAB Pre-Standard
Paraprevotella	0.014	HAB vs. NAB Pre-Standard
Prevotellaceae UCG 001	0.027	HAB vs. NAB Pre-Standard

Chlamydia	0.007	HAB vs. NAB Pre-Standard
Gastranaerophilales uncultured rumen bacterium	0.011	HAB vs. NAB Pre-Standard
Staphylococcus	0.014	HAB vs. NAB Pre-Standard
Enterococcus	0.014	HAB vs. NAB Pre-Standard
Christensenellaceae R 7 group	0.011	HAB vs. NAB Pre-Standard
Christensenellaceae uncultured	0.014	HAB vs. NAB Pre-Standard
Candidatus Arthromitus	0.014	HAB vs. NAB Pre-Standard
Clostridium sensu stricto 1	0.014	HAB vs. NAB Pre-Standard
Acetitomaculum	0.029	HAB vs. NAB Pre-Standard
Incertae Sedis	0.014	HAB vs. NAB Pre-Standard
Lachnoclostridium	0.014	HAB vs. NAB Pre-Standard
Lachnospiraceae NC2004 group	0.014	HAB vs. NAB Pre-Standard
Lachnospiraceae UCG 005	0.027	HAB vs. NAB Pre-Standard
Lachnospiraceae UCG 006	0.014	HAB vs. NAB Pre-Standard
Roseburia	0.027	HAB vs. NAB Pre-Standard
Lachnospiraceae uncultured	0.014	HAB vs. NAB Pre-Standard
Peptococcus	0.027	HAB vs. NAB Pre-Standard
Ruminococcaceae UCG 011	0.014	HAB vs. NAB Pre-Standard
Erysipelotrichaceae uncultured	0.014	HAB vs. NAB Pre-Standard
Desulfovibrio	0.014	HAB vs. NAB Pre-Standard
Anaeroplasma	0.014	HAB vs. NAB Pre-Standard

No significant differences in alpha diversity were observed when comparing normal anxiety-related behaviour and high anxiety-related behaviour mice pre-environmental enrichment (Figure 12) or pre standard housing (Figure 13).

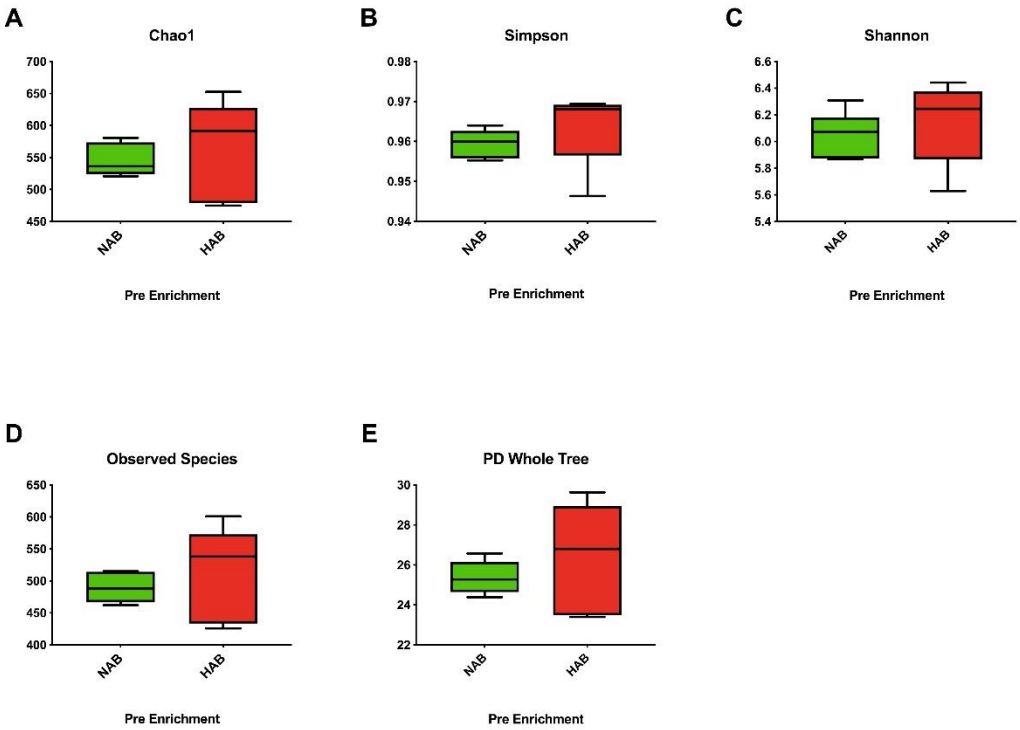


Figure 12: Alpha diversity estimates for enriched environment and mouse phenotype based on Chao1, Simpson, Shannon, Observed Species, and PD Whole Tree indices. Normal anxiety-related behaviour pre-enrichment is shown in green, and high anxiety-related behaviour pre-enrichment is shown in red.

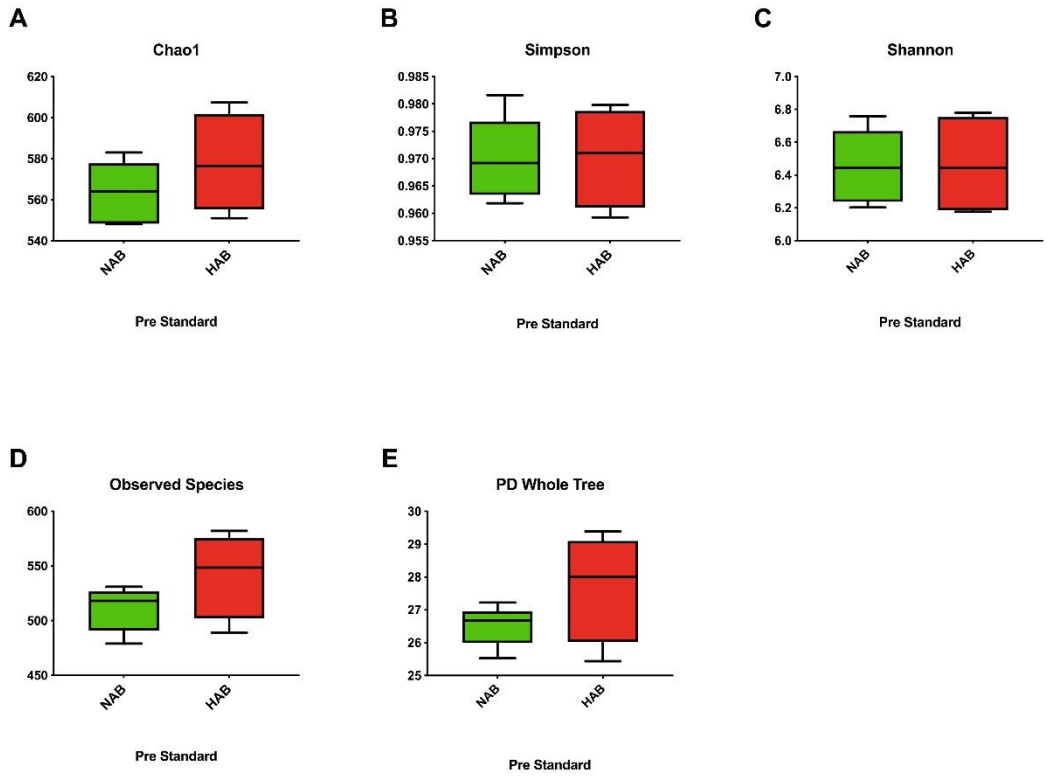


Figure 13: Alpha diversity estimates for standard environment and mouse phenotype based on Chao1, Simpson, Shannon, Observed Species, and PD Whole Tree indices. Normal anxiety-related behaviour pre standard is shown in green, and high anxiety-related behaviour pre standard is shown in red.

Differences in beta diversity were observed based on mouse phenotype. Principal component analysis was performed to examine changes in the relative abundance of all bacterial genera in high anxiety-related behaviour and normal anxiety-related behaviour mice pre enriched (Figure 14A) or standard environment (Figure 14B). To determine if any significant differences in beta diversity occurred based on mouse phenotype, PCoA plots were constructed based on Bray Curtis distance matrices. We sought to investigate if samples from the same mouse phenotype would cluster together once the confounding variable of the CCB cohort was removed. Adonis tests showed no significant differences in beta diversity between high anxiety-related behaviour and normal anxiety-related behaviour mice pre-enrichment (Figure 14A). However, there were significant differences observed between the

standard housing groups (Figure 14B). There was a significant difference between high anxiety-related behaviour and normal anxiety-related behaviour mice in standard housing conditions at baseline (p = 0.035), indicating that mouse phenotype may have an influence on microbial beta diversity. Clustering was also seen on PCoA plots comparing all standard groups where samples clustered based on mouse phenotype as samples from high anxiety-related behaviour mice clustered separately to samples from normal anxiety-related behaviour mice.

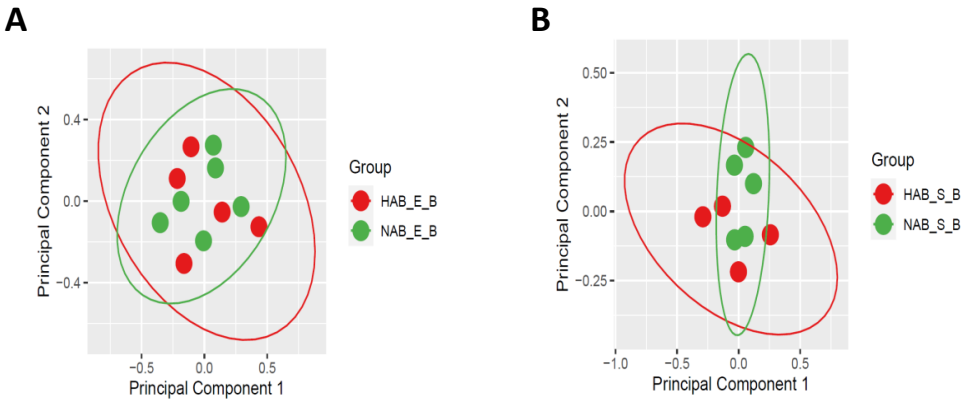


Figure 14: Visualisation of the PCoA analysis based on Bray-Curtis distance matrices at genus level to determine if there is separation of samples based on mouse phenotype for mice in the enriched (A) or standard (B) cohorts. Red represents high anxiety-related behaviour mice pre-environmental intervention, while green represents normal anxiety-related behaviour mice pre-environmental intervention.

4.4.7 Environmental enrichment resulted in significant differences in behaviour as assessed using the Light Dark Test and Open Field Test

Significant differences were observed in the Light Dark Test between high anxiety-related behaviour and normal anxiety-related behaviour mice after both enriched and standard conditions (Table 3). When comparing high anxiety versus normal anxiety mice post enrichment, there were significant differences in number of visits

into the light ($p = 0.035$), distance in the light ($p = 0.018$) and total distance in the Light Dark Test ($p = 0.028$) whereby normal anxiety-related behaviour mice had a greater number of visits into the light, travelled a greater distance in the light as well as in the Light Dark Test in total compared to high anxiety-related behaviour mice (Figure 15). Similarly, significant differences were observed between high anxiety and normal anxiety mice post standard conditions in latency to enter the light ($p = 0.037$), time in the light ($p = 0.014$), number of visits into the light ($p = 0.014$), distance in the light ($p = 0.008$) and distance total ($p = 0.008$). Normal anxiety-related behaviour mice took less time to enter the light, spent more time in the light, had more visits to the light, travelled a greater distance in the light and in the Light Dark Test in total compared to high anxiety-related behaviour mice as expected (Figure 16). When comparing high anxiety-related behaviour mice post enrichment to high anxiety-related behaviour mice post standard conditions, only one difference was observed where high anxiety mice who had been exposed to environmental enrichment spent a greater amount of time in the light compared to the high anxiety cohort in standard housing ($p = 0.05$). No differences were observed in normal anxiety-related behaviour mice post enrichment compared to post standard.

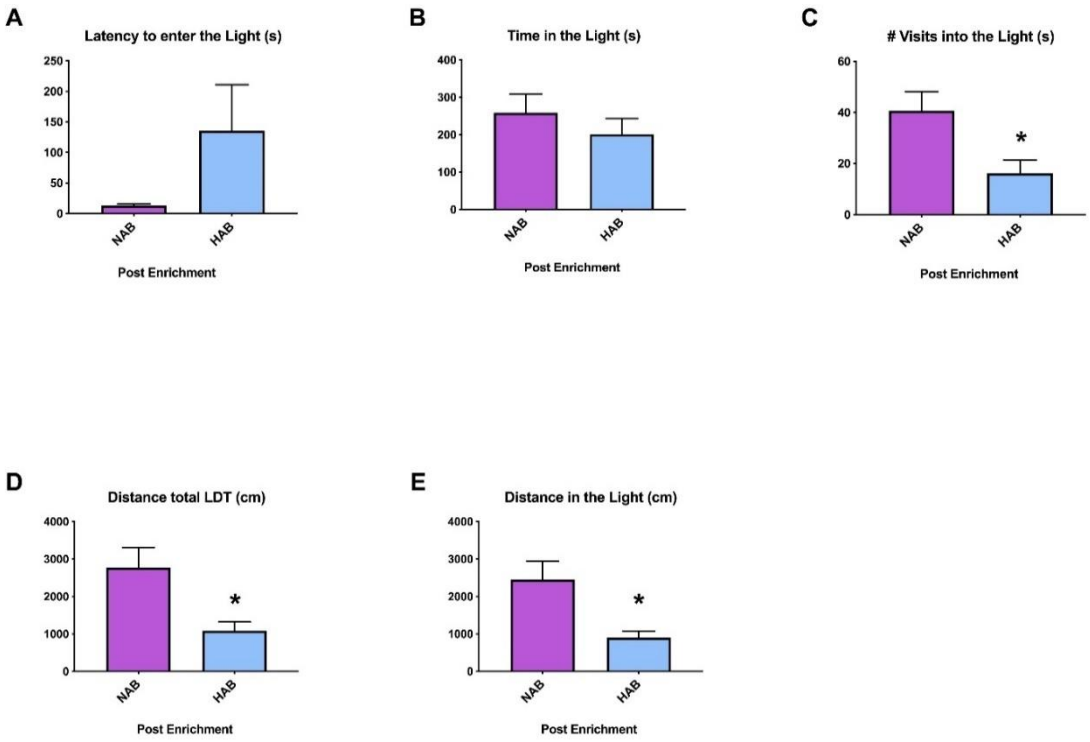


Figure 15: High anxiety-related behaviour mice display increased anxiety-like behaviour in the Light Dark test compared to normal anxiety-related behaviour mice following environmental enrichment. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the light (s), (B) time in the light (s), (C) visits into the light (#), (D) distance total Light Dark Test (cm), (E) distance in the light (cm). * $p < 0.05$ high anxiety-related behaviour post enrichment versus normal anxiety-related behaviour post enrichment.

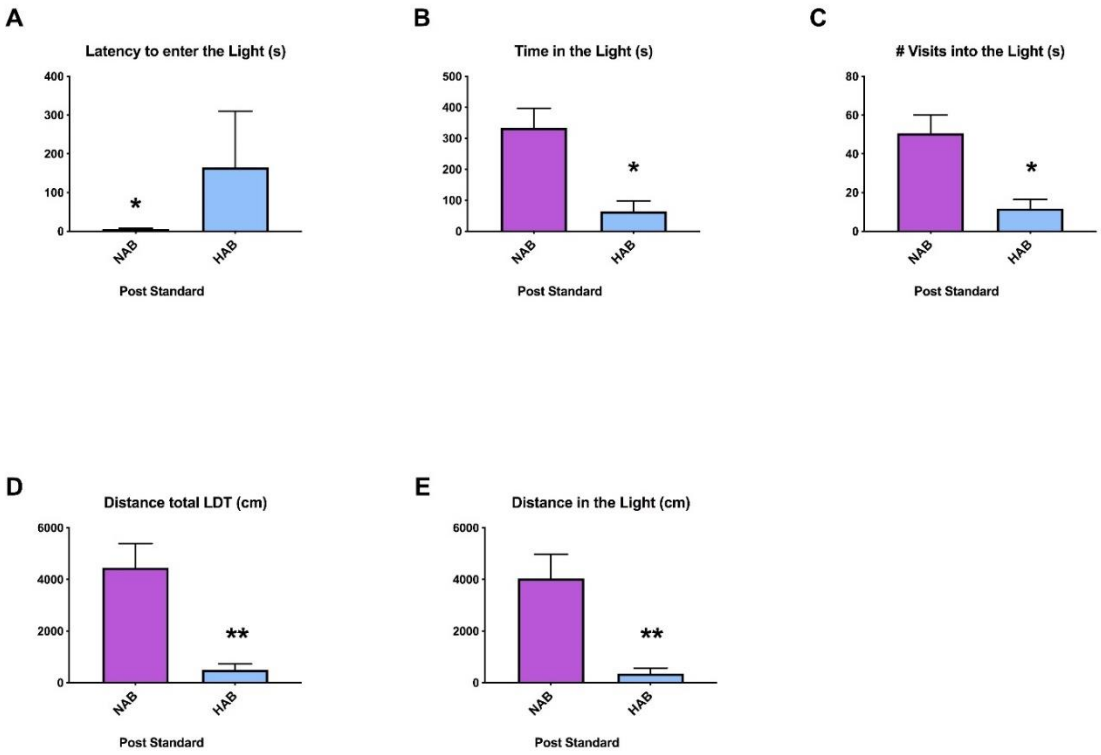


Figure 16: High anxiety-related behaviour mice display increased anxiety-like behaviour in the Light Dark test compared to normal anxiety-related behaviour mice following standard conditions. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the light (s), (B) time in the light (s), (C) visits into the light (#), (D) distance total Light Dark Test (cm), (E) distance in the light (cm). * $p < 0.05$, ** $p < 0.01$ high anxiety-related behaviour post standard versus normal anxiety-related behaviour post standard.

The Open Field Test was also carried out to further investigate the differences in anxiety-like behaviour between groups (Figure 17). Surprisingly, high anxiety-related behaviour mice post environmental enrichment had an increased number of visits to the centre ($p = 0.013$) and travelled a greater distance in the centre compared to normal anxiety-related behaviour mice post enrichment ($p = 0.028$), possibly indicating that environmental enrichment had a greater effect in reducing anxiety-like behaviour in the high anxiety-related behaviour phenotype compared to the normal anxiety phenotype. When comparing high anxiety-related behaviour and normal anxiety-related behaviour mice post standard conditions (Figure 18),

normal anxiety mice travelled a greater total distance in the Open Field Test compared to high anxiety-related behaviour mice post standard ($p = 0.011$). The enriched high anxiety-related behaviour cohort took significantly less time to enter the centre of the Open Field Test ($p = 0.014$) compared to the high anxiety-related behaviour cohort post standard conditions. They also had an increased number of visits to the centre ($p = 0.027$) and travelled a significantly greater distance in the centre ($p = 0.049$). No differences were observed between normal anxiety-related behaviour mice post enrichment versus post standard conditions.

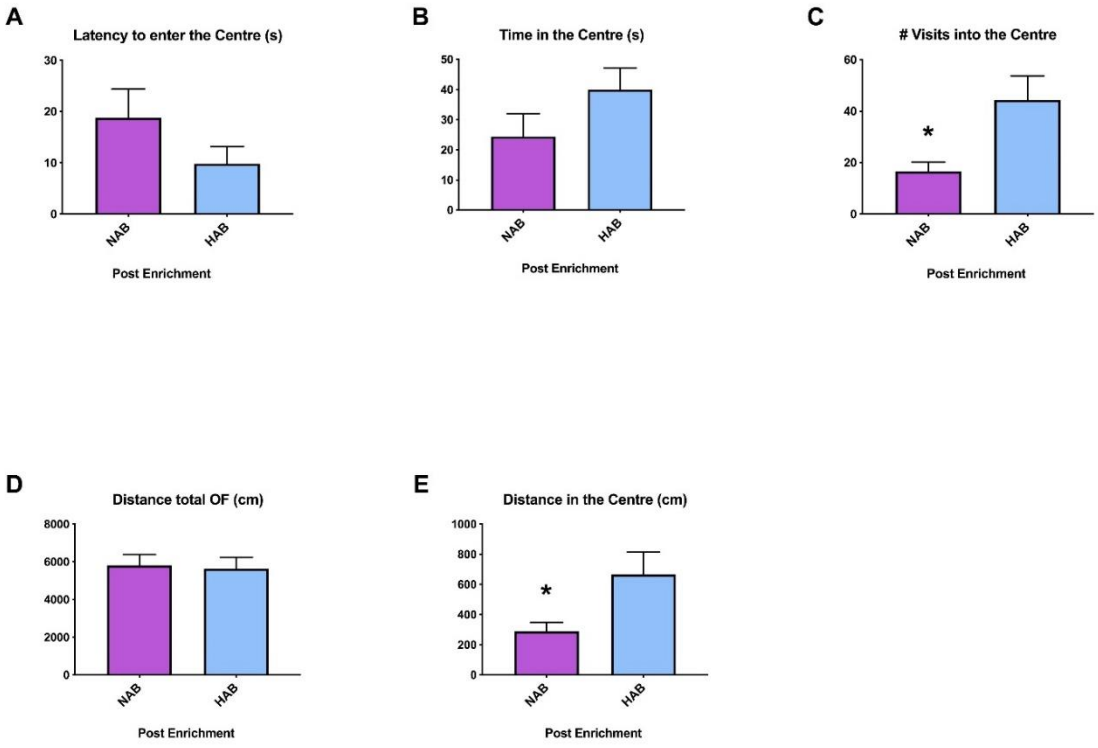


Figure 17: High anxiety-related behaviour mice display reduced anxiety-like behaviour in the Open Field Test compared to normal anxiety-related behaviour mice following environmental enrichment. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the centre (s), (B) time in the centre (s), (C) visits into the centre (#), (D) distance total Open Field Test (cm), (E) distance in the centre (cm). * $p < 0.05$ high anxiety-related behaviour post enrichment versus normal anxiety-related behaviour post enrichment.

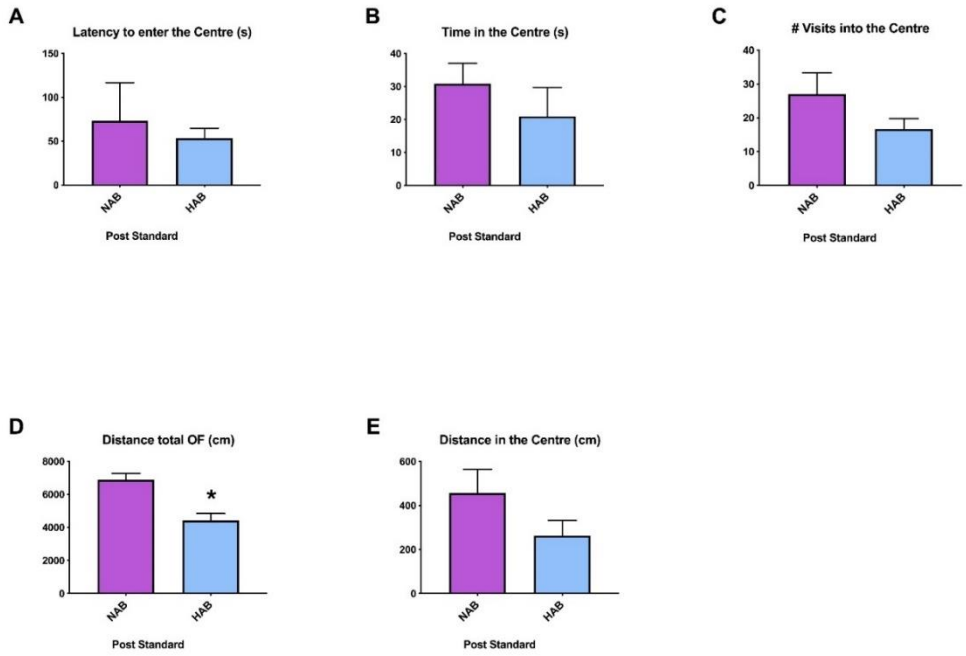


Figure 18: High anxiety-related behaviour mice display reduced anxiety-like behaviour in the Open Field Test compared to normal anxiety-related behaviour mice following standard conditions. Anxiety-like behaviour was assessed in both strains through (A) latency to enter the centre (s), (B) time in the centre (s), (C) visits into the centre (#), (D) distance total Open Field Test (cm), (E) distance in the centre (cm). * $p < 0.05$ high anxiety-related behaviour post standard versus normal anxiety-related behaviour post standard.

Table 3: Comparison of anxiety behaviour in high anxiety-related behaviour versus normal anxiety-related behaviour mice in both enriched and standard conditions as investigated using Light Dark Test and the Open Field Test.

Behaviour Test	Measurement	NAB vs HAB Post Enrichment	NAB vs HAB Post Standard

Light Dark Test	Latency to Enter the Light (s)	0.1	0.037 *
	Time in the Light (s)	0.361	0.014 *
	# Visits into the Light	0.035 *	0.014 *
	Distance in the Light (cm)	0.018 *	0.008 **
	Distance Total Light Dark Test (cm)	0.028 *	0.008 **
Open Field Test	Latency to Enter the Centre (s)	0.201	0.394
	Time in the Centre (s)	0.144	0.522
	# Visits into the Centre	0.013 *	0.201
	Distance in the Centre (cm)	0.028 *	0.201
	Distance Total Open Field (cm)	1	0.011 *

4.4.8 Environmental enrichment had little effect on microbiota composition and diversity of normal anxiety-related behaviour mice

At phylum level, normal anxiety-related behaviour mice shared common phyla pre- and post-enrichment. The most prevalent phyla observed in normal anxiety-related behaviour mice were Bacteroidetes, Firmicutes and Proteobacteria. As expected, no significant differences were observed at phylum level between normal anxiety-related behaviour mice pre- and post-enrichment and between normal anxiety-related behaviour mice pre- and post-standard conditions. When looking at genus level, again non-anxious mice shared similar genera over time and between environmental conditions, including *Bacteroides* (5.7%), *Bacteroidales S24-7* group uncultured (11.6%), *Lachnospiraceae NK4A136* group (10.7%), *Alistipes* (9%), *Helicobacter* (8%), and *Prevotellaceae UCG001* (7.6%). Limited differences were observed between normal anxiety-related behaviour mice pre- and post-enrichment. *Coprococcus 2* (p = 0.03), *Ruminiclostridium* (p = 0.008), and

Ruminiclostridium 5 (p = 0.03) were significantly different but significance was lost when the false discovery rate correction was applied. Similarly, 13 genera were significantly different in normal anxiety-related behaviour mice pre versus post standard environment, but significance was lost when the false discovery rate correction was applied. The significant genera for each group comparison and their respective p values are outlined in Table 4.

Table 4: Significant differences at genus level between normal anxiety-related behaviour mice pre versus post enrichment and normal anxiety-related behaviour mice pre versus post standard ($p<0.05$) as calculated using the Wilcox test for paired groups.

Genus	P value	FDR	Comparison
Coprococcus 2	0.031	0.961	NAB Pre vs. Post Enrichment
Ruminiclostridium	0.008	0.889	NAB Pre vs. Post Enrichment
Ruminiclostridium 5	0.032	0.961	NAB Pre vs. Post Enrichment
Rikenella	0.008	0.226	NAB Pre vs. Post Standard
Rikenellaceae RC9 gut group	0.032	0.278	NAB Pre vs. Post Standard
Enterococcus	0.032	0.278	NAB Pre vs. Post Standard
Clostridiales vadinBB60 group uncultured bacterium	0.032	0.278	NAB Pre vs. Post Standard
Clostridiales Eubacterium brachy group	0.016	0.226	NAB Pre vs. Post Standard
Clostridiales Eubacterium	0.032	0.278	NAB Pre vs. Post

nodatum group			Standard
Acetitomaculum	0.025	0.278	NAB Pre vs. Post Standard
Tyzzerella	0.008	0.226	NAB Pre vs. Post Standard
Peptococcaceae uncultured	0.008	0.226	NAB Pre vs. Post Standard
Ruminiclostridium 6	0.016	0.226	NAB Pre vs. Post Standard
Ruminococcaceae UCG 011	0.008	0.226	NAB Pre vs. Post Standard
Ruminococcaceae uncultured	0.016	0.226	NAB Pre vs. Post Standard
Desulfovibrio	0.016	0.226	NAB Pre vs. Post Standard

To determine if alterations in microbial diversity occurred as a result of environmental enrichment or standard housing in normal anxiety-related behaviour mice, alpha and beta diversity were investigated. No significant differences in alpha diversity were evident between normal anxiety-related behaviour mice pre versus post enrichment or between normal anxiety mice pre versus post standard housing using the Shannon's index, Simpson's index, Observed Species, PD Whole Tree or Chao1 indices. To determine if any significant differences in beta diversity occurred based on environmental enrichment in normal anxiety-related behaviour mice, PCoA plots were constructed based on Bray Curtis distance matrices. Again, no significant differences were observed between normal anxiety-related behaviour mice pre- and post-enrichment and between normal anxiety-related behaviour mice pre- and post-standard conditions (Figure 19). Together, these results indicate that environmental enrichment had no effect on microbial diversity in normal anxiety-related behaviour mice.

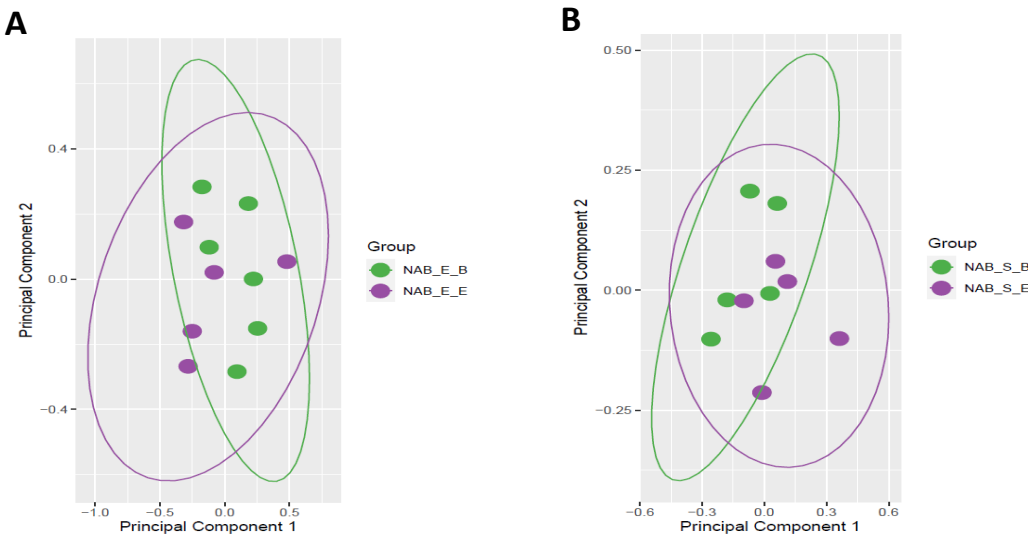


Figure 19: Visualisation of the PCoA analysis based on Bray-Curtis distance matrices at genus level to determine if there is separation of samples based on mouse phenotype and time for mice in the normal anxiety-related behaviour cohort. Normal anxiety-related behaviour baseline samples are presented in green while normal anxiety-related behaviour mice endpoint samples are shown in purple for both enriched (A) and standard (B) environments.

4.4.9 Effect of environmental enrichment on microbiome composition in high anxiety-related behaviour mice

At phylum level, high anxiety-related behaviour and normal anxiety-related behaviour mice shared common phyla pre- and post-enrichment. The most prevalent phyla in all enriched groups were Bacteroidetes, Firmicutes and Proteobacteria, with average abundances of 49.5%, 36.6% and 9.8% respectively. Actinobacteria, Deferribacteres, Tenericutes, Verrucomicrobia, Chlamydiae, Cyanobacteria and Saccharibacteria contributed smaller proportions of the sequencing reads (Figure 20). Many of the significant differences were lost after removal of the CCB mice, however there was a significant difference in Chlamydiae ($p = 0.003$) and *Other* ($p = 0.033$) between all four enriched groups, with a higher

abundance present in high anxiety-related behaviour mice. When comparing mice housed in a standard environment, again high anxiety and normal anxiety mice shared common phyla at baseline and at the end time point of standard housing (Supplementary Figure 3). The most prevalent phyla in all groups were Bacteroidetes, Firmicutes and Proteobacteria, with average abundances of 47%, 40% and 9% respectively. There was a significant difference in Chlamydiae (p = 0.002), Actinobacteria (p = 0.03) and Saccharibacteria (p = 0.016) between all four groups, with a higher abundance present in high anxiety-related behaviour mice.

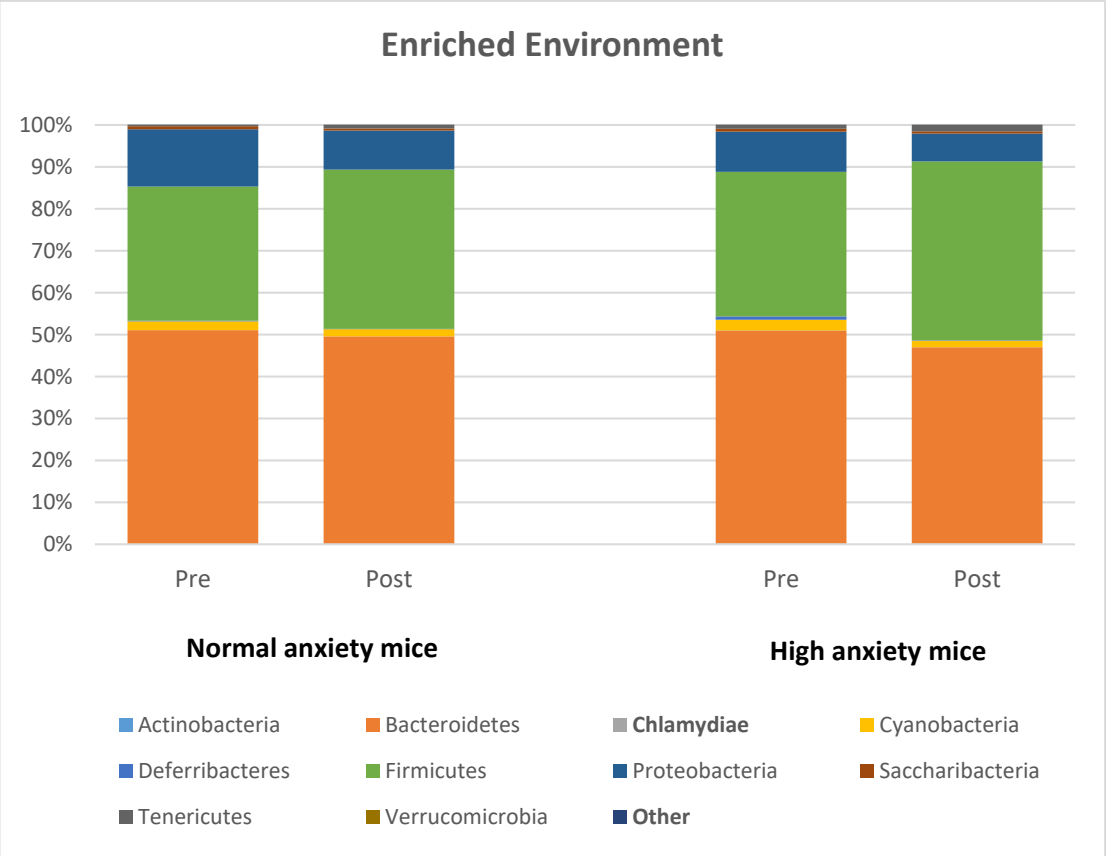


Figure 20: Relative abundances (%) distribution of bacterial phyla in normal anxiety-related behaviour and high anxiety-related behaviour mice samples pre versus post environmental enrichment. Statistically significant differences in phyla are highlighted in bold ($p<0.05$).

At genus level, high anxiety-related behaviour and normal anxiety-related behaviour mice shared common genera pre- and post-enrichment (Figure 21). The most prevalent genera were *Bacteroides* (6.7%), *Bacteroidales S24-7* group uncultured (12.5%), *Lachnospiraceae NK4A136* group (11.5%), *Alistipes* (8.3%), *Lachnospiraceae* uncultured (8%), *Helicobacter* (8%), and *Prevotellaceae UCG001* (8.4%). Overall, 20 genera were significantly different between all enrichment groups. When comparing high anxiety-related behaviour mice pre versus post enrichment, eight differences were observed, however significance was lost when false discovery rate was applied: *Coriobacteriaceae UCG 002* ($p = 0.025$), *Lactobacillus* ($p = 0.008$), *Christensenellaceae uncultured* ($p = 0.02$), *Clostridiales Eubacterium nodatum group* ($p = 0.03$), *Ruminiclostridium* ($p = 0.008$), *Ruminococcaceae UCG 013* ($p = 0.008$), *Bilophila* ($p = 0.03$) and *Mollicutes RF9 uncultured bacterium* ($p = 0.008$) (Table 5). Similarly, when comparing mice housed in standard environmental conditions, high anxiety and normal anxiety mice shared common genera pre- and post-enrichment (Supplementary Figure 4). The most prevalent genera were *Bacteroidales S24-7* group uncultured (14.3%), *Lachnospiraceae NK4A136* group (10%), *Alistipes* (8.8%), *Lachnospiraceae* uncultured (7.6%), *Helicobacter* (6.9%), and *Prevotellaceae UCG001* (7%). Overall, 39 genera were significantly different between all standard groups. When comparing high anxiety-related behaviour mice pre versus post standard, six differences were observed, however significance was lost when false discovery rate was applied. 29 significant differences were observed post enrichment between high anxiety-related behaviour and normal anxiety-related behaviour phenotypes. The significant genera for each group comparison and their respective FDR-corrected p values are outlined in Table 5.

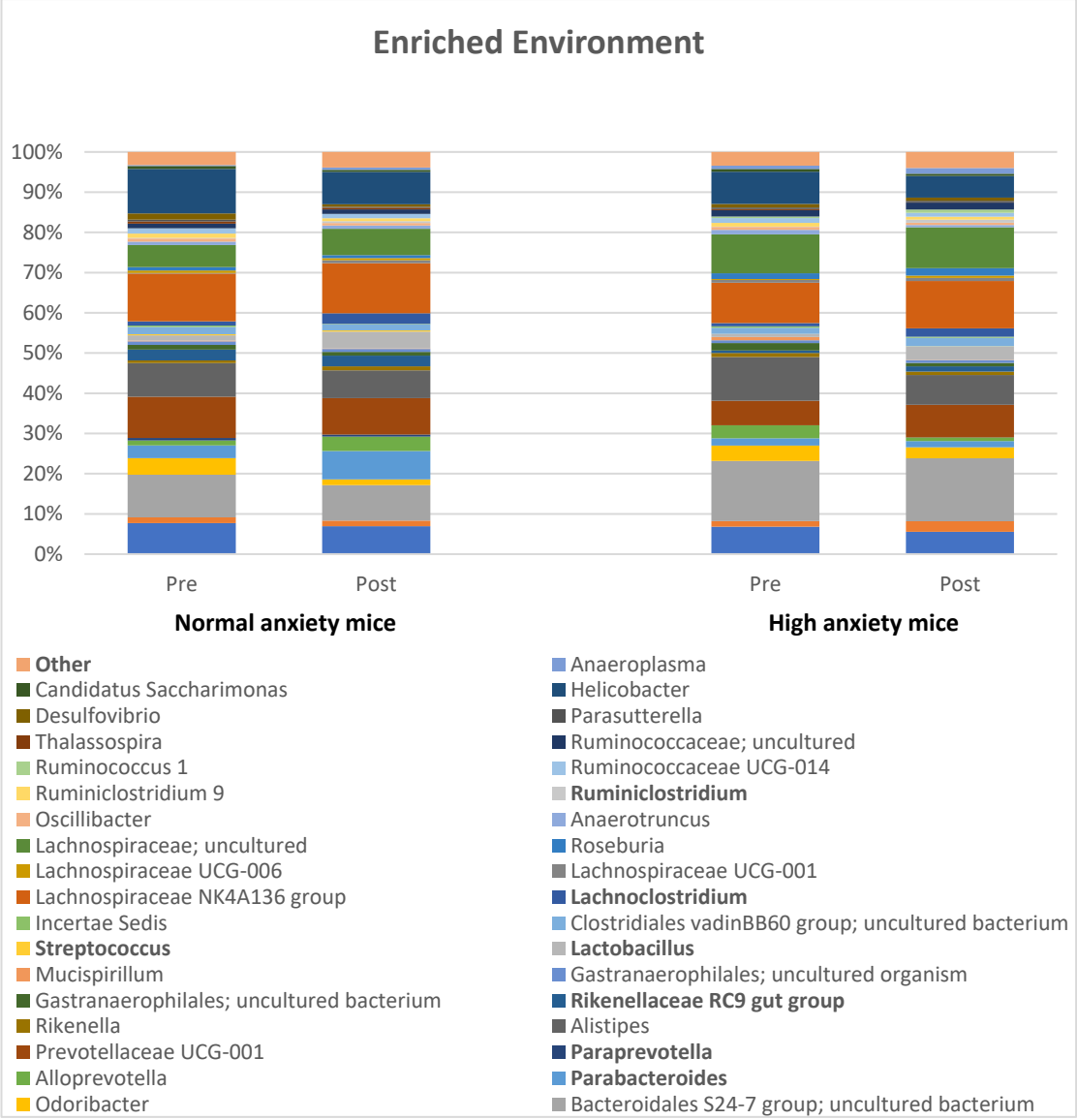


Figure 21: Relative abundances (%) distribution of bacterial genera in high anxiety-related behaviour and normal anxiety-related behaviour mice samples pre versus post environmental enrichment. Statistically significant differences are highlighted in bold ($p<0.05$). Represented only taxa >1% in at least one group. The Other category contains all other genera present at <1% of assignable reads at genus level.

Table 5: Significant differences at genus level between all groups ($p<0.05$) as calculated using the Wilcox test for paired data and the Kruskal Wallis test for non-paired groups.

Genus	FDR-corrected p value	Comparison
Coriobacteriaceae UCG 002	0.448	HAB Pre vs. Post Enrichment
Lactobacillus	0.224	HAB Pre vs. Post Enrichment
Christensenellaceae uncultured	0.448	HAB Pre vs. Post Enrichment
Clostridiales Eubacterium nodatum group	0.448	HAB Pre vs. Post Enrichment
Ruminiclostridium	0.224	HAB Pre vs. Post Enrichment
Ruminococcaceae UCG 013	0.224	HAB Pre vs. Post Enrichment
Bilophila	0.448	HAB Pre vs. Post Enrichment
Mollicutes RF9 uncultured bacterium	0.224	HAB Pre vs. Post Enrichment
Bacteroidetes VC2.1 Bac22 uncultured bacterium	0.013	HAB vs. NAB Post Enrichment
Parabacteroides	0.028	HAB vs. NAB Post Enrichment
Alloprevotella	0.028	HAB vs. NAB Post Enrichment
Paraprevotella	0.009	HAB vs. NAB Post Enrichment
Chlamydia	0.005	HAB vs. NAB Post Enrichment
Gemella	0.019	HAB vs. NAB Post Enrichment
Streptococcus	0.009	HAB vs. NAB Post Enrichment
Acetitomaculum	0.019	HAB vs. NAB Post Enrichment
Coprococcus.2	0.007	HAB vs. NAB Post Enrichment
Marvinbryantia	0.016	HAB vs. NAB Post Enrichment
Roseburia	0.047	HAB vs. NAB Post Enrichment
Tyzzerella.3	0.009	HAB vs. NAB Post Enrichment
Lachnospiraceae uncultured	0.016	HAB vs. NAB Post Enrichment
Other	0.016	HAB vs. NAB Post Enrichment
Coriobacteriaceae UCG 002	0.449	HAB Pre vs. Post Standard
Bacteroidales BS11 gut group uncultured bacterium	0.449	HAB Pre vs. Post Standard
Gemella	0.449	HAB Pre vs. Post Standard

Clostridium sensu stricto 1	0.449	HAB Pre vs. Post Standard
Anaerovorax	0.449	HAB Pre vs. Post Standard
Ruminococcaceae Eubacterium coprostanoligenes group	0.449	HAB Pre vs. Post Standard
Coriobacteriaceae UCG 002	0.007	HAB vs. NAB Post Standard
Bacteroidetes VC2 1 Bac22 uncultured bacterium	0.013	HAB vs. NAB Post Standard
Bacteroidales BS11 gut group uncultured bacterium	0.014	HAB vs. NAB Post Standard
Parabacteroides	0.014	HAB vs. NAB Post Standard
Paraprevotella	0.011	HAB vs. NAB Post Standard
Rikenella	0.014	HAB vs. NAB Post Standard
Chlamydia	0.007	HAB vs. NAB Post Standard
Gastranaerophilales uncultured rumen bacterium	0.011	HAB vs. NAB Post Standard
Gemella	0.007	HAB vs. NAB Post Standard
Staphylococcus	0.011	HAB vs. NAB Post Standard
Enterococcus	0.014	HAB vs. NAB Post Standard
Lactococcus	0.027	HAB vs. NAB Post Standard
Streptococcus	0.05	HAB vs. NAB Post Standard
Christensenellaceae R 7 group	0.014	HAB vs. NAB Post Standard
Clostridiales vadinBB60 group uncultured Clostridiales bacterium	0.032	HAB vs. NAB Post Standard
Clostridiales Family XIII AD3011 group	0.027	HAB vs. NAB Post Standard
Clostridiales Eubacterium nodatum group	0.027	HAB vs. NAB Post Standard
Acetitomaculum	0.014	HAB vs. NAB Post Standard
Coprococcus 2	0.027	HAB vs. NAB Post Standard
Tyzzerella 3	0.014	HAB vs. NAB Post Standard

Lachnospiraceae uncultured	0.014	HAB vs. NAB Post Standard
Ruminococcaceae NK4A214 group	0.014	HAB vs. NAB Post Standard
Ruminococcaceae UCG 009	0.014	HAB vs. NAB Post Standard
Ruminococcaceae UCG 011	0.011	HAB vs. NAB Post Standard
Ruminococcaceae UCG 014	0.027	HAB vs. NAB Post Standard
Ruminococcaceae Eubacterium coprostanoligenes group	0.013	HAB vs. NAB Post Standard
Erysipelatoclostridium	0.014	HAB vs. NAB Post Standard
Candidatus Saccharimonas	0.027	HAB vs. NAB Post Standard
Other	0.027	HAB vs. NAB Post Standard

4.4.10 Alpha diversity was not different between high anxiety-related behaviour and normal anxiety-related behaviour phenotypes and was not affected by housing type

To determine if alterations in microbial diversity occurred as a result of environmental enrichment, alpha and beta diversity were investigated. Five different measures of alpha diversity were used as response variables to test the effect of diet on the microbiome community: Shannon's index, Simpson's index, Observed Species, PD Whole Tree and Chao1. The Shannon index was employed to measure the overall diversity of the community, including both the number of present taxa and evenness, while Chao1 was used to quantify the number of species present in a community, omitting the abundance of each species. Chao1 is an estimate of richness, while Shannon and Simpson indices are indicators of evenness. Richness measures the number of present taxa, while evenness measures how evenly abundant the present taxa are. The alpha diversity of microbial community was not different between high anxiety-related behaviour and normal anxiety-related behaviour groups once the CCB mice were removed and was not affected by housing type (Figure 22). There was no significant difference in alpha

diversity when comparing all groups using the Chao1, Observed Species or Simpson indexes. No significant differences were observed in highly anxious mice pre versus post enrichment, or between highly anxious and non-anxious mice post enrichment. Similarly, there was no significant difference in alpha diversity between the standard housing groups (Supplementary Figure 5). Taken together, these results indicate that environmental enrichment had no effect on the alpha diversity of high anxiety-related behaviour mice.

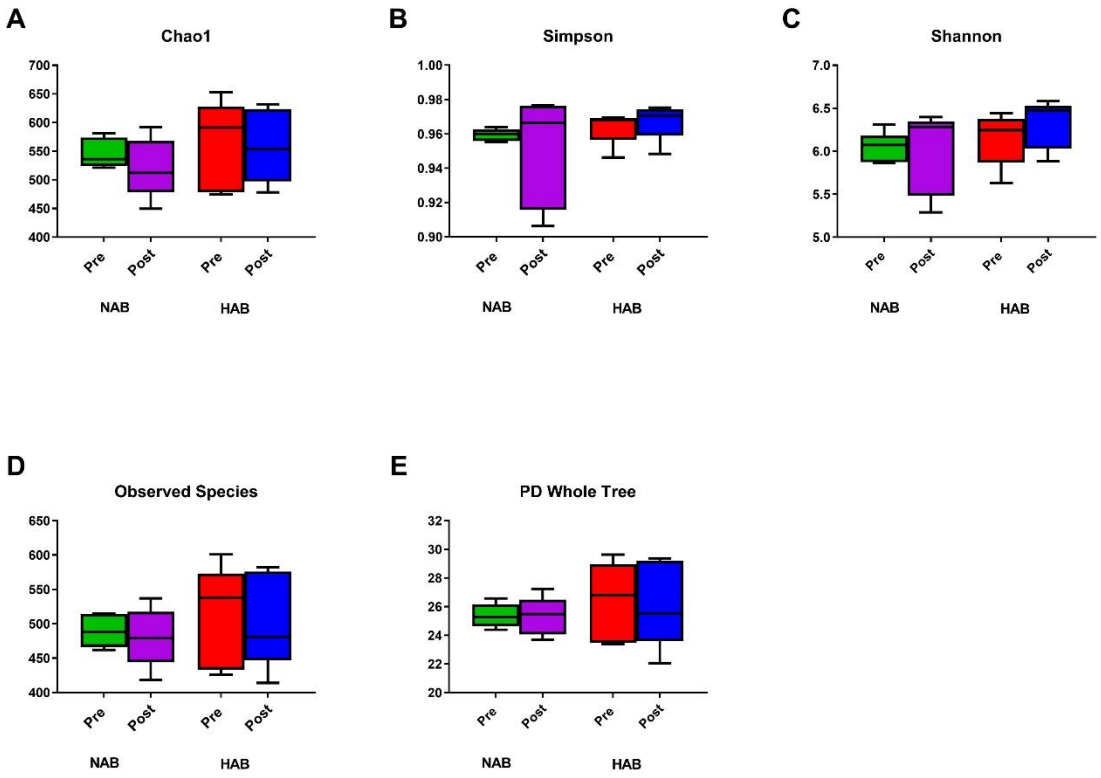


Figure 22: Alpha diversity estimates for enriched environment and mouse phenotype based on Chao1 (A), Simpson (B), Shannon (C), Observed Species (D) and PD Whole Tree (E) indices. Non-anxious mice at baseline and at endpoint are shown in green and purple respectively, while highly anxious mice at baseline are shown in red and are shown in blue at endpoint.

4.4.11 Differences in beta diversity of high anxiety-related behaviour mice were observed based on mouse phenotype but not environmental conditions

Principal coordinate analysis was performed to examine changes in the relative abundance of all bacterial genera in high anxiety-related behaviour and normal anxiety-related behaviour mice housed in either enriched or standard environment. To determine if any significant differences in beta diversity occurred based on environmental enrichment, PCoA plots were constructed based on Bray Curtis distance matrices. We sought to investigate if samples from the same mouse phenotype or environmental housing would cluster together once the confounding variable of the CCB cohort was removed. Adonis tests showed no significant differences in beta diversity between high anxiety and normal anxiety mice pre versus post enrichment (Figure 23), although there was a trend towards significance between the highly anxious and non-anxious mice post environmental enrichment ($p = 0.055$, 23B) where highly anxious mice clustered separately to normal anxiety mice. However, there were significant differences observed between the standard housing groups. There was a significant difference between high anxiety-related behaviour and normal anxiety-related behaviour mice in standard housing conditions at baseline ($p = 0.035$) and between highly anxious mice and non-anxious mice post standard conditions ($p = 0.009$, Figure 23D), while no separation was visible between high anxiety-related behaviour mice pre versus post standard. These results indicate that mouse phenotype may have a greater influence on microbial beta diversity than housing in an enriched environment versus a standard environment because samples clustered based on phenotype but not based on environment. However, no significant differences were observed between high anxiety-related behaviour and normal anxiety-related behaviour mice post enrichment, whereas significant differences were found between high anxiety and normal anxiety mice post standard housing, suggesting that environmental enrichment may reduce differences in beta diversity between high anxiety and normal anxiety mice compared to those mice not exposed to enrichment. This will be an interesting finding to investigate further. However, these results should be interpreted cautiously due to the low n number of this study. Furthermore,

differences in beta diversity which were previously observed between high anxiety-related behaviour and normal anxiety-related behaviour mice post enrichment were lost once mice from the CCB facility were removed, suggesting that this separation was due to the different facilities and not due to the study variables.

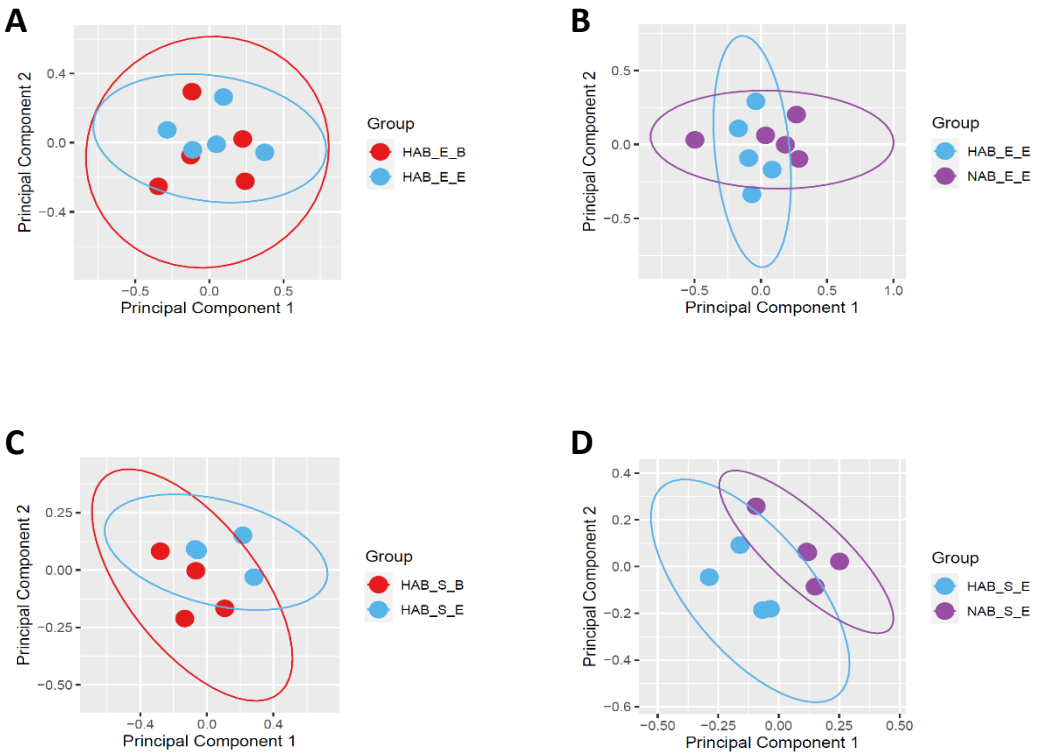


Figure 23: Visualisation of the PCoA analysis based on Bray-Curtis distance matrices at genus level to determine if there is separation of high anxiety-related behaviour mice based on phenotype and time between high anxiety-related behaviour pre vs. post enrichment (A), high anxiety-related behaviour vs. normal anxiety-related behaviour post enrichment (B), high anxiety-related behaviour pre vs. post standard (C), and high anxiety-related behaviour vs. normal anxiety-related behaviour post standard (D). Red represents high anxiety-related behaviour mice pre-environmental intervention, while blue represents high anxiety-related behaviour mice post environmental intervention, and normal anxiety-related behaviour mice post environmental intervention are presented in purple.

To further interrogate the factors associated with the clustering of samples from mice in enriched environment groups based on diversity, a number of additional multivariate analyses were carried out, including Canonical Correspondence Analysis, Canonical Correspondence Analysis+, Principal Component Analysis, Principal Component Analysis+, Redundancy Analysis and Redundancy Analysis+ to further investigate the complex associations between community composition and various explanatory variables (Figure 24). Principal Component Analysis allows data visualisation as a 2D plot, identifying sample clusters and determining outliers, which may need to be excluded from downstream analysis, while Canonical Correspondence Analysis tests if variations in the data matrix can be explained by selected variables, and Redundancy Analysis indicates how well samples can be separated according to the defined explanatory variables. Distinct separation of all four enriched groups is clearly visible in Canonical Correspondence Analysis, Canonical Correspondence Analysis+, Redundancy Analysis and Redundancy Analysis+ plots, where high anxiety-related behaviour mice cluster tightly together away from normal anxiety-related behaviour mice which also cluster tightly together on both the Principal Component Analysis and Principal Component Analysis+ plots (Figure 24). Significant differences were observed based on strain using both Canonical Correspondence Analysis+ ($p = 0.001$) and Redundancy Analysis+ ($p = 0.001$), while timepoint was significantly different using Redundancy Analysis+ ($p = 0.002$). Significance was accepted as $p<0.05$. These additional multivariate tests further suggest that the microbiome differences in this study were as a result of mouse phenotype rather than environment.

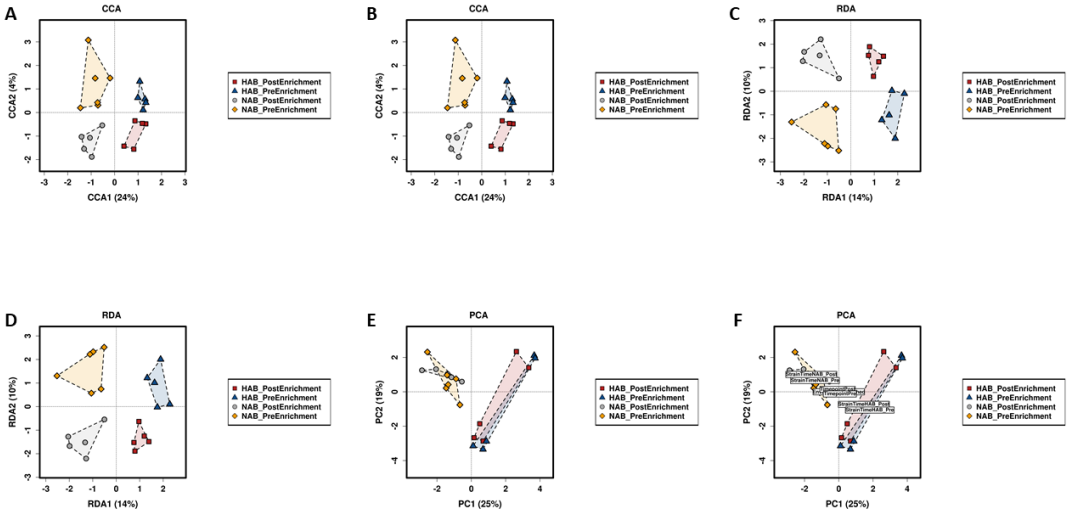


Figure 24: Multivariate Analysis performed in Calypso: (A) Canonical Correspondence Analysis, (B) Canonical Correspondence Analysis+, (C) Redundancy Analysis, (D) Redundancy Analysis+, (E) Principal Component Analysis, and (F) Principal Component Analysis+ for all groups to further investigate the complex associations between community composition and various explanatory variables. The high anxiety-related behaviour mice pre-enrichment group is shown in blue, high anxiety-related behaviour mice post enrichment in red, the normal anxiety-related behaviour mice pre-enrichment group in yellow and the normal anxiety-related behaviour post enrichment group in grey.

To determine whether there were bacteria present in the gut microbiota that could discriminate based on enriched environment between high anxiety normal anxiety mice, a feature selection statistical analysis (LDA Effect Size (LEfSe)) was used, which determined the genera most likely to explain differences between groups. LEfSe identified one discriminating genus for the normal anxiety-related behaviour pre enrichment group (*Rikenellaceae RC9 gut group*), two discriminating genera for the normal anxiety-related behaviour post enrichment group (*Parabacteroides* and *Streptococcus*), one discriminating genus for the high anxiety-related behaviour pre enrichment group (uncultured bacterium), and three discriminating taxa for the

high anxiety-related behaviour post enrichment group (*Ruminiclostridium*, *Tyzzerella* and uncultured bacterium) (Figure 25).

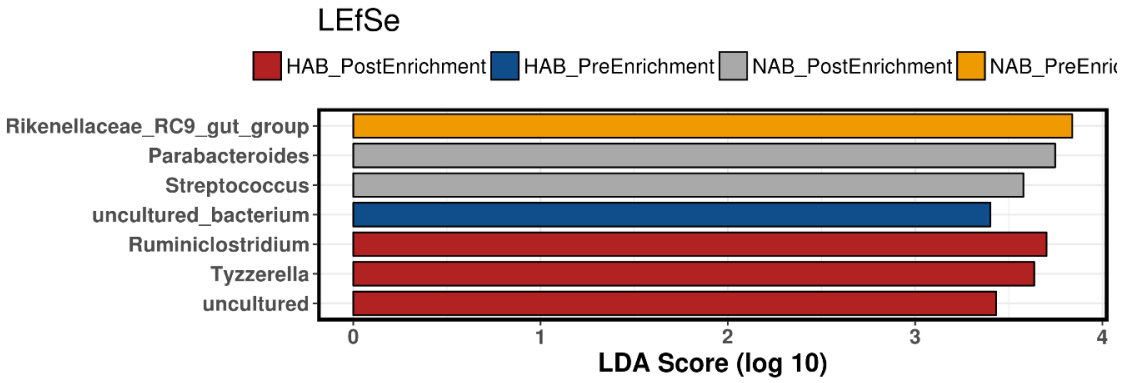


Figure 25: Feature selection statistical analysis (LDA Effect Size (LEfSe)) was used to determine the genera most likely to explain differences between groups. LEfSe identified one discriminating bacteria for the normal anxiety-related behaviour pre-enrichment group (yellow), two discriminating taxa for the normal anxiety-related behaviour post enrichment group (grey), one discriminating genus for the high anxiety-related behaviour pre-enrichment group (blue) and three discriminating genera for the high anxiety-related behaviour post enrichment group (red).

LEfSe analysis was repeated for all groups (Figure 26) and identified six discriminating bacteria for the normal anxiety-related behaviour pre-enrichment group (black), three discriminating taxa for the normal anxiety-related behaviour post enrichment group (green), four discriminating genera for the high anxiety-related behaviour pre-enrichment group (grey) and five discriminating genera for the high anxiety-related behaviour post enrichment group (red). LEfSe identified four discriminating bacteria for the normal anxiety-related behaviour pre standard (purple), five for normal anxiety-related behaviour post standard (yellow), two for high anxiety-related behaviour pre standard (orange) and six for high anxiety-related behaviour post standard (blue) as outlined below.

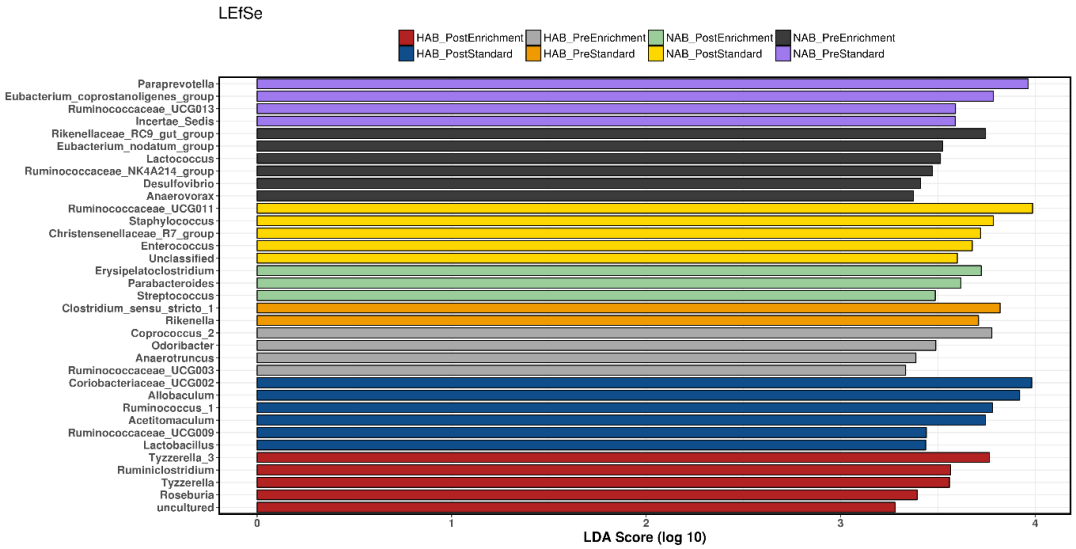


Figure 26: Feature selection statistical analysis (LDA Effect Size (LEfSe)) was used to determine the genera most likely to explain differences between all groups. LEfSe identified six discriminating bacteria for the normal anxiety-related behaviour pre-enrichment group (black), three discriminating taxa for the normal anxiety-related behaviour post enrichment group (green), four discriminating genera for the high anxiety-related behaviour pre-enrichment group (grey) and five discriminating genera for the high anxiety-related behaviour post enrichment group (red). LEfSe identified four discriminating bacteria for the normal anxiety-related behaviour pre standard (purple), five for normal anxiety-related behaviour post standard (yellow), two for high anxiety-related behaviour pre standard (orange) and six for high anxiety-related behaviour post standard (blue).

Finally, hierarchical clustering of the 50 most abundant genera in the enriched groups ($p < 0.05$; analysis of variance) were visualised using a heatmap to determine patterns in gut microbiota based on mouse phenotype, environment, and time (Figure 27). Rows represent explanatory variables, columns represent taxa, red represents a positive correlation and blue represents a negative correlation. Some notable differences are highlighted below, for example, genera highlighted in the first row were present at greater abundances in the normal anxiety-related behaviour pre-enrichment group, while genera highlighted in the

seventh row were present at greater abundances in the normal anxiety-related behaviour post enrichment group and genera highlighted in the third row were present at greater abundances in normal anxiety-related behaviour phenotype. Genera highlighted in the fourth row were present in higher abundances in the high anxiety-related behaviour pre-enrichment group, genera highlighted in the sixth row were lower in the high anxiety-related behaviour mice post enrichment and genera highlighted in the fifth row were higher (red) and lower (blue) in highly anxious mice.

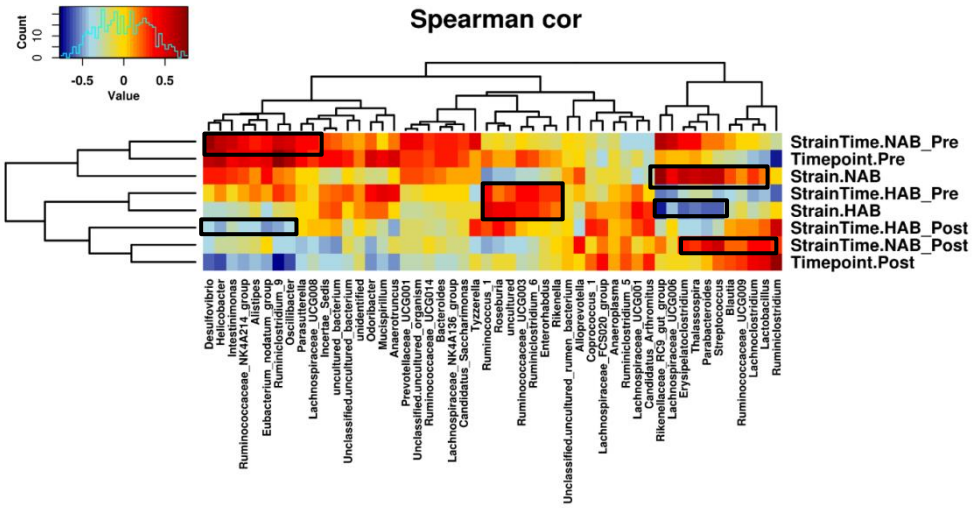


Figure 27: Microbiota associated with normal anxiety-related behaviour or high anxiety-related behaviour phenotype and with environmental enrichment. Heatmap based on genus relative abundance data. Rows represent explanatory variables, columns represent taxa, red represents a positive correlation and blue represents a negative correlation. Black boxes highlight positive and negative correlations observed based on explanatory variables.

4.5 Discussion

It has been well established that high anxiety-related behaviour mice display enhanced anxiety-like behaviour compared to normal anxiety-related behaviour mice, as evidenced by increased risk assessment behaviour in the Elevated Plus Maze Test, Open Arm Exposure Test, Light/Dark Avoidance Test and Ultrasound Vocalisation Test as well as a preference for immobility/passive stress-coping strategies in the Forced Swim and Tail Suspension tests and anhedonia assessed in the Sucrose Preference Test (Sah *et al.*, 2012; Schmuckermair *et al.*, 2013). Previous studies have also shown altered c-Fos response in several brain areas of the anxiety/fear circuitry in high anxiety mice including prefrontal-cortical, limbic and hypothalamic areas, indicating altered neuronal activation (Muigg *et al.*, 2009); aberrant sleep patterns and alterations in electroencephalogram activities (Jakubcakova *et al.*, 2012); reduced serotonergic transmission at presynaptic and postsynaptic levels (Keck *et al.*, 2005); reduced hippocampal neurogenesis (Sartori, Landgraf and Singewald, 2011); and dysregulation of circadian rhythms in high anxiety-related behaviour mice (Griesauer *et al.*, 2014). Environmental enrichment has been shown to provide several beneficial physiological, neurobiological and behavioural effects such as altering brain structure, promoting superior learning (Renner and Rosenzweig, 1987a) and cognitive function, reducing anxiety (Benaroya-Milshtein *et al.*, 2004; Chamove, 1989; Nilsson *et al.*, 1999), facilitating recovery from brain lesions (Will *et al.*, 1977), combatting addiction (Thiel *et al.*, 2009), and reversing the detrimental effects of past stressors (Escorihuela, Tobeña and Fernández-Teruel, 1994) such as prenatal stress by increasing social interaction, decreasing corticosterone secretion and strengthening the immune system (Laviola *et al.*, 2004; Morley-Fletcher *et al.*, 2003). The impact of environmental conditions on behaviour, particularly anxiety and stress are also well-known. Multiple studies have previously shown that enrichment increases the number of open arm entries and time spent in open arms in the Elevated Plus Maze, which is indicative of reduced anxiety (Chapillon *et al.*, 1999; Fernandez-Teruel *et al.*, 1997; Friske and Gammie, 2005). The protective effects of environmental enrichment are particularly apparent in animals that are highly anxious or when the task is

especially challenging for the subject, for example, environmental enrichment reduced freezing behaviour in the highly anxious BALB/c mice (Chapillon *et al.*, 1999). Furthermore, the influence of environmental conditions on gut microbiome diversity and composition has also been demonstrated previously. Rothschild *et al.* (2018) found that genetically unrelated individuals who share a house possess significant similarities in their microbiome composition compared to relatives who are from different households. Similarly, a twin study demonstrated that the gut microbiomes of twins became more genetically dissimilar when living apart over time (Xie *et al.*, 2016). While numerous studies have explored the impact of environmental enrichment on psychological and physiological measures, there has been limited investigation into the effects of environmental enrichment on gut microbiome composition and diversity, and whether these changes may play an underlying role in mediating the effects of environmental enrichment. Therefore, the aim of this study was to investigate whether environmental enrichment can attenuate enhanced anxiety and depression-like behaviour in high anxiety mice compared to normal anxiety mice and, if so, whether alterations in gut microbial composition and diversity could be involved in the underlying mechanisms. Additionally, we aimed to assess whether the gut microbiota profile differed between high anxiety-related behaviour and normal anxiety-related behaviour mice.

At the beginning of the study, enhanced anxiety-like behaviour was confirmed in high anxiety mice compared to normal anxiety mice using the Elevated Plus Maze. As expected, there was a clear impact of mouse phenotype identified in the Elevated Plus Maze with respect to the number of open arm entries and the time spent in open arms for the high anxiety mice, compared with the normal anxiety mice whereby high anxiety mice had fewer open arm entries and spent less time in open arms. This is in agreement with a previous study by Salome *et al.* (2002) who found that high anxiety-related behaviour mice spent less time on the open arms and had fewer percentage entries than their low anxiety-related behaviour counterparts. Anxiety behaviour was then assessed post enrichment using the Light Dark Test and Open Field Test. Interestingly, the high anxiety-related behaviour

mice did not display any significant differences to normal anxiety-related behaviour mice post enrichment in the Light Dark Test, while multiple differences were observed between normal anxiety and high anxiety mice post standard conditions including latency to enter the light, time in the light, number of visits into the light, distance in the light and total distance in the Light Dark Test whereby normal anxiety mice had a greater number of visits into the light, travelled a greater distance in the light as well as in the Light Dark Test in total compared to high anxiety mice. Similarly, high anxiety mice showed reduced anxiety-like behaviour in the Open Field Test following enriched but not standard housing. When comparing normal anxiety-related behaviour and high anxiety-related behaviour mice post environmental enrichment, significant differences were observed in number of visits into the centre whereby high anxiety mice visited the centre more than normal anxiety-related behaviour mice following environmental enrichment. They also tended to travel a greater distance in the centre and spend greater time in the centre compared to normal anxiety-related behaviour mice, although these findings failed to reach significance. In contrast, when comparing high anxiety and normal anxiety mice post standard housing, a significant difference in total distance travelled in the Open Field Test was observed whereby normal anxiety mice travelled a greater distance than high anxiety mice. Together, the results from these behavioural tests suggest that environmental enrichment reduced anxiety-like behaviour of highly anxious mice to levels comparable to or even lower than non-anxious mice. This is a very interesting finding, so we then investigated whether the gut microbiota had a role to play in the underlying mechanisms involved. However, when examining associations with the microbiome results, we noticed patterns of responders and non-responders and it was determined that this may be due to a colonisation effect from where the animals were reared. Upon further investigation it was found that a subsection of the high anxiety-related behaviour mice cohort was born in a different facility (CCB) to the remainder of the high anxiety-related behaviour mice and the entire normal anxiety-related behaviour cohort (PM1), which was consequently skewing the results. High anxiety-related behaviour mice born in the CCB facility maintained a very different microbiota profile despite being raised in the PM1 facility and clustered separately

from the remainder of the high anxiety-related behaviour mice on PCoA plots. Therefore, it was decided to re-analyse the results with the high anxiety mice from the CCB facility omitted which resulted in a low n number, reducing the power of this study so consequently these results should be regarded as preliminary.

The behavioural analysis was repeated with the high anxiety mice from the CCB facility cohort omitted and similar findings were observed in the Elevated Plus Maze test, reaffirming the altered anxiety profile of highly anxious mice compared to non-anxious mice. There was a significant difference between normal anxiety and high anxiety phenotypes pre-enrichment in number of open arm entries, percentage of open arm entries and time spent in open arms where highly anxious mice had fewer open arm entries and spent less time in open arms than non-anxious mice. To determine whether enrichment influenced anxiety behaviour in high anxiety mice, the Light Dark Test and Open Field Test were again analysed without the highly anxious from the CCB facility. However, many of the positive effects of enrichment observed in the Light Dark Test and Open Field Test with the full dataset were lost. Significant differences were observed in the Light Dark Test between high anxiety-related behaviour and normal anxiety-related behaviour mice for both post enriched and post standard conditions whereby normal anxiety mice had a greater number of visits into the light, travelled a greater distance in the light as well as in the Light Dark Test in total compared to high anxiety-related behaviour mice. Similarly, significant differences were observed between high anxiety-related behaviour and normal anxiety-related behaviour mice post standard conditions as normal anxiety mice took less time to enter the light, spent more time in the light, had more visits to the light, travelled a greater distance in the light and in the Light Dark Test in total compared to high anxiety mice. An interesting finding was observed when high anxiety mice that had been exposed to environmental enrichment spent a greater amount of time in the light compared to high anxiety mice who were housed in standard conditions. No differences were observed in normal anxiety mice post enrichment compared to post standard. Interestingly, high anxiety mice post enrichment demonstrated improved anxiety-like behaviour in the Open Field Test compared to normal anxiety mice post enrichment as they

had an increased number of visits to the centre and travelled a greater distance in the centre compared to normal anxiety mice post enrichment, possibly indicating that environmental enrichment had a greater effect in reducing anxiety-like behaviour in the highly anxious phenotype compared to the non-anxious phenotype. When comparing high anxiety-related behaviour and normal anxiety-related behaviour mice post standard conditions, normal anxiety mice travelled a greater total distance in the Open Field Test compared to high anxiety mice post standard. The enriched high anxiety cohort took significantly less time to enter the centre of the Open Field Test compared to the high anxiety cohort post standard conditions. They also had an increased number of visits to the centre and travelled a significantly greater distance in the centre. As expected, no differences were observed between normal anxiety mice post enrichment versus post standard conditions. These diverging behavioural results after the removal of the high anxiety mice from the CCB facility compared to the full dataset could indicate that the facility factor skewed the results or that the power is too low to obtain reliable results. In order to determine this, the present study would need to be repeated with a larger dataset of mice from the same facility and work is currently underway to carry this out.

The influence of mouse phenotype on microbiome diversity and composition was also investigated at baseline. Many of the significant differences were lost after removal of the CCB mice, however there was a significant difference at phylum level in Chlamydiae between high anxiety-related behaviour and normal anxiety-related behaviour mice pre-enrichment and between high anxiety-related behaviour and normal anxiety-related behaviour mice pre standard housing, with a higher abundance present in highly anxious mice. Similarly, when comparing high anxiety and normal anxiety phenotypes at genus level pre-enrichment, 21 significant differences at genus level were observed including *Staphylococcus*, *Lactococcus*, and *Tyzzerella*. When comparing high anxiety and normal anxiety mice housed in standard environmental conditions, 25 significant differences were observed such as *Enterococcus*, *Roseburia*, and *Parabacteroides*. No significant differences in alpha diversity were observed when comparing normal anxiety and

high anxiety mice pre-environmental enrichment or pre standard housing, indicating that phenotype does not have a substantial influence on microbial alpha diversity. However, differences in beta diversity were observed based on mouse phenotype. Adonis tests showed no significant differences in beta diversity between high anxiety-related behaviour and normal anxiety-related behaviour mice pre-enrichment. However, there were significant differences observed between high anxiety and normal anxiety mice in standard housing conditions at baseline, indicating that mouse phenotype may have an influence on microbial beta diversity. The influence of facility was also evident when examining the microbiome results. When comparing high anxiety and normal anxiety mice pre-enrichment, there were significant differences in Firmicutes, Proteobacteria and Other between highly anxious and non-anxious mice. Firmicutes and Other were increased in the high anxiety-related behaviour group, while Proteobacteria was higher in the normal anxiety-related behaviour group pre-enrichment. This contrasts with just one significant difference with the high anxiety mice from the CCB facility omitted. Similarly, at genus level, 41 genera were significantly different between the high anxiety-related behaviour and normal anxiety-related behaviour phenotypes when examining the full dataset in contrast to 21 differences with the CCB mice omitted. Some of these included *Bacteroides, Staphylococcus, Enterococcus, Lactococcus, Streptococcus, Desulfovibrio*, and *Helicobacter*. *Roseburia* was increased in the high anxiety-related behaviour group, while *Staphylococcus, Streptococcus, Bacteroides, Enterococcus, Lactococcus, Desulfovibrio* and *Helicobacter* were higher in the normal anxiety-related behaviour group pre-enrichment. Contrasting findings for beta diversity were also observed whereby significant differences between high anxiety-related behaviour mice pre-enrichment and normal anxiety-related behaviour mice pre-enrichment were evident with the full dataset but lost when the CCB mice were removed. Clustering was also seen on PCoA plots comparing all enriched groups where samples clustered based on mouse phenotype. Again, it is unclear whether the greater number of differences observed from the full dataset were as a result of the differences in facility of origin, or whether the n number of the revised dataset is too low to distinguish these differences and further studies will be required to investigate this query.

As expected, environmental enrichment had little impact on the microbiome of normal anxiety-related behaviour mice as minimal differences were observed between normal anxiety mice pre versus post enrichment. At phylum level, normal anxiety mice shared common phyla pre- and post-enrichment and no significant differences were observed at phylum level between normal anxiety mice pre- and post-enrichment, or between normal anxiety mice pre- and post-standard conditions. When looking at genus level, again normal anxiety-related behaviour mice shared similar genera over time and between environmental conditions, including *Bacteroides*, *Bacteroidales S24-7* group uncultured, *Lachnospiraceae* NK4A136 group, *Alistipes*, *Helicobacter*, and *Prevotellaceae* UCG001. Limited differences were observed between normal anxiety-related behaviour mice pre and post enrichment. *Coprococcus 2*, *Ruminiclostridium*, and *Ruminiclostridium 5* were significantly different but significance was lost when the false discovery rate correction was applied. Similarly, 13 genera were significantly different in normal anxiety-related behaviour mice pre versus post standard environment, but significance was lost when the false discovery rate correction was applied. No significant differences in alpha diversity were evident between non-anxious mice pre versus post enrichment or between non-anxious mice pre versus post standard housing using the Shannon's index, Simpson's index, Observed Species, PD Whole Tree or Chao1 indices. To determine if any significant differences in beta diversity occurred based on environmental enrichment in normal anxiety-related behaviour mice, PCoA plots were constructed based on Bray Curtis distance matrices. Again, no significant differences were observed between normal anxiety-related behaviour mice pre- and post-enrichment and between normal anxiety-related behaviour mice pre- and post-standard conditions. Together, these results indicate that environmental enrichment had no effect on microbial diversity in normal anxiety mice, which is in agreement with other studies which suggest that the protective effects of environmental enrichment are particularly apparent in animals that are highly anxious or when the task is especially challenging for the subject. For example, environmental enrichment reduced freezing behaviour in the highly anxious BALB/c mice (Chapillon *et al.*, 1999). Similarly, environmental enrichment

reduced freezing behaviour following predator exposure only in females, which are more emotionally reactive to predator exposure (Klein *et al.*, 1994). Furthermore, a more challenging task highlights the anxiolytic effect of environmental enrichment (Fernández-Teruel *et al.*, 2002), for example, enrichment improved maze exploration in the relatively more complex eight-arm maze but not in the four-arm maze (Janus *et al.*, 1995). These results suggest that environmental enrichment may be therapeutically anxiolytic only in situations of pronounced anxiety (Fox, Merali and Harrison, 2006).

When examining the effect of environmental enrichment on high anxiety-related behaviour mice, many of the significant differences were lost after removal of the CCB mice. However, there was a significant difference in Chlamydiae and Other between enriched cohorts, with a higher abundance present in high anxiety-related behaviour mice. Conversely, when comparing mice housed in a standard environment, there was a significant difference in Chlamydiae, Actinobacteria and Saccharibacteria between all four groups, with a higher abundance present in high anxiety-related behaviour mice. When comparing high anxiety-related behaviour mice pre versus post enrichment, eight differences were observed, however significance was lost when false discovery rate was applied. Similarly, when comparing high anxiety-related behaviour mice pre versus post standard, six differences were observed, however significance was lost when false discovery rate was applied. Interestingly, 29 significant differences were observed post enrichment between high anxiety and normal anxiety phenotypes, indicating that environmental enrichment had little impact on the microbiome composition of high anxiety mice while phenotype had a substantial influence. No significant differences were observed in high anxiety mice pre versus post enrichment, or between high anxiety and normal anxiety mice post enrichment. Similarly, there was no significant difference in alpha diversity between the standard housing groups. Taken together, these results indicate that environmental enrichment had minimal influence on the alpha diversity of high anxiety-related behaviour mice. Adonis tests showed no significant differences in beta diversity between high anxiety and normal anxiety mice pre versus post enrichment, although there was a trend

towards significance between the highly anxious and non-anxious mice post environmental enrichment where highly anxious mice clustered separately to non-anxious mice. However, there were significant differences observed between the standard housing groups. There was a significant difference between high anxiety normal anxiety mice in standard housing conditions at baseline and between high anxiety and normal anxiety mice post standard conditions, while no separation was visible between high anxiety mice pre versus post standard. These results indicate that mouse phenotype may have a greater influence on microbial beta diversity than housing in an enriched environment versus a standard environment because samples clustered based on phenotype but not based on environment. However, no significant differences were observed between high anxiety and normal anxiety mice post enrichment, whereas significant differences were found between high anxiety versus normal anxiety mice post standard housing, indicating that environmental enrichment may reduce differences in beta diversity between highly anxious and non-anxious mice compared to those mice not exposed to enrichment. This will be an interesting finding to investigate further. However, these results should be interpreted cautiously due to the low n number of this study. Furthermore, differences in beta diversity which were previously observed between high anxiety-related behaviour and normal anxiety-related behaviour mice post enrichment were lost once mice from the CCB facility were removed, suggesting that this separation was due to the different facilities and not due to the study variables.

In terms of future studies, this work will need to be repeated with a higher n number using mice born and raised within the same facility in order to determine whether the observed differences in behaviour and microbiome were due to the facility factor or due to the environmental enrichment itself. Once this is determined, it will be important to identify species of bacteria that are strongly associated with these changes and future studies could employ strains which were present in a higher abundance in normal anxiety-related behaviour mice compared to high anxiety-related behaviour mice, or which increased in the high anxiety cohort following enrichment, to test for probiotic potential as these could be

providing protective effects. A significant challenge in microbiome research is determining whether microbiota alterations are causal or consequential of behavioural changes. Future studies could administer these strains to examine whether they are causal or protective in these effects. LEfSe analysis was carried out to identify key phylotypes that contributed to the differences between the high anxiety-related behaviour and normal anxiety-related behaviour gut microbiome profiles and which were most likely to explain the differences between individual groups in order to identify potential candidates for future probiotic studies. *Rikenellaceae* RC9 gut group was associated with normal anxiety-related behaviour mice prior to environmental enrichment. The specific function of this taxa remains poorly understood, however it has previously shown to be decreased in rats with hypertriglyceridemia-related acute necrotising pancreatitis, and high *Rikenellaceae* abundance is reportedly associated with healthy metabolic states (Huang *et al.*, 2017; Lippert *et al.*, 2017). Furthermore, members of the *Rikenellaceae* family can produce propionate, acetate and/or succinate as a fermentation end product (Graf, 2014). Other genera which were discriminating in this group include *Lactococcus*, which has been demonstrated to have anti-inflammatory effects and was consequently identified as a potential treatment for Irritable Bowel Disease (Luerce *et al.*, 2014). It has probiotic characteristics such as gastric acid resistance and survival in NaCl and bile salts. It has also been identified as a bacteriocin producer against *Listeria monocytogenes* and a producer of neuroactive bioamines such as dopamine, noradrenaline, serotonin (Stoyanova *et al.*, 2017), and GABA (Song *et al.*, 2017). Furthermore, several studies have demonstrated various strains of this genus improve depressive- and anxiety-like behaviour (Ramalho *et al.*, 2019; Colica *et al.*, 2017; Liu, Walsh and Sheehan, 2019). For example, Surzenko *et al.* (2019) demonstrated that prenatal exposure to *Lactococcus* exerted sex-specific long-term effects on cortical development and anxiety-related behaviour in the offspring, suggesting a beneficial role in anxiety-like behaviour for future studies. *Parabacteroides* was one of the discriminating genera in normal anxiety-related behaviour mice post enrichment and certain strains have been shown to possess beneficial metabolic effects such as reducing obesity, inflammation and insulin resistance (Wu *et al.*, 2019). *Parabacteroides* has also been demonstrated to be

inversely associated with stress, depression and anxiety in adults (Taylor *et al.*, 2019).

Ruminiclostridium was identified as a discriminating genus in high anxiety-related behaviour mice post enrichment. Decreased levels of *Ruminiclostridium* 5 were found in patients with kidney stones (Tang *et al.*, 2018) and in a rat model of acute necrotising pancreatitis (Chen *et al.*, 2017), potentially indicating an important role for this bacterium in inflammatory diseases. Similarly, *Roseburia* was also identified as a discriminating factor of the high anxiety-related behaviour mice post enrichment. This genus has been identified as a butyrate producer (Tamanai-Shacoori *et al.*, 2017), is negatively associated with Irritable Bowel Syndrome severity (Liu *et al.*, 2020), and is reduced in ulcerative colitis patients (Machiels *et al.*, 2014). Regarding anxiety, *Roseburia* has been found to be more abundant in healthy controls compared to people with generalised anxiety disorder (Jiang *et al.*, 2015). When looking at the discriminating genera in the normal anxiety-related behaviour group prior to standard housing, *Ruminococcaceae* has been shown to be more abundant in healthy controls compared to people with generalised anxiety disorder and correlated negatively with the severity of anxiety, depression and anxiety scale scores and positively with anxiety reduction (Chen *et al.*, 2019). *Paraprevotella* was also identified as a discriminating genus in this group and is a known propionate producer (Schwiertz *et al.*, 2010). Furthermore, a recent study by Marroquin et al. found *Paraprevotella* to be associated with low anxiety participants (Marroquin *et al.*, 2020). *Christensenellaceae* was found to be a discriminating genus of the normal anxiety-related behaviour post standard group. This bacteria is associated with a healthy phenotype and longevity in humans, has a strong inverse relationship with body mass index and is also related to a healthy status in a number of other different disease contexts, including obesity and inflammatory bowel disease (Waters and Ley, 2019). Any of the above mentioned bacteria could be contributing to the reduced anxiety profile in normal anxiety-related behaviour mice and in high anxiety-related behaviour mice post environmental enrichment and it would be interesting to investigate these further in future animal studies examining environmental enrichment, particularly the

strains which have previously been shown to be beneficial for anxiety and depression-like behaviour.

Future studies will also need to take into account the general challenges associated with environmental enrichment and microbiome studies such as differences in the procedures prior to and/or during testing, including use of animals from different breeders, use of different sex, and differences in the experimental setup in the order in which the tests were carried out (Sartori, Landgraf and Singewald, 2011). Firstly, there is considerable variability in the enrichment protocols used by different investigators, which may account for some of the inconsistencies observed in the effect of environmental enrichment on behavioural (e.g. anxiety) and endocrine (e.g. plasma corticosterone) responses. The design and composition of the environment presents an important variable which needs to be fully addressed. There is substantial variance in the standards for enrichment between studies; what is considered highly enriched in one study could be referred to as standard enrichment in others. Furthermore, a large variety of stimulus objects and settings have been utilised in previous enrichment studies, which provides an additional source of variation because different objects provide different types of experiences (e.g. cognitive, social, acrobatic, or sensory). Some studies have provided toys and blocks (Belz *et al.*, 2003; Friske and Gammie, 2005), while others have used running wheels, ladders and tunnels (Briones, Klintsova and Greenough, 2004; Faherty *et al.*, 2005; Moncek *et al.*, 2004; Naka *et al.*, 2005; Passineau, Green and Dietrich, 2001; Van Waas and Soffié, 1996).

It has been suggested that successful enrichment designs vary between species, sexes and ages (Galef, 1999), adding to the difficulty of standardising the design and composition of environmental enrichment. An additional confounding variable in these types of studies is the procedural differences in environmental enrichment protocols, for example how regularly toys are changed. Other variables such as duration of enrichment, strain, and number of animals housed together also varied between studies. For example, studies have indicated that exposure for just two hours a day can be as effective in recovery from lesions and reducing anxiety as a regimen of 24 hours a day (Widman and Rosellini, 1990; Will *et al.*, 1977). The

number of animals housed together also differs between studies, ranging from five to 14 animals (Benaroya-Milshtein *et al.*, 2004; Faherty *et al.*, 2005; Pham *et al.*, 2002; Will *et al.*, 2004). However, comparison of two studies that used a similar test of object exploration in rats revealed that environmental enrichment enhanced the quality of exploration regardless of strain, number of stimulus objects or their characteristics and how often they were changed (Van Waas and Soffié, 1996; Widman and Rosellini, 1990). Behaviourally, the majority of studies indicate that environmental enrichment reduces resting or basal anxiety levels. Similarly, most studies reported that animals exposed to environmental enrichment expressed relatively less anxiety in response to a stressor compared to matched controls (Huck and Price, 1975; Martínez-Cué *et al.*, 2005; Widman, Abrahamsen and Rosellini, 1992). Therefore, despite such inconsistencies in the literature, much of the animal research data indicates that environmental enrichment is capable of exerting substantial behavioural and physiological anxiolytic effects against psychogenic and neurogenic stressors. However, care needs to be taken when providing an appropriate species-specific 'dose' of enrichment (Huff *et al.*, 2003; Van de Weerd *et al.*, 1994). In order to fully understand and utilise environmental enrichment for improving the physical and psychological health of experimental subjects, future studies which compare the anxiolytic effects of various environmental enrichment conditions are required for standardisation of the environmental enrichment manipulation. Understanding the contribution of various enrichment characteristics may aid in explaining the variability in the results of environmental enrichment studies. Future studies should also compare the effects of pre- and post-stress enrichment, and compare the effects of environmental enrichment and stress inoculation on recovery from acute stress exposure (Fox, Merali and Harrison, 2006).

4.6 Conclusion

In the present study, we have shown that high anxious mice display a very heterogeneous and unstable gut microbiota community, which differs from the

normal anxious microbiota both in the structure and in the responsiveness to the environment. This study also highlights the important effect that the origin facility has on preclinical mouse trials such as this as it was determined separation observed on PCoA plots was as a result of different facilities of origin of the mice and not due to environmental enrichment. However, the differential anxiety-like behaviour in highly anxious mice compared to non-anxious mice was robust and differences in behavioural tests based on phenotype remained evident despite different facilities of origin and persisted when the CCB mice were removed, further confirming the robust nature of this mouse line. However, microbiome composition and diversity were more affected by facility of origin and differences were observed once the CCB mice were removed, strengthening the hypothesis that environment has a strong influence on the gut microbiome. Nevertheless, interesting results were observed from this study so future studies will be required to further investigate the impact of environmental enrichment on anxiety-like behaviour and the potential underlying role of the gut microbiota, ensuring that facility of origin remains constant and subsequent studies are sufficiently powered. Once this is further understood, it will be interesting to carry out subsequent preclinical studies administering potential probiotics identified from this work to determine whether they can improve anxiety and depression-like behaviour. A significant challenge in microbiome research remains determining whether microbiota alterations are causal or consequential of behavioural changes, but future studies administering candidate probiotics which were altered as a result of environmental intervention may provide clarity on this.

Author Contributions

N. Singewald designed the study; A. Sah designed the study, performed animal experiments, collected and analysed behavioural and molecular data; N. Wiley carried out DNA extractions, 16S rRNA sequencing, data analysis, drafted the manuscript and assisted with interpretation; A.V. Golubeva and T. Bastiaanssen assisted with data analysis and interpretation; F. Fouhy ran 16S sequences through the Teagasc Moorepark pipeline to create OTU tables; J.F. Cryan, T.G. Dinan, R.P.

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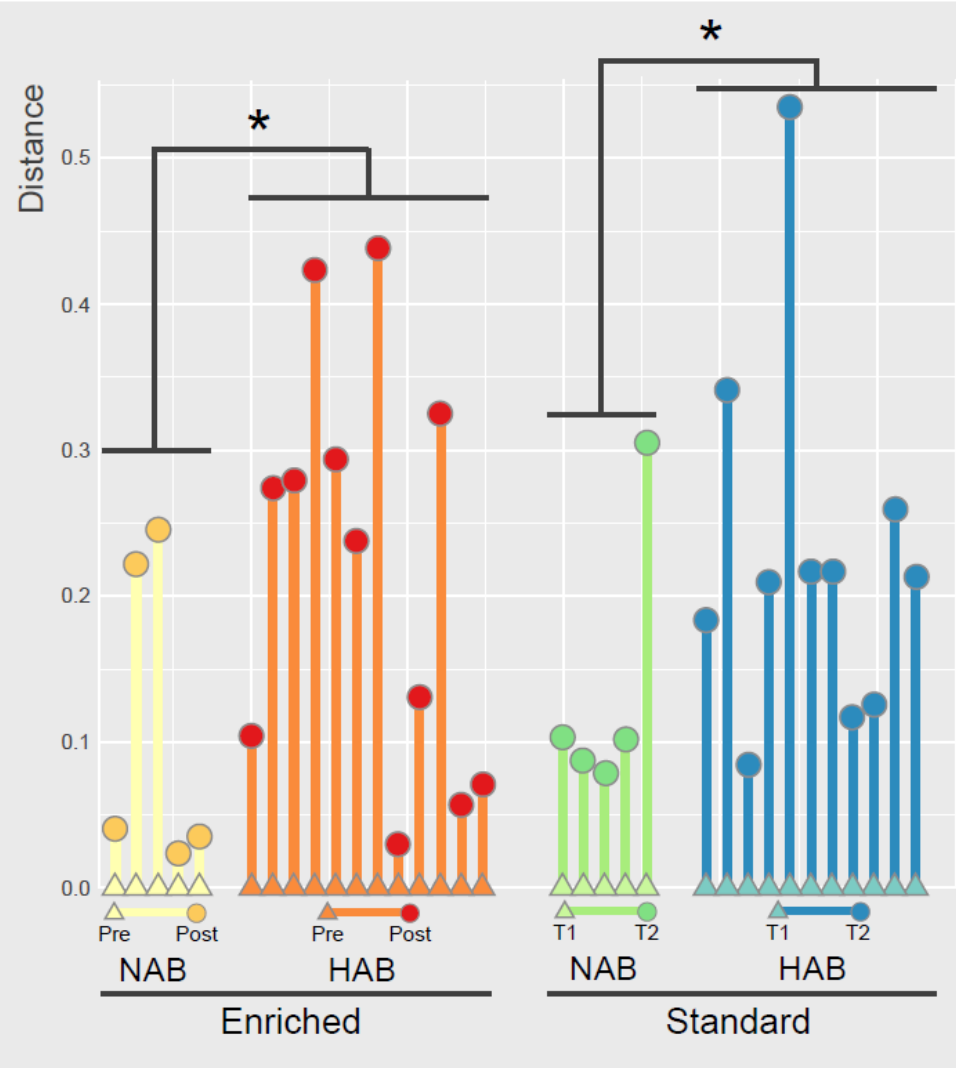
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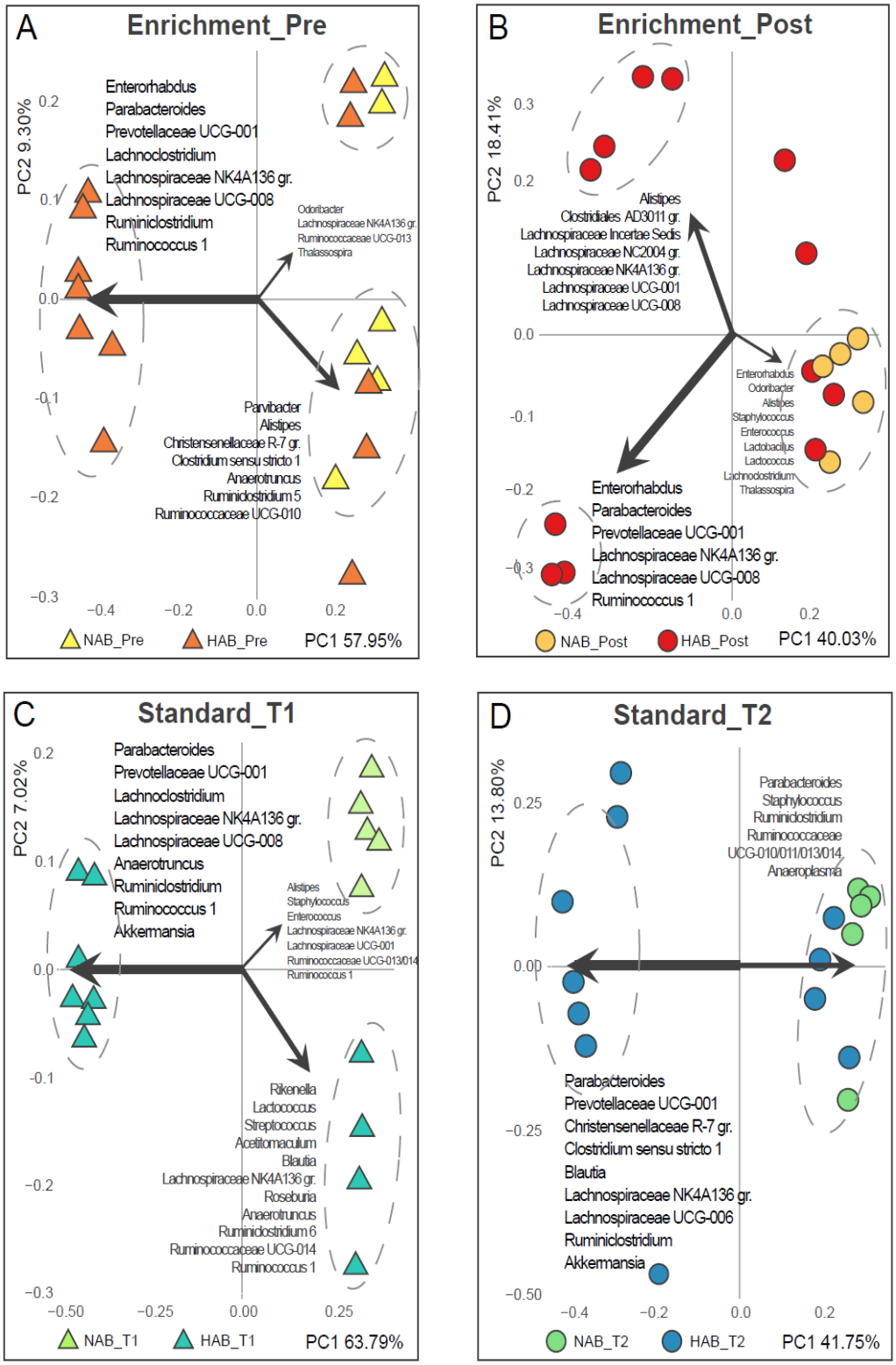
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4.8 Supplementary Material



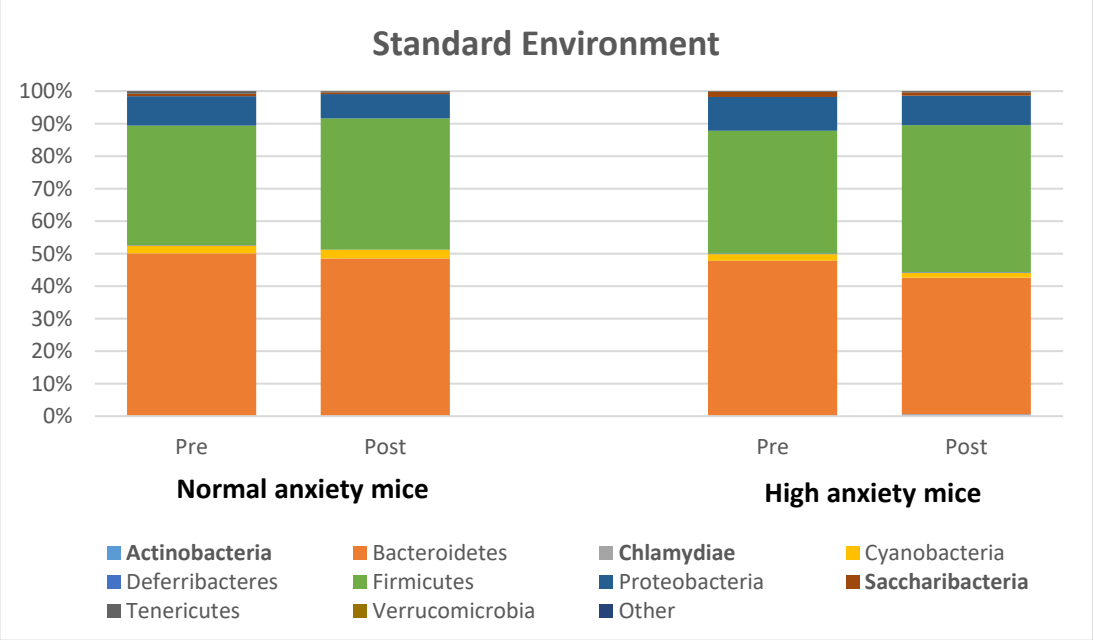
p = 0.041 for HAB vs. NAB in Enriched housing
p = 0.045 for HAB vs. NAB in Standard housing
p = 1.000 for NAB in Enriched vs. Standard housing
p = 1.000 for HAB in Enriched vs. Standard housing
Mann-Whitney test

Supplementary Figure 1: High-anxious mice demonstrate a more pronounced shift in microbiota composition in either standard or enriched environment. PCoA distances between two time points (as a measure of shift in microbiota composition) were plotted for each animal, and distances between groups were compared with unpaired Mann-Whitney test.

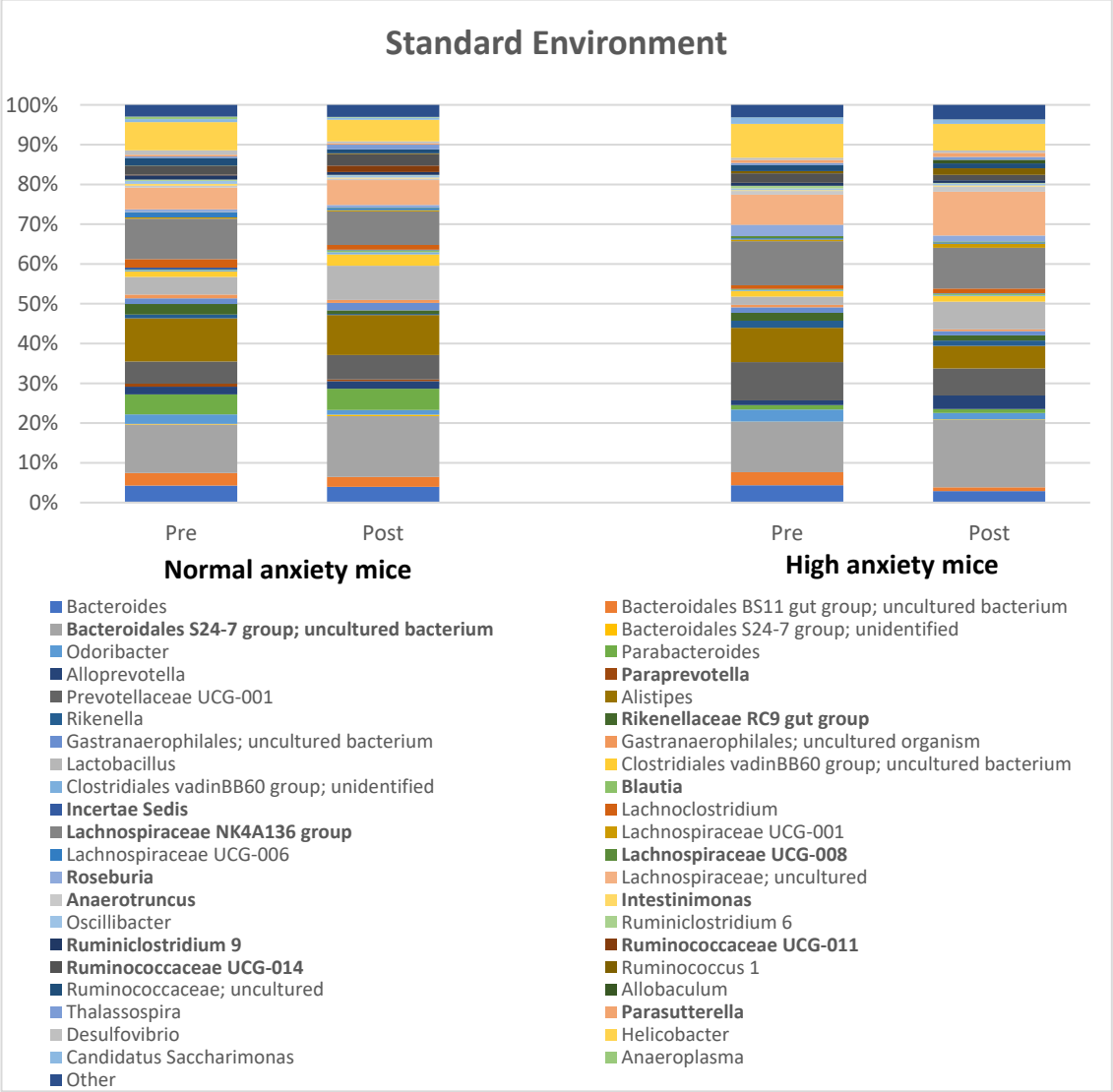


Supplementary Figure 2: Differential re-structuring of the high anxiety-related behaviour microbiota in response to standard and enriched housing. High anxiety-

related behaviour and normal anxiety-related behaviour mice were clustered based on their position on the PCoA plot (dashed ellipses). Bacterial species were correlated with two main components of the PCoA plot; the OTUs displaying the strongest correlation with the location of each cluster of interest are shown on the plots. The size of the font and the thickness of arrows correspond to the strength of the observed associations. (A-B) high anxiety-related behaviour and normal anxiety-related behaviour mice prior to and four weeks after the environmental enrichment. (C-D) high anxiety-related behaviour and normal anxiety-related behaviour mice at the beginning and following four weeks of standard housing.



Supplementary Figure 3: Relative abundances (%) distribution of bacterial phyla in normal anxiety-related behaviour and high anxiety-related behaviour mice samples pre versus post standard environment excluding CCB mice. Statistically significant differences in phyla are highlighted in bold ($p<0.05$).



Supplementary Figure 4: Relative abundances (%) distribution of bacterial genera in high anxiety-related behaviour and normal anxiety-related behaviour mice samples pre versus post standard conditions excluding CCB mice. Statistically significant differences are highlighted in bold ($p<0.05$). Represented only taxa >1% in at least one group. The Other category contains all other genera present at <1% of assignable reads at genus level.

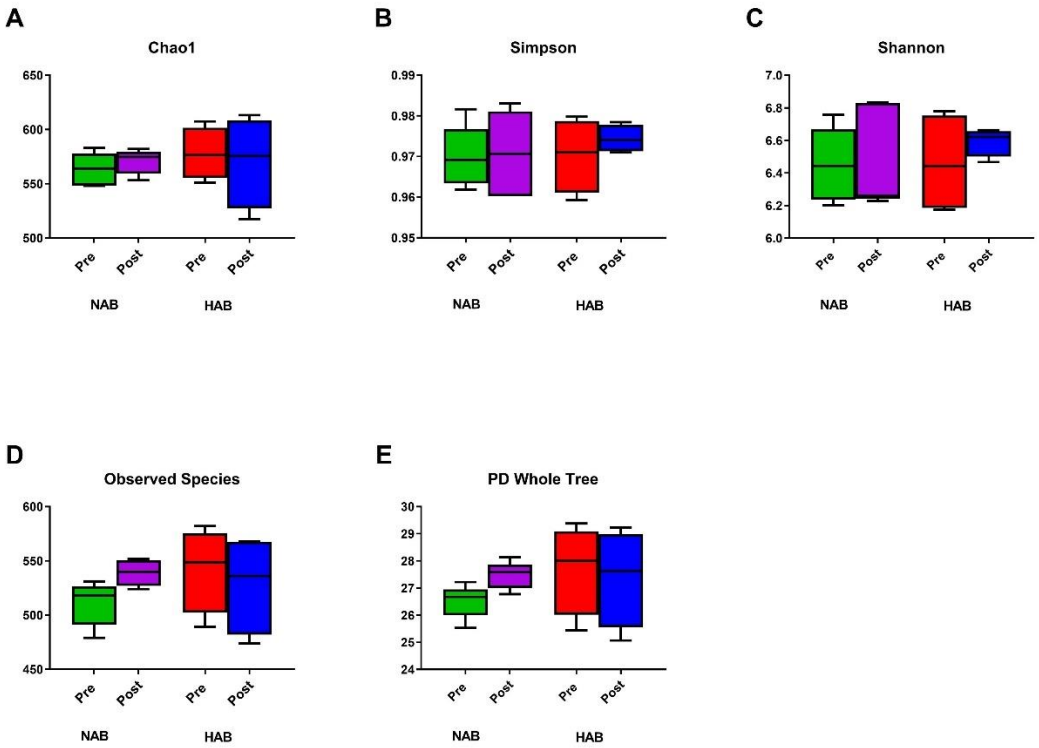
Supplementary Table 1: Significant differences at genus level between all standard environment groups ($p<0.05$) as calculated using the Wilcox test for paired data and the Kruskal Wallis test for non-paired groups excluding CCB mice.

Genus	FDR-corrected p value	Comparison
Coriobacteriaceae UCG 002	0.449	HAB Pre vs. Post Standard
Bacteroidales BS11 gut group uncultured bacterium	0.449	HAB Pre vs. Post Standard
Gemella	0.449	HAB Pre vs. Post Standard
Clostridium sensu stricto 1	0.449	HAB Pre vs. Post Standard
Anaerovorax	0.449	HAB Pre vs. Post Standard
Ruminococcaceae Eubacterium coprostanoligenes group	0.449	HAB Pre vs. Post Standard
Rikenella	0.226	NAB Pre vs. Post Standard
Rikenellaceae RC9 gut group	0.278	NAB Pre vs. Post Standard
Enterococcus	0.278	NAB Pre vs. Post Standard
Clostridiales vadinBB60 group uncultured bacterium	0.278	NAB Pre vs. Post Standard
Clostridiales Eubacterium brachy group	0.226	NAB Pre vs. Post Standard
Clostridiales Eubacterium nodatum group	0.278	NAB Pre vs. Post Standard
Acetitomaculum	0.278	NAB Pre vs. Post Standard
Tyzzerella	0.226	NAB Pre vs. Post Standard
Peptococcaceae uncultured	0.226	NAB Pre vs. Post Standard
Ruminiclostridium 6	0.226	NAB Pre vs. Post Standard
Ruminococcaceae UCG 011	0.226	NAB Pre vs. Post Standard
Ruminococcaceae uncultured	0.226	NAB Pre vs. Post Standard
Desulfovibrio	0.226	NAB Pre vs. Post Standard

Bacteroidetes VC2.1 Bac22 uncultured bacterium	0.013	HAB vs. NAB Pre Standard
Parabacteroides	0.014	HAB vs. NAB Pre Standard
Paraprevotella	0.014	HAB vs. NAB Pre Standard
Prevotellaceae UCG 001	0.027	HAB vs. NAB Pre Standard
Chlamydia	0.007	HAB vs. NAB Pre Standard
Gastranaerophilales uncultured rumen bacterium	0.011	HAB vs. NAB Pre Standard
Staphylococcus	0.014	HAB vs. NAB Pre Standard
Enterococcus	0.014	HAB vs. NAB Pre Standard
Christensenellaceae R 7 group	0.011	HAB vs. NAB Pre Standard
Christensenellaceae uncultured	0.014	HAB vs. NAB Pre Standard
Candidatus Arthromitus	0.014	HAB vs. NAB Pre Standard
Clostridium sensu stricto 1	0.014	HAB vs. NAB Pre Standard
Acetitomaculum	0.029	HAB vs. NAB Pre Standard
Incertae Sedis	0.014	HAB vs. NAB Pre Standard
Lachnoclostridium	0.014	HAB vs. NAB Pre Standard
Lachnospiraceae NC2004 group	0.014	HAB vs. NAB Pre Standard
Lachnospiraceae UCG 005	0.027	HAB vs. NAB Pre Standard
Lachnospiraceae UCG 006	0.014	HAB vs. NAB Pre Standard
Roseburia	0.027	HAB vs. NAB Pre Standard
Lachnospiraceae uncultured	0.014	HAB vs. NAB Pre Standard
Peptococcus	0.027	HAB vs. NAB Pre Standard
Ruminococcaceae UCG 011	0.014	HAB vs. NAB Pre Standard
Erysipelotrichaceae uncultured	0.014	HAB vs. NAB Pre Standard
Desulfovibrio	0.014	HAB vs. NAB Pre Standard
Anaeroplasma	0.014	HAB vs. NAB Pre Standard
Coriobacteriaceae UCG 002	0.007	HAB vs. NAB Post Standard
Bacteroidetes VC2 1 Bac22 uncultured bacterium	0.013	HAB vs. NAB Post Standard

Bacteroidales BS11 gut group uncultured bacterium	0.014	HAB vs. NAB Post Standard
Parabacteroides	0.014	HAB vs. NAB Post Standard
Paraprevotella	0.011	HAB vs. NAB Post Standard
Rikenella	0.014	HAB vs. NAB Post Standard
Chlamydia	0.007	HAB vs. NAB Post Standard
Gastranaerophilales uncultured rumen bacterium	0.011	HAB vs. NAB Post Standard
Gemella	0.007	HAB vs. NAB Post Standard
Staphylococcus	0.011	HAB vs. NAB Post Standard
Enterococcus	0.014	HAB vs. NAB Post Standard
Lactococcus	0.027	HAB vs. NAB Post Standard
Streptococcus	0.05	HAB vs. NAB Post Standard
Christensenellaceae R 7 group	0.014	HAB vs. NAB Post Standard
Clostridiales vadinBB60 group uncultured Clostridiales bacterium	0.032	HAB vs. NAB Post Standard
Clostridiales Family XIII AD3011 group	0.027	HAB vs. NAB Post Standard
Clostridiales Eubacterium nodatum group	0.027	HAB vs. NAB Post Standard
Acetitomaculum	0.014	HAB vs. NAB Post

		Standard
Coprococcus 2	0.027	HAB vs. NAB Post Standard
Tyzzerella 3	0.014	HAB vs. NAB Post Standard
Lachnospiraceae uncultured	0.014	HAB vs. NAB Post Standard
Ruminococcaceae NK4A214 group	0.014	HAB vs. NAB Post Standard
Ruminococcaceae UCG 009	0.014	HAB vs. NAB Post Standard
Ruminococcaceae UCG 011	0.011	HAB vs. NAB Post Standard
Ruminococcaceae UCG 014	0.027	HAB vs. NAB Post Standard
Ruminococcaceae Eubacterium coprostanoligenes group	0.013	HAB vs. NAB Post Standard
Erysipelatoclostridium	0.014	HAB vs. NAB Post Standard
Candidatus Saccharimonas	0.027	HAB vs. NAB Post Standard
Other	0.027	HAB vs. NAB Post Standard



Supplementary Figure 5: Alpha diversity analyses of faecal microbiota of mice from standard environment groups using Chao1 (A), Simpson (B), Shannon (C), Observed Species (D) and PD Whole Tree (E) indices.

5. Investigating the Link between the Microbiome, Diet, Stress Response and Exploration Behaviour in a Wild Bird

Niamh Wiley[a,b], Gabrielle Davidson[c,d], Amy C. Cooke[c], Crystal N. Johnson[a,b], Fiona Fouhy[a,b], Michael S. Reichert[c,e], Ivan de la Hera[c], Jodie M.S. Crane[c,f], Ipek G. Kulahci[c], John L. Quinn[c] , R. Paul Ross[a,b], Catherine Stanton[a,b]

a. APC Microbiome Ireland, University College Cork, Cork, Ireland.

b. Teagasc Food Research Centre, Moorepark, Fermoy, Ireland.

c. School of Biological, Earth and Environmental Sciences, Distillery Fields, North Mall, University College Cork, Cork, Ireland.

d. Department of Psychology, Downing Street, University of Cambridge, Cambridge, UK.

e. Department of Integrative Biology, Oklahoma State University, USA

f. Kākāpō Recovery Programme, Department of Conservation, 7th Floor, 33 Don Street, Invercargill 9810, New Zealand.

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5.1 Abstract

The microbial community in the gut is influenced by environmental factors, especially diet, which can moderate host behaviour and cognition through the microbiome-gut-brain axis. Research into the underlying mechanisms involved is a rapidly growing field which incorporates physiological, behavioural, and evolutionary investigations, however, the ecological relevance of microbiome-mediated behavioural plasticity in wild animals is largely unexplored. We manipulated the gut microbiome of wild caught great tits (*Parus major*) by administering one of two diets that differed in their relative levels of fat, protein, and fibre content: an insect only diet (low content), or a seed, nut, and beef suet diet (high content). Exploration behaviour and corticosterone response were also assessed to gain an insight into the interactions between the gut microbiome, behaviour, and the stress response in this ecologically relevant model. Microbial communities changed substantially and were less diverse among individuals given the insect but not the seed diet. Few differences were observed in microbial composition or diversity between fast and slow exploration groups, apart from seven differences at genus level. Although the effect on behaviour may not be solely or directly a consequence of the gut microbiota manipulation, this nevertheless provides evidence for an association between behaviour, diet and the gut microbiome in a wild animal system, and points to an overlooked but potentially crucial mechanism underlying individual behavioural plasticity in evolutionary ecology and suggests the gut-brain axis is conserved across species to some extent.

5.2 Introduction

Experimental targeting of the gut microbiome through diet manipulation, infection, stress and exercise has been shown to impact important behaviours and cognition, including learning, memory, anxiety, activity levels and social interactions (Clarke *et al.*, 2013; Diaz Heijtz *et al.*, 2011; Desbonnet *et al.*, 2014; Hoban *et al.*, 2018; Magnusson *et al.*, 2015), as well as cause changes to neurogenesis (Ogbonnaya *et*

al., 2015) and protein expression in the brain (Clarke *et al.*, 2013; Gareau *et al.*, 2011; Hoban *et al.*, 2018). However, our understanding of these processes in natural populations is extremely limited as evidence of the microbiome-gut-brain axis' influence on behaviour is predominantly derived from experiments on model laboratory animals, correlational studies on mental health in humans (Cryan and Dinan, 2012; Foster and McVey Neufeld, 2013), and from captive animals in laboratories or zoos (Colston and Jackson, 2016). Similarly, individual differences in cognition are often quantified in environments where subjects are either reared in captivity or brought temporarily into captivity from the wild and the effect of captivity on the microbiome is rarely accounted for. This is a significant limitation to understanding the impact of the gut microbiome on cognition and behaviour as individual performance in cognitive tasks may be influenced by several aspects of captivity such as neophobia, habituation and stress (Davidson *et al.*, 2018). It is largely unknown whether these findings are also relevant in natural populations, where microbiome-host interactions are predicted to influence traits that directly impact animal fitness (Davidson *et al.*, 2018), including cognition (Morand-Ferron, Cole and Quinn, 2016) and foraging behaviour (Stephens and Krebs, 1986). Davidson *et al.* (2018) argue that the microbiome is an important trait that should be investigated as a mechanism explaining individual differences in cognition, which are of relevance for selection pressures that favour specific cognitive mechanisms, such as spatial memory in food-hoarding birds.

This lack of knowledge into the gut microbiome of wild animals is even more pronounced in non-mammalian vertebrates as the gut microbial communities of amphibians, reptiles, birds and fish are poorly described. Birds in particular are highly diverse, with 10,425 described species and more than 20,000 subspecies varieties. They are of particular interest to microbiome research due to the wide variety of diets and life history strategies they employ. While our understanding of the avian gut microbiome is greater than other non-mammalian vertebrates, the majority of avian microbiome studies have focused on economically important species such as turkey and chicken (Colston and Jackson, 2016). The avian gut microbiome is influenced by several factors including host genes (Benson *et al.*,

2010; McKnite *et al.*, 2012), developmental conditions (Koenig *et al.*, 2011), natal environment (e.g. birth canal (Bokulich *et al.*, 2016) and nest environment (Jacob *et al.*, 2015)), and current environmental effects (such as diet, infection or acute stress (Li *et al.*, 2009; Gareau *et al.*, 2011)), all of which can determine its resilience to perturbations that may alter community composition. Most published studies of avian endogenous microbiomes have used culture-dependent approaches or Sanger sequencing of 16S rRNA gene clone libraries which revealed that Bacteroidetes, Firmicutes and Proteobacteria are typically the most common phyla, while Firmicutes are present in higher proportions in captive poultry compared to wild birds (Waite and Taylor, 2015). The site of sampling is a significant factor to consider for microbiome analysis in birds. Ordination analyses revealed that gastrointestinal tract microbial communities group by sampling region (crop, caeca, cloaca, and faecal (Waite and Taylor, 2014)). Functions such as food storage, acid secretion and trituration, which are normally carried out in the stomach of other vertebrates are divided among the crop, proventriculus and gizzard in birds. The site of microbial fermentation is known to vary substantially within herbivorous bird species and may occur in the crop, midgut, caeca, or colon. Characterisation of the bird gut microbiome has overwhelmingly occurred using faecal samples and occasionally using cloacal swabs or intestinal tissue. One of the first culture-independent studies to investigate the gut microbiome of wild birds examined seasonal changes in the gut microbial community of both wild and captive capercaillie (Wienemann *et al.*, 2011). Bacterial community composition in wild birds differed in summer versus winter months compared to captive individuals, likely due to dietary shifts not experienced by captive animals (Wienemann *et al.*, 2011).

The gut microbiota can have both a positive and negative influence on bird health and reproductive success. Infectious diseases and intoxication by bacterial by-products can reduce fitness or even result in mortality in wild bird populations (Hudson and Dobson, 1991; Pinowski, Kavanagh and Gorski; Clayton and Moore, 1997). Furthermore, antibiotic treatment temporarily enhanced nestling growth in Magellanic penguins by modifying gut bacterial communities (Potti *et al.*, 2002).

However, the gut microbiota also plays a positive role in nutrition, growth performance, detoxification, and protection against pathogenic bacteria (Mead, 1997). For example, intestinal inoculation of one day old broiler chicks with adult fowl faeces enhanced the chicks' resistance to Salmonella colonisation (Nurmi and Rantala, 1973). Moreover, because microbiome diversity is associated with fitness in mammals (Suzuki, 2017) and life-history traits such as lay date, clutch size and adult survival in birds (Benskin *et al.*, 2015), an adaptive component to microbiome variation may exist which could function to optimise food preferences, immune functions, social behaviour, stress responses, and cognitive abilities to mutually benefit both the host and their microbiota (Davidson *et al.*, 2018). While host genetics strongly influence gut microbiota structure in mammals, fish and reptiles (Ley *et al.*, 2008; Arumugam *et al.*, 2011; Wong and Rawls, 2012), avian host-specific gut bacterial communities appear to be more dependent on diet and geography (Hird *et al.*, 2014; Waite and Taylor, 2014) rather than phylogeny. The microbiome has been linked to changes in host growth rate and metabolism (De Winter, Stratford and Chapman, 2015), host phylogeny (Anderson *et al.*, 2012; Colman, Toolson and Takacs-Vesbach, 2012; Phillips *et al.*, 2012), host ecology, life history (Wong and Rawls, 2012; Coon *et al.*, 2014; Dill-McFarland *et al.*, 2014), geography (Hird *et al.*, 2014), habitat characteristics (Costa *et al.*, 2016; Knutie, Chaves and Gotanda, 2019), and seasonality (Amato *et al.*, 2015; Hicks *et al.*, 2018; Maurice *et al.*, 2015). Fluctuations in the microbiome can occur through pathogen infection (Gareau *et al.*, 2011), dietary changes (Magnusson *et al.*, 2015) and exercise (Kang *et al.*, 2014), all of which vary significantly between species, for example in animals that are prone to contracting infections as a result of social interactions, in animals that are subjected to geographical and temporal changes in food availability, and in animals that vary their energy expenditure during migration or breeding seasons (Kohl, Skopec and Dearing, 2014; Clayton *et al.*, 2016; Davidson *et al.*, 2018). This is especially relevant for wild animal populations, which are more exposed to infectious diseases and are impacted by food availability, as these are important factors which can alter the microbiome of individuals.

Colonisation of nestlings by environmental microbes begins soon after hatching as demonstrated by Mills *et al.* (1999) who detected cloacal micro-organisms in tree swallow nestlings two days after hatching. Inoculation of nestlings by microbes may occur from the environment via ingestion of adult saliva, food provided by the parents or nest materials (Kyle and Kyle, 1993; Singleton and Harper, 1998; Mills, Lombardo and Thorpe, 1999; Berger, Disko and Gwinner, 2003), as demonstrated by Lucas and Heeb (2005) who aimed to examine the relative contributions of genetic background versus parental and nest-based environmental factors in shaping nestling cloacal microbiota in two avian species. They performed partial cross-fostering between great tit and blue tit broods from the same study areas and found that nestlings raised in the same nest shared significantly similar bacterial communities, and also observed a higher microbial similarity between these nestlings compared to between biological siblings raised in separate nests. Many hatchling birds feed on regurgitated food items from their parents, which potentially provides a mechanism for vertical transmission of gut microbiomes across generations (van Dongen *et al.*, 2013). Several studies have also revealed an age effect on the microbiota composition. Bacterial diversity increases with nestling age and stabilises at adult age (Mills, Lombardo and Thorpe, 1999; Knarreborg *et al.*, 2002; Wielen *et al.*, 2002).

Host diet has a profound effect on the gut microbiome, as demonstrated by Dewar *et al.* (2013) who utilised a combination of qPCR (to measure the overall bacterial abundance) and Next Generation Sequencing analysis to examine the gut microbiota of penguins, which is of interest to microbiome research due to their ability to store food for long periods (Colston and Jackson, 2016). The same phyla (Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria) were dominant among the four penguin species studied (King, Gentoo, Macaroni, and Little Penguin), however the proportion of each phylum varied greatly between species. In addition to harbouring the most distinct gut microbial community, King penguins also displayed the lowest microbial diversity, which is consistent with findings showing that higher predators tend to have more prey-associated microbiota and lower overall diversity. Human studies indicate a role for Bacteroidetes members of

the GI tract in carbohydrate fermentation and the production of short-chain fatty acids that can be absorbed by the host and contribute to nutrition (Walter and Ley, 2011). In contrast, Firmicutes are associated with protein degradation, and the gut bacterial communities of terrestrial carnivorous mammals contain greater proportions of Firmicutes than those of terrestrial herbivorous mammals (Nelson, Rogers and Brown, 2013). While reports of direct effects of dietary manipulations on the gut microbiota in birds to date have been restricted to poultry (Pan and Yu, 2014), changes in diet can lead to a significant alteration to the gut microbiome within 24 hours in humans (David *et al.*, 2014). These changes are dependent on dietary features such as protein levels (Clarke *et al.*, 2014; David *et al.*, 2014), fat levels (Fava *et al.*, 2013), and whether plants are present in the diet (Wu *et al.*, 2016; Zimmer *et al.*, 2012). Thus, microbiome plasticity should be particularly important in wild populations (Davidson *et al.*, 2018), many of which are subject to regular changes in food availability (Kartzinel *et al.*, 2019), and in which individuals typically differ in their foraging strategies (Bolnick *et al.*, 2014).

Diet-related changes to the gut microbiome have repeatedly been linked to changes in host cognition and behaviour and have been demonstrated in animals that engage in coprophagy (Soave and Brand, 1991), migratory birds (Lewis, Moore and Wang, 2017), wild mice across seasons (Maurice *et al.*, 2015) and animals brought into captivity (Kohl, Skopec and Dearing, 2014). Multiple phenotypic traits such as innovation, cognitive traits including learning and generalisation, and animal personality traits such as competitive ability, potentially influence the microbiome composition mediated through diet, which in turn may influence cognition. For example, competitive individuals may have greater access to easily accessible foods such as bird feeders, while less competitive individuals are more likely to use alternative, more diverse food sources through innovative foraging behaviours (Davidson *et al.*, 2018). Studies in model lab organisms demonstrated that phenotypic plasticity in cognitive performance occurred in parallel with diet-related gut microbiome alterations (Magnusson *et al.*, 2015; Li *et al.*, 2009). For example, mice fed beef-chow had a higher microbial diversity than those fed on normal chow, and showed improved working and reference memory (Li *et al.*,

2009). High fat and high sugar diets caused differential alterations to gut microbial composition in mice at genus level. Both diets resulted in poorer behavioural flexibility on a reversal learning task, with the high sugar diet also impacting spatial memory (Magnusson *et al.*, 2015). Conversely, protein has been shown to increase microbial diversity and improve performance on tests of working and spatial memory (Li *et al.*, 2009). Four day old nestlings which were preferentially fed taurine-rich spiders by their parents displayed improved spatial memory (Arnold *et al.*, 2007). Observational studies also point to a relationship between diet, cognition, and mood in humans (Psaltopoulou *et al.*, 2013; Carlson *et al.*, 2018), effects that may be mediated by the host gut microbiome. However, the causal direction of these correlations remains unclear, and there is an increasing need for manipulative studies using model ecological organisms in natural populations to understand the ecological and evolutional significance of microbiota-gut-brain axis mechanisms and how dietary induced changes in the microbiome might affect functionally significant behaviours in the wild. There is also a need to investigate the potential role of the microbiome in mediating cognitive differences as a result of stress and infection in wild populations. Changes in faecal glucocorticoid metabolites and the microbiome of wild red squirrels (*Tamiasciurus hudsonicus*) were found to be associated with an increase in Pasteurellaceae, a group of potential pathogens (Stothart *et al.*, 2016). To further understand the mechanisms involved, manipulative studies of the microbiome through dietary intervention before measuring corticosterone (CORT) concentrations are required both in the natal environment and in adulthood, in order to identify the causal effects and to understand individual differences in response to these alterations (Davidson *et al.*, 2018).

Anxiety is a behavioural measure that is often associated with the microbiome and can be measured in terms of activity or exploratory behaviour in birds. Exploration behaviour is a measure of animal personality (consistent individual differences across time and contexts) and is defined as an individual's reaction to a new situation which includes a new habitat, new food, or novel objects. It is regarded as one of the five major personality traits: shyness-boldness, exploration-avoidance,

activity, sociability and aggressiveness (Réale *et al.*, 2007), many of which can be considered to be related to differences in how animals cope hormonally with stress. Personalities can be classified as proactive or reactive and have a strong influence on individual CORT and behaviour responses. Birds with proactive personalities display relatively active behavioural responses and low CORT stress responses. Conversely, birds with reactive personalities generally have passive behavioural responses accompanied by large CORT responses (Cockrem, 2007). Exploration in particular has important consequences for the survival of the animal, especially in the post-fledgling period when mortality is high, because it allows them to locate food, water and hiding places. Individuals that explore more are considered "fast" personality types, and those that explore less are considered "slow" personality types, and differences in these traits are consistent and repeatable over time (Bell, Hankison and Laskowski, 2009). Exploratory behaviour predicts how individuals manage predation risk when foraging (Van Oers *et al.*, 2004; Quinn and Cresswell, 2005; Arnold *et al.*, 2007; Wolf *et al.*, 2007; Quinn *et al.*, 2012), competitive foraging ability (David *et al.*, 2012; Riebli *et al.*, 2011; Cole and Quinn, 2012), and foraging flexibility (Verbeek, Drent and Wiepkema, 1994). Regarding great tits, this species typically lose weight over the course of captivity due to stress, with slower explorers displaying a more reactive endocrine stress response (Baugh *et al.*, 2013). Exploration behaviour of a novel environment and boldness toward a novel object are phenotypically correlated, repeatable within individuals, and artificial selection experiments have observed a heritable component (Drent, Oers and Noordwijk, 2003). Exploratory behaviour has also been correlated with dominance in wild great tits, which is a strong predictor of diet quality in social animals (Toscano *et al.*, 2016). Furthermore, exploratory behaviour might also inform dietary preference as Serrano-Davies *et al.* (2017) found a link between this personality trait and diet preference in captivity whereby faster explorers consumed a higher proportion of sunflower seeds, while slower great tits included a higher proportion of peanuts. Exploratory behaviour is also linked to the novel object recognition test, an additional measure of personality. Those that approach objects quicker are "bolder" and those that take longer are "shy". Birds show consistent behavioural responses when these tests are repeated using different exploration rooms or

objects. Interestingly, these different single measures of personality correlate such that fast birds are bolder, and slow birds are shy. This can extend to other personality measures such as aggression and sociality, which are known as behavioural Syndromes (when several personality traits correlate). Some studies suggest that hormonal differences may correlate with these personalities.

The HPA axis is the endocrine system which is most studied for mechanisms regulating personality variation and the main glucocorticoid in birds is CORT, which can influence several regulatory and behavioural processes simultaneously. For example, elevated CORT levels promote locomotor activity and stimulate foraging when food reserves are low, but can also suppress reproduction (Romero, 2004; Sapolsky, Romero and Munck, 2000). However, while an acute rise in CORT in response to a stressor is considered to be beneficial, if the CORT response to a stressor is larger than normal, if it persists for longer than usual, or if basal CORT levels are high then this may have negative effects (Cockrem, 2007). CORT responses are usually measured in birds by capture and handling, as it is expected that birds experience this as a form of predation (Silverin, 1998). CORT concentrations rise rapidly for 10-15 minutes after capture of free-living birds before a more gradual rise for the remainder of the sampling period, which typically lasts for 30-60 minutes (Wingfield, O'Reilly and Astheimer, 1995; Wingfield, Vleck and Moore, 1992). High stress is expected to be associated with slow explorers and low stress to be associated with fast explorers (Baugh *et al.*, 2012). Shy or reactive individuals are predicted to have higher HPA axis reactivity, lower sympathetic adrenomedullary reactivity, higher cardiac parasympathetic activity, a stronger innate and adaptive immune response, and a higher susceptibility to stress-related illness compared to bolder (proactive) individuals (Carere and Van Oers, 2004; Koolhaas *et al.*, 1999; Korte *et al.*, 1997; Martins *et al.*, 2007; Satterlee and Johnson, 1988; Pinxten *et al.*, 2004). Baugh *et al.* (2012) found evidence which supports this hypothesis, observing higher circulating CORT concentrations following exposure to a standardised stressor in great tits artificially selected for a combination of slow exploratory behaviour and shyness compared to individuals from the fast-bold selection line, indicating a genetic correlation between exploratory behaviour and

stress physiology. They concluded that reactivity of the HPA system and not basal regulation correlates with personality because baseline plasma CORT levels did not differ, while stress-induced levels did. An additional study selected Japanese quail for low and high CORT responses (Cockrem *et al.*, 2010) and demonstrated variation in open field behaviour, whereby birds from the line selected for low CORT responses showed less freezing behaviour and began exploring sooner than did birds selected for high CORT responses (Jones, Satterlee and Ryder, 1992), indicating a link between exploration behaviour and CORT.

To test the relationship between diet, behaviour, and the gut microbiota, we performed a dietary manipulation on wild great tits and examined their gut microbiota profiles as well as exploration behaviour and CORT response. Here we tested whether diet predicted the gut microbiome of wild-caught great tits, and whether natural and manipulated gut microbiome profiles correlated with exploration behaviour and the stress response. Great tits are an ideal species to study the relationship between microbiome, diet, exploration behaviour and the stress response. It is a highly innovative species that has long been a model organism in field studies on behaviour, ecology, and evolution. It is also ideal for short-term laboratory studies because they adapt well to temporary captivity (Dunn, Cole and Quinn, 2011; Cole *et al.*, 2012). They experience seasonal dietary changes (Perrins, 1991; Vel'ký, Kaňuch and Krištín, 2011), and are opportunistic foragers that vary individually in their problem solving performance (Cole, Cram and Quinn, 2011). Furthermore, the great tit lacks sex differences in both exploration behaviour (Carere *et al.*, 2005) and CORT concentrations (Stöwe *et al.*, 2010) so is therefore a useful model for studying correlations. First we sampled birds from both rural and urban areas, known to differ in their quality as habitat for great tits (Biard *et al.*, 2017), and therefore we expected host gut microbiota may differ in diversity, community structure similarity/dissimilarity (i.e. beta diversity) and/or changes in taxa-level abundance (Teyssier *et al.*, 2018), though our replication at the site level was limited. Second, we manipulated the diet of wild-caught great tits to test whether microbiota community structures became more dissimilar, and whether phylum-level and genus-level taxa changed within and

between treatment groups. Given the seed diet was higher in fat, protein and fibre content than the insect only diet, we expected these individuals to have higher alpha diversity, higher Firmicutes and lower Bacteroidetes if similar dietary effects occur in great tits as have been reported in humans (Clarke *et al.*, 2014). Third, we tested whether a change in the gut microbiome as a consequence of the manipulated diet influenced exploration behaviour and CORT response. We predicted that if the gut microbiome influenced host behaviour, rather than the dietary treatment itself doing so, then a change in behaviour should specifically be associated with the same metrics of the gut microbiome as those that were changed as a result of dietary manipulation.

5.3 Materials and Methods

5.3.1 Subjects

Thirty-six great tits were captured using a mist net between January and March 2017 across four sites. Two sites were within Cork city (urban), 1.6 km apart (51°54'32.4"N 8°28'12.0"W and 51°53'56.1"N 8°29'15.4"W), and two were in deciduous woodlands (rural) 23 km apart (51˚43'08.4"N 8˚35'52.8"W and 51˚51'37.5"N 8˚47'01.1"W) and located at least 23km from the urban sites. Birds were lured using feeders containing sunflower seeds and peanuts. All birds were banded with rings issued by the British Trust of Ornithology for individual identification. Birds were sexed (male/female) and aged (adult/juvenile) from plumage characteristics. Upon capture, birds were transported to the aviary facilities at University College Cork and singly housed in wire cages (45 × 50 × 60 cm) containing two wooden perches. Birds were provided with food and water ad libitum. Permission to conduct fieldwork was provided by the National Parks and Wildlife Services (C11/2017), Coillte Forestry and private landowners.

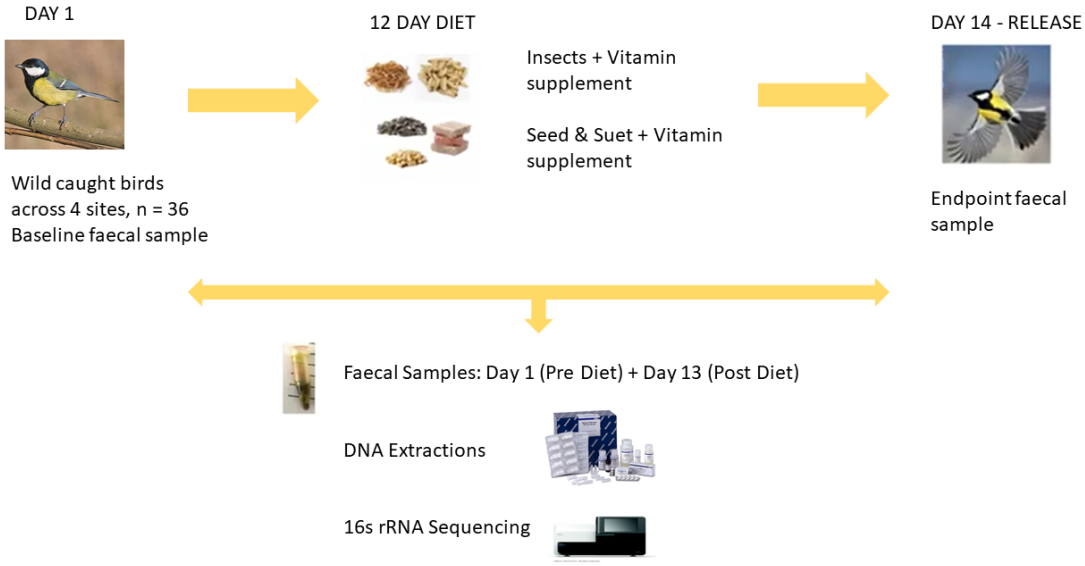


Figure 1: Schematic outlining study design. Birds were captured from the wild across four sites and baseline blood and faecal samples were collected on day 1 before birds underwent a 12-day dietary manipulation of either insect or seed and suet-based diets. Endpoint blood and faecal samples were taken on day 14 and birds were released.

5.3.2 Dietary manipulation

From day 2-13 of captivity, birds were given one of two different dietary treatments designed to reflect seasonal differences and ecological variation seen in the wild, for example changes in the availability of seed or animal food sources (Perrins, 1991; Vel'ký, Kaňuch and Krištín, 2011), or reflecting potential individual differences in dietary specialisations (O'Shea, Serrano-Davies and Quinn, 2017): 1) Seed and suet, n = 17; and 2) Insect diet, n = 19. The insect diet consisted of wax moth larvae (*Achroia grisella*) and mealworm larvae (*Tenebrio molitor*). Notably, these diets differed in the relative content of protein, fat and fibres (Table S1), features of diets that have previously been associated with differences in host microbiota in mammals (Singh *et al.*, 2017). Mealworms were provided ad libitum, and five wax worms were provided each morning and each evening. The seed diet consisted of

sunflower hearts, peanuts, and suet. We provided birds in the seed diet five mealworms and one wax worm on day seven of captivity for welfare reasons as we did not want to deprive the birds of a preferred food item as preference for mealworms/wax worms was observed in our population, and on the basis of previous work (Aplin, Sheldon and McElreath, 2017). To limit more general nutritional deficiencies, all birds received vitamin powder mixed with their food and drops mixed in their water (AviMix®). Birds were assigned to treatment groups randomly, counterbalanced for age and sex.

5.3.3 Faecal sampling

Faecal samples were collected within one hour of arrival into the aviary, and again on Day 13 of captivity. A clean sheet of brown paper was placed on the floor of each cage for faecal collection. Paper was used in order to soak liquid urea away from the faecal matter as urea can act as a downstream inhibitor to amplification (Khan *et al.*, 1991). Using sterile inoculation loops, we transferred the faecal matter into tubes containing 1ml of 100% ethanol and stored tubes at -20°C.

5.3.4 DNA Extraction

Microbial DNA was extracted using the Qiagen QIAamp DNA Stool Kit, following the "Isolation of DNA from Stool for Pathogen Detection" protocol with modifications described in Zeale *et al.* (2011) to increase DNA yield and remove excess inhibitors expected to be present in the uric acid of bird faeces. Briefly, a 0.1 - 0.2 g aliquot of faecal sample was added to 1.4 ml of buffer ASL supplied in the kit together with 0.4 g of zirconia beads. The samples were homogenised for one minute using a vortex. The suspension was then heated for 30 minutes at 70°C following the addition of Proteinase K. Following centrifugation, one InhibitEx tablet was added to 1.2 ml of the supernatant and the suspension was incubated for one minute at room temperature to allow inhibitors to absorb to the InhibitEx matrix. The samples were centrifuged twice and 20 µl of Proteinase K was added to 400 µl of the

supernatant along with 400 µl of AL. The suspension was homogenised and incubated at 70°C for 15 minutes. 400 µl of ethanol (96-100%) was added to the lysate and thoroughly mixed using a vortex. Subsequent removal of RNA, protein and purification of the DNA was completed according to the manufacturer's instructions. DNA was then stored at -20°C.

5.3.5 16S Sequencing

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Klindworth *et al.*, 2013). Each PCR reaction contained 23 µl DNA template, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 25 µl 2X Kapa HiFi Hotstart ready mix (Roche, Ireland), to a final volume of 50 µl. Two negative controls were run in parallel: one from the DNA extraction and one containing PCR water instead of DNA template. PCR amplification was carried out as follows: heated lid 110°C, 95°C x 3 minutes, 30 cycles of 95°C x 30 seconds, 55°C x 30 seconds, 72°C x 30 seconds, then 72°C x 5 minutes and held at 4°C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V). Ten samples failed to amplify and were not pooled for sequencing (Day 1: n = 1 (seed), Day 13: n = 3 (seed), n = 6 (insect)). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5 µl) to index each of the samples, allowing samples to be pooled for sequencing on the same flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2x Kapa HiFi

Hot Start Ready mix, 10 µl PCR grade water. PCRs were completed as described above, but with eight amplification cycles. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit (Bio-Sciences, Dublin, Ireland), along with the high sensitivity DNA quantification assay kit (Bio-Sciences, Dublin, Ireland) and samples were then pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. The sample pool was prepared following Illumina guidelines. Samples were sequenced at the Teagasc Sequencing Centre on an Illumina MiSeq sequencing platform using a 600 cycle v3 kit according to the manufacturer's instructions and following standard Illumina sequencing protocols.

5.3.6 Bioinformatics Analysis

Three hundred base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Alpha diversities were generated in QIIME 4. Samples with <15,000 reads were excluded from the analysis; therefore, excluding our two negative control samples.

5.3.7 Statistical analyses

16S rRNA sequencing resulted in between ~16,000 and 180,000 reads per sample; 17 samples had less than 40,000 sequencing reads, so were therefore interpreted

cautiously. The 16S rRNA gene sequencing revealed approximately 798 bacterial genera of 37 different phyla in insect and seed groups. Statistical analysis was carried out in SPSS, R software environment (version 1.0.136), and Calypso. We included all taxa in the dataset, most notably those that are often viewed as 'dietary contaminants' (i.e. microbes originating from ingested food, such as cyanobacteria present in plants). This is because not all cyanobacteria are dietary contaminants (Di Rienzi *et al.*, 2013) and because our study specifically tested differences between diets. Therefore, removal of one so-called 'dietary contaminant' specific to plants (i.e. cyanobacteria), but none specific to insects (e.g. insect microbiome) could systematically bias our results. Relative abundance of bacterial taxa was expressed as % of identified sequences. Between-group differences in alpha-diversity indices and in the relative abundance of bacterial phyla and genera were analysed with paired-sample Wilcoxon test in R Studio for the following comparisons: Pre-Insect diet vs. Post Insect diet, and Pre-Seed diet vs. Post Seed diet. Kruskal Wallis test was used to estimate the differences between groups for the following comparisons: Post Insect diet vs. Post Seed diet, Pre-Insect diet vs. Pre-Seed diet, Post Insect diet vs. Pre-Seed diet, Pre-Insect diet vs. Post Seed diet, and Fast exploration vs. Slow exploration behaviour. p value < 0.05 was deemed significant; Benjamini-Hochberg adjustment with Q=0.2 was used to correct p values for multiple testing.

Four different measures of alpha diversity were used as response variables to test the effect of diet on the microbiome community: Shannon's index, Simpson's index, Abundance-based Coverage Estimator (ACE), and Chao1. The Shannon index was employed to measure the overall diversity of the community, including both the number of present taxa and evenness, while Chao1 was used to quantify the number of species present in a community, omitting the abundance of each species. Chao1 and ACE are both estimates of richness, while Shannon and Simpson indices are indicators of evenness. Richness measures the number of present taxa, while Evenness measures how evenly abundant the present taxa are.

PCoA plots based on Bray-Curtis distance matrices were constructed using R Studio (version 1.0.136), and the "vegan" package (vector size and orientation was

determined with the envfit() function. Base images were generated using the "ggplot2" package. The adonis () function in the 'vegan' package (version 2.3-2) for R Studio (version 1.0.136) was used to analyse beta diversity. A p value of <0.05 was considered significant. These are measures of the global shift in microbiota composition, a non-significant p value means there is no overall difference but there may still be changes in the Relative Abundance of individual taxa. Several multivariate analyses were also carried out, including Canonical Correspondence Analysis (CCA), CCA+, Principal Component Analysis (PCA), PCA+, Redundancy Analysis (RDA) and RDA+ to further investigate the complex associations between community composition and various explanatory variables. PCA analysis allows data visualisation as a 2D plot, identifying sample clusters and determining outliers, which may need to be excluded from downstream analysis, while CCA tests if variations in the data matrix can be explained by selected variables, and RDA indicates how well samples can be separated according to the defined explanatory variables. Feature selection statistical analysis (LDA Effect Size (LEfSe)) was used to determine the genera most likely to explain differences between groups. Finally, hierarchical clustering of the 50 most abundant genera ($p < 0.05$; analysis of variance) were visualised using a heatmap to determine patterns in gut microbiota based on explanatory variables such as diet, exploration behaviour, habitat, age, and sex. Heatmaps of the top 50 genera were constructed in Calypso based on the Spearman correlation metric.

5.3.8 Exploration Behaviour Assay

On the morning after capture, birds underwent an exploratory behaviour assay as a measure of the reactive proactive axis (Verbeek, Drent and Wiepkema, 1994). To avoid unnecessary handling of the birds, each housing cage was connected to the novel assay room by a sliding trapdoor, so that birds could be moved between rooms without handling. The assay room was based on the design used in Verbeek, Drent and Wiepkema (1994). The trial lasted two minutes, beginning from when the bird entered the room. The number of flights and hops performed by each bird was

recorded throughout the trial and exploration scores were then expressed by the sum of all the movements recorded during the first two minute trial undertaken by the birds (Dingemanse *et al.*, 2002). Exploratory behaviour is a significantly repeatable trait in our populations (adjusted for age: p = 0.04, R2 = 0.40). Exploration score was taken the morning after their arrival in the aviary, and after 12 days of captivity.

5.3.9 Corticosterone Sampling

The concentration of CORT in response to a standard capture/restraint stressor (Wingfield and Romero, 2010) was quantified at the point of capture in the field, and 13 days following captivity. All blood samples were taken between the hours of 8 am and 12 pm by pricking the brachial vein with a 27-gauge needle tip to obtain approximately 40 µl of blood. Two samples were collected at each sampling day to quantify both the basal CORT concentration and stressor-induced CORT concentrations. Blood was collected in a capillary tube before being transferred to a microcentrifuge tube on wet ice. Baseline CORT was sampled within three minutes of the first stressor, which was when they hit the mist net in the field, or upon experimenters entering the aviary in captivity. Birds were held in a cloth bag until a second sample was taken from the opposite brachial vein 30 minutes from the initial stressor. From the field, birds were held in a cloth bag and transported to the aviary at the University. If sampled in captivity, birds were returned to their home cage for approximately two hours, before being released at the site of capture. Blood samples were centrifuged within two hours of collection to separate the plasma from the red blood cells. The plasma was extracted and stored in a separate microcentrifuge tube at -20°C.

5.3.10 Corticosterone Hormone Assay

Blood plasma (6 µl - 29 µl) was quantified via radioimmunoassay (Spencer, Evans and Monaghan, 2009). The assays were performed using anti-CORT antiserum

(Esoterix Endocrinology, USA, B3-163; 1:15,000 dilution in assay buffer) and [1,2,6,7-3H]-CORT label (Perkin Elmer, UK). Bound and free portions were separated using 500 µl of a dextran coated charcoal suspension (0.25% dextran, 0.5% charcoal). Samples were run in one assay and in duplicate. Extraction efficiency was quantified from a 50 µl aliquot taken from each of the reconstituted samples (mean = 66 ± 0.06 %). Calculations of hormone concentrations were corrected for variation in initial aliquot volume and individual recoveries. ED50 for the assay was 0.68 ng/ml, % binding was 28.45% and non-specific binding was 1.74%.

5.4 Results

5.4.1 Alpha Diversity

To determine if alterations in microbial diversity occurred as a result of consuming an insect diet compared to a seed-based diet, alpha and beta diversity were investigated in Calypso and in R Studio (version 1.0.136). The Shannon index was employed to measure the overall diversity of the community, including both the number of present taxa and evenness, while Chao1 is a quantification measurement with respect to the number of species present in a community, omitting the abundance of each species. Chao1 and ACE are both estimates of richness, while Shannon and Simpson indices are indicators of evenness. Richness measures the number of present taxa, while Evenness measures how evenly abundant the present taxa are. Diet was found to have a significant effect on alpha diversity (Figure 2) according to ACE (p=0.003, A), Chao1 (p=0.00796, B), and Simpson (p=0.00427, D) indexes at OTU level. Birds fed the insect diet had significantly different microbiome profile at alpha diversity level compared to birds post seed diet using using Chao1 (p=0.021) and Shannon (p=0.045) indexes, where there was a decrease in alpha diversity observed. There was no significant difference in alpha diversity between pre insect and post insect diet, however the Simpson index was significant before FDR correction was applied (p=0.047). There was no significant difference in alpha diversity between pre seed and post seed diet. Finally, no significant differences in alpha diversity were observed based on

exploration behaviour, categorised by fast versus slow behaviour (Figure 3). Sex, sample day and habitat do not predict exploration behaviour, while adults were more exploratory than juveniles (p = 0.04). Overall, no microbiome metrics were found to predict exploration behaviour.

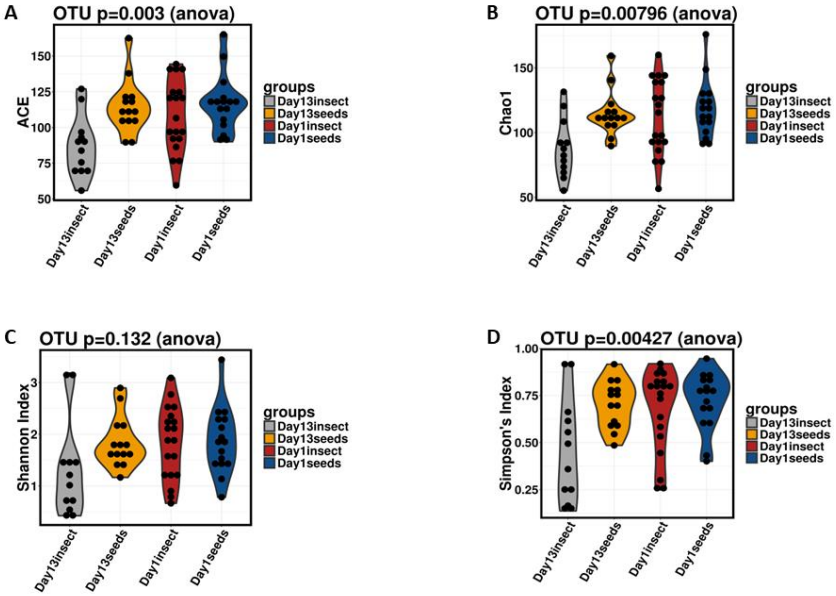


Figure 2: Diet had a significant effect on alpha diversity, as significant differences were observed between insect and seed diets. Alpha diversity estimates for insect and seed diets, before and after intervention, for ACE (A), Chao1 (B), Shannon (C) and Simpson (D) indices.

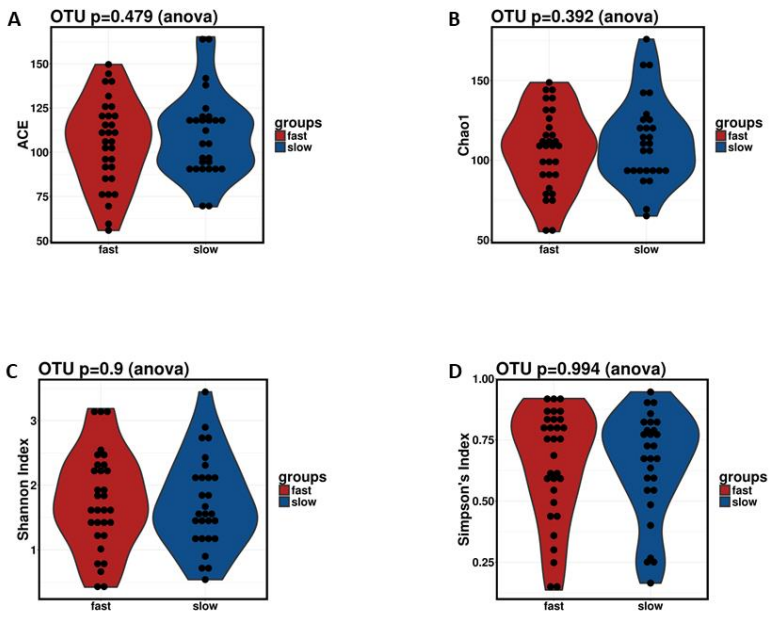


Figure 3: There were no significant differences in alpha diversity based on exploration behaviour (fast versus slow). Alpha diversity estimates for fast versus slow exploration behaviour based on ACE (A), Chao1 (B), Shannon (C) and Simpson (D) indices.

5.4.2 Beta Diversity

To determine if any significant differences in beta diversity occurred across dietary treatments, PCoA plots were constructed based on Bray Curtis distance matrices. We sought to investigate if samples from the same dietary groups would cluster together, and if there would be separation based on insect and seed diets. We also aimed to determine whether differences in beta diversity were evident based on exploration behaviour (fast versus slow). Adonis tests showed dietary treatment significantly influenced beta diversity between insect and seed diets (p=0.012, Figure 4A). Upon further investigation it was found that consumption of an insect-based diet significantly altered beta diversity at genus level (p=0.018, Figure 4B), with the post insect diet group clustering separately from the other groups. However, consumption of a seed-based diet had no significant impact on beta diversity at genus level (p=0.425, Figure 4C). There was a trend towards significance

in beta diversity at genus level between the insect and seed groups post dietary manipulation (p=0.051, Figure 4D).

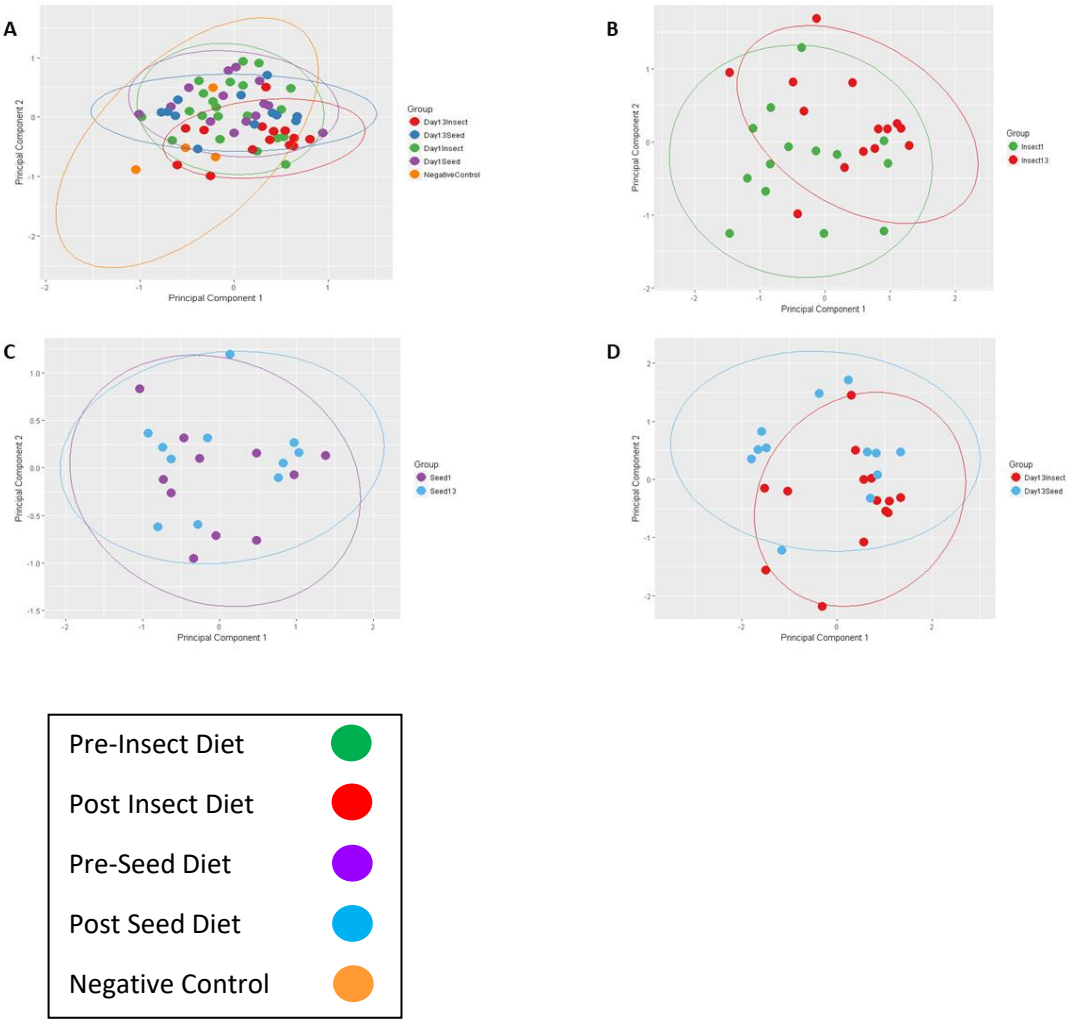
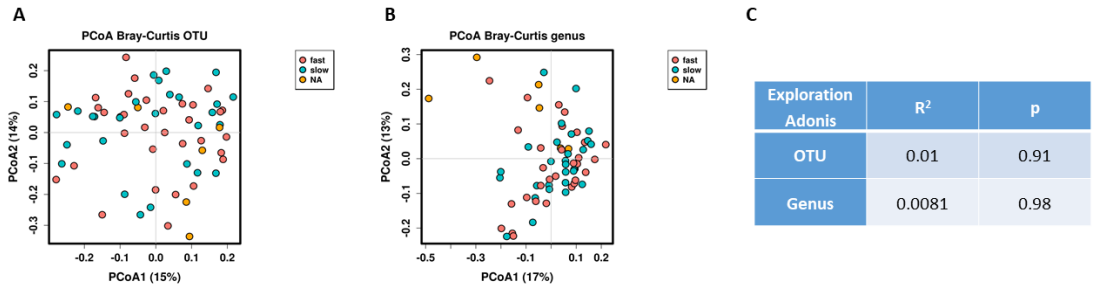


Figure 4: Visualisation of the PCoA analysis based on Bray-Curtis distance matrices at genus level to determine if there is separation of samples based on diet and time for (A) all groups, (B) insect pre vs. post, (C) seed pre vs. post, (D) post insect vs. post seed diet. Green represents pre insect diet, purple denotes pre seed diet, red represents post insect diet and blue denotes post seed diet. Adonis variance analysis based on Bray-Curtis distance matrices at genus level comparing insect versus seed dietary manipulation and sample date. A p value <0.05 is considered significant.

No clustering or separation between fast and slow exploration was observed on PCoA plots constructed based on Bray Curtis distance matrices (Figure 5). Furthermore, there was no significant difference in beta diversity at OTU (p=0.91) or genus level (p=0.981) based on exploration behaviour as measured using Adonis (Figure 5C). Finally, no significant differences in beta diversity were observed based on either sex or age (data not shown).



C

Exploration Adonis	R^2	p
OTU	0.01	0.91
Genus	0.0081	0.98

Figure 5: Visualisation of PCoA analysis based on Bray-Curtis distance matrices at OTU (A) and genus level (B) to determine if there was separation of samples based on fast versus slow exploration behaviour. Red represents fast exploration behaviour, while blue denotes slow exploration behaviour. Adonis results based on Bray Curtis distance matrices at OTU and genus level (C). A p value <0.05 is considered significant. R^2 is the proportion of inter-group variance explained by the microbiota composition. These are measures of the global shift in microbiota composition, a non-significant p value means there is no overall difference but there may still be changes in the Relative Abundance of individual taxa. Performed using the adonis () function in the 'vegan' package (version 2.3-2) for R (version 3.2.3).

To further interrogate the factors associated with the clustering of samples based on diversity, a number of additional multivariate analyses were carried out, including Canonical Correspondence Analysis (CCA), CCA+, Principal Component Analysis (PCA), PCA+, Redundancy Analysis (RDA) and RDA+ to further investigate

the complex associations between community composition and various explanatory variables (Figure 6). PCA analysis allows data visualisation as a 2D plot, identifying sample clusters and determining outliers, which may need to be excluded from downstream analysis, while CCA tests if variations in the data matrix can be explained by selected variables, and RDA indicates how well samples can be separated according to the defined explanatory variables. Significant differences were observed based on site, time, and diet in these analytical tests (Table 1). No significant differences were observed based on sex, age, and exploration behaviour. Significance was accepted as $p<0.05$.

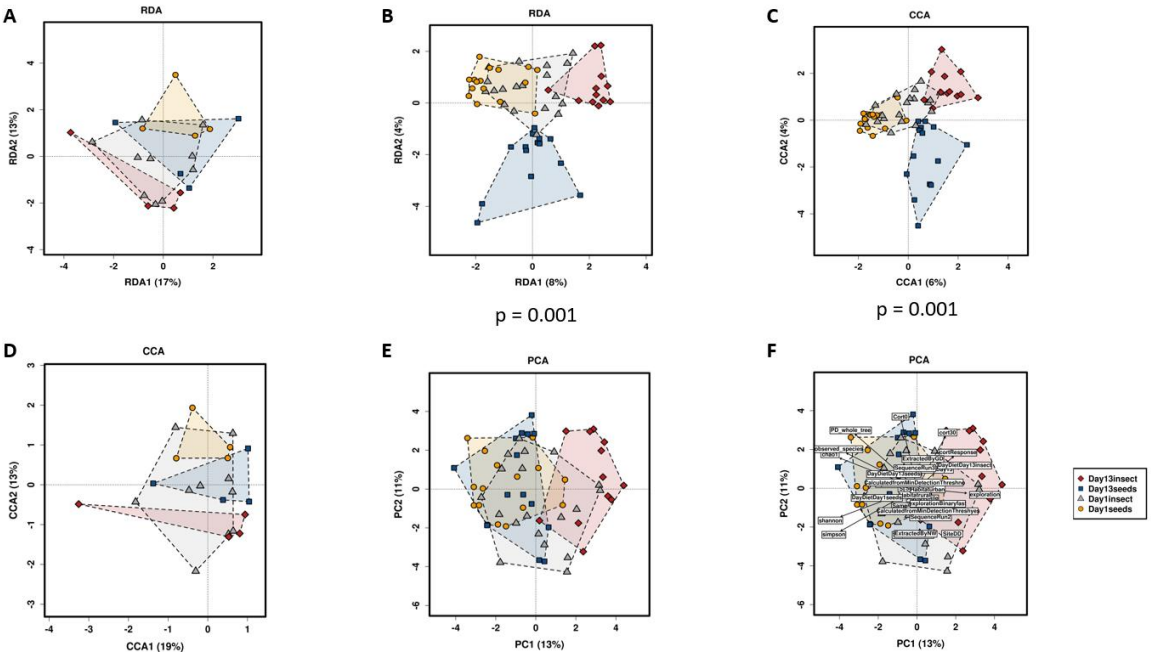


Figure 6: Multivariate Analysis performed in Calypso: (A) Redundancy Analysis + (RDA+), (B) RDA, (C) Canonical Correspondence Analysis (CCA), (D) CCA+, (E) Principal Component Analysis (PCA), and (F) PCA+ for all groups to further investigate the complex associations between community composition and various explanatory variables. The pre insect group is shown in grey, the post insect group in red, the pre seed group in yellow and the post seed group in blue.

Table 1: P values from diet RDA+ and CCA+ multivariate analysis in Calypso based on site, sex, age, time, diet and exploration. Significance is accepted as p<0.05.

Group	RDA+	CCA+
Site	0.033	0.033
Sex	0.303	0.295
Age	0.414	0.468
Time	0.005	0.01
Diet	0.013	0.106
Exploration	0.474	0.471

Multivariate analysis was again carried out based on exploration behaviour (Figure 7). Significant differences were observed based on site, time, and diet, while no significant differences were observed based on sex, age and exploration behaviour (Table 2). Significance was accepted as p<0.05.

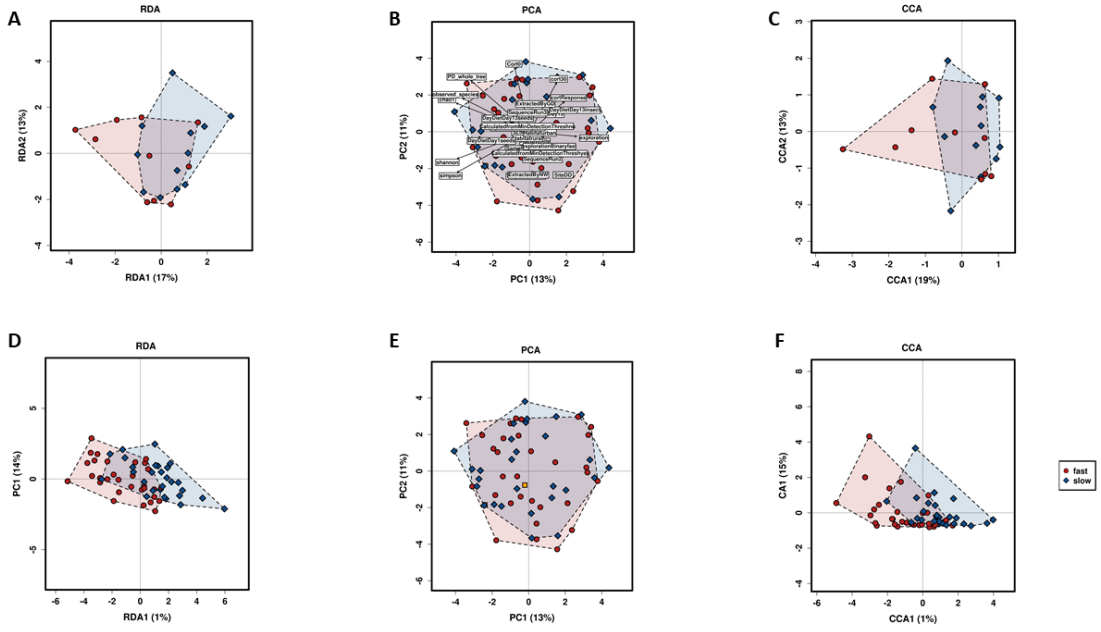


Figure 7: Multivariate Analysis performed in Calypso: (A) Redundancy Analysis + (RDA+), (B) Principal Component Analysis + (PCA+), (C) Canonical Correspondence Analysis + (CCA+), (D) RDA, (E) PCA (F) CCA for all groups to further investigate the complex associations between community composition and various explanatory variables. The fast exploration group is shown in red and the slow exploration group in blue.

Table 2: P values from exploration RDA+ and CCA+ multivariate analysis in Calypso based on site, sex, age, time, diet and exploration. Significance is accepted as $p<0.05$.

Group	RDA+	CCA+
Site	0.029	0.016
Sex	0.292	0.270
Age	0.410	0.422
Time	0.002	0.007
Diet	0.021	0.083
Exploration	0.480	0.424

5.4.3 Relative Abundance

At phylum level, insect and seed groups shared common phyla pre- and post-dietary manipulation. The most prevalent phyla across all samples were Proteobacteria (~57.3%), Cyanobacteria (~15%), Firmicutes (~10%), and Tenericutes (~9.45%), with Actinobacteria (~3%), and Bacteroidetes (~2%) contributing smaller proportions of the sequencing reads. Figure 8 depicts relative abundance across dietary treatments. At phylum level, fast and slow exploration groups shared common phyla including Proteobacteria (~57%), Cyanobacteria (~15%), Firmicutes

(~10%) and Tenericutes (~9%). Figure 9 outlines relative abundance across exploration behaviours.

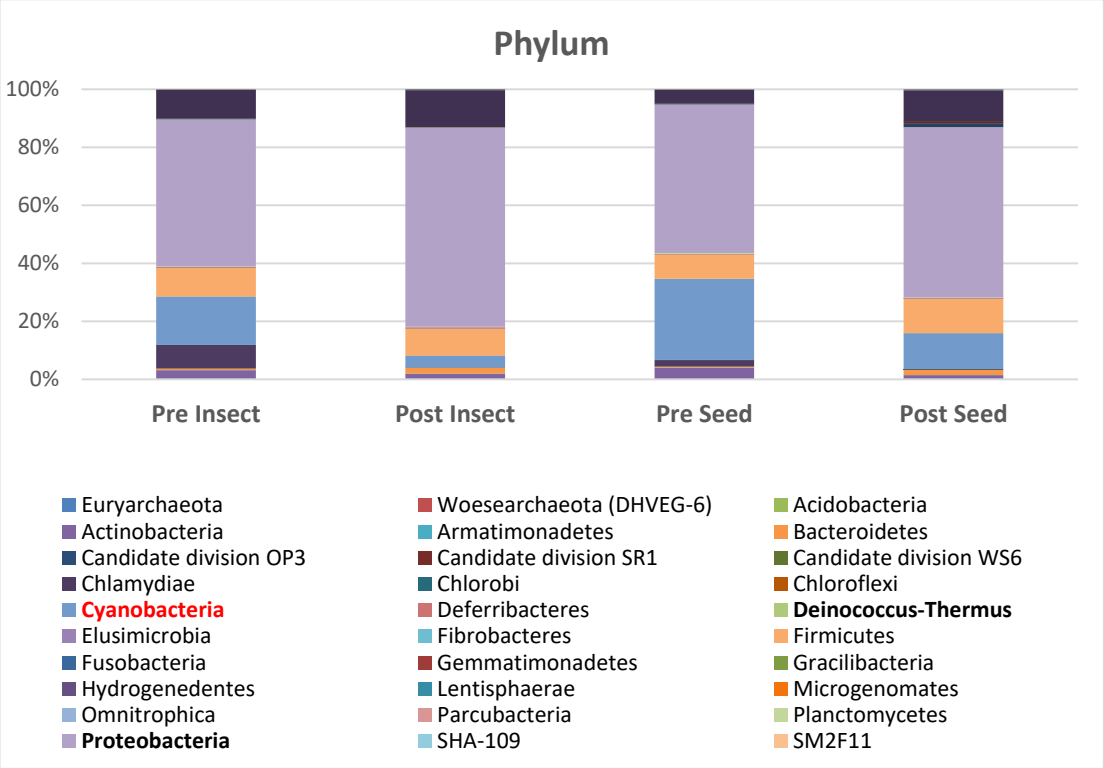


Figure 8: Relative abundances (%) distribution of bacterial phyla across dietary treatments and sample dates. Statistically significant differences are highlighted in bold and red ($p<0.05$). Statistically significant differences before FDR correction are highlighted in bold.

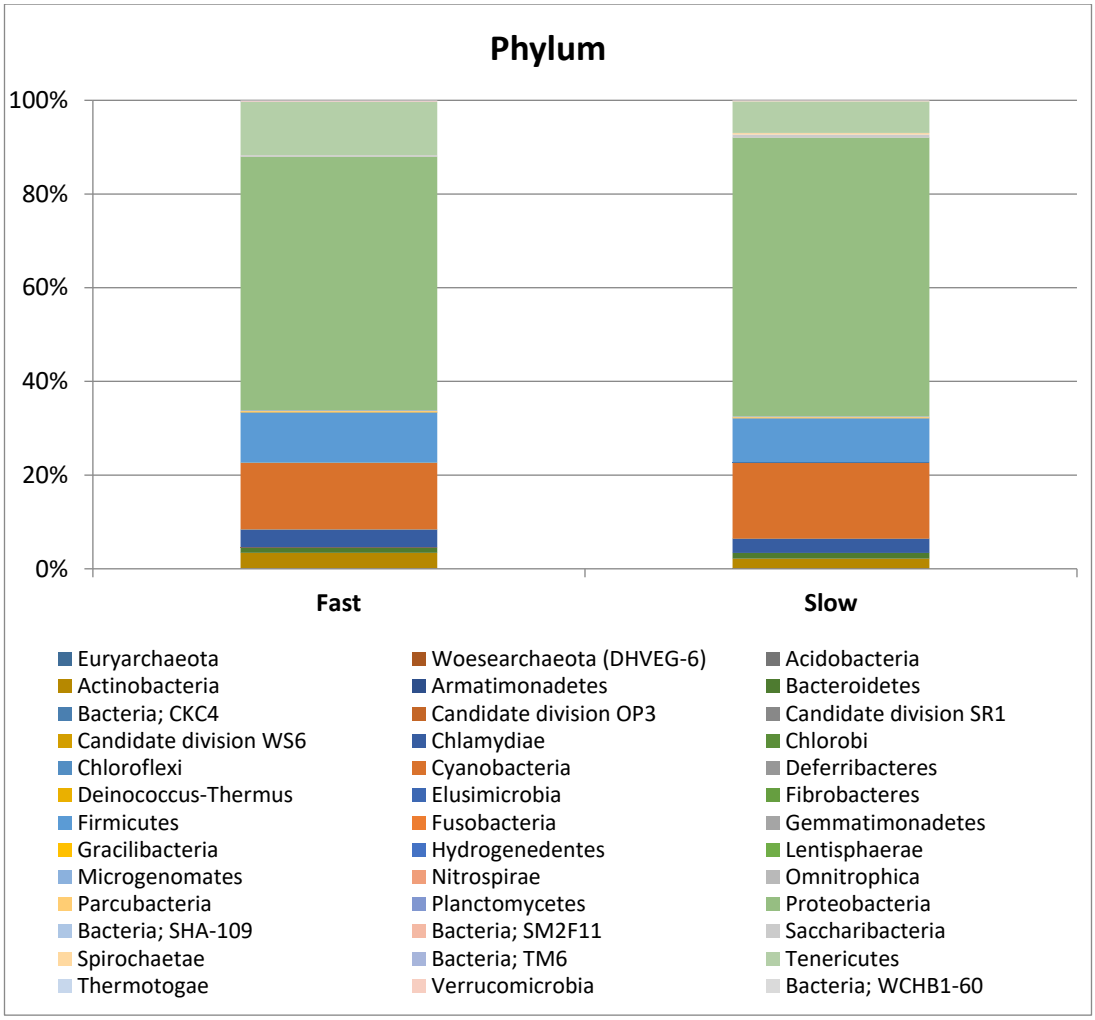


Figure 9: Relative abundances (%) distribution of bacterial phyla based on fast versus slow exploration behaviour. No statistically significant differences in phyla were observed.

To determine if statistically significant differences in relative abundance occurred at phylum level between insect and seed diets and between fast and slow exploration, the Kruskal Wallis and Wilcox tests were used (Table 3). Statistical significance was accepted as $p<0.05$ and data was adjusted using False Discovery Rate (FDR). Proteobacteria increased significantly over the course of captivity in the insect diet group ($p=0.04$), but not in the seed diet group ($p=0.9$) and tended to be higher in adults than juveniles ($p=0.07$). Cyanobacteria was significantly decreased in the post insect group compared to the pre insect group ($p=0.000269$, FDR=0.01), while

Deinococcus Thermus (p=0.02, FDR=0.36) and Bacteria WD272 (p=0.04, FDR=0.36) were also significantly different between pre- and post-insect dietary manipulation before FDR correction was applied. As expected, seed intervention significantly increased Cyanobacteria relative abundance compared to insect intervention (p<0.001). Spirochaetae (p=0.048) and Other (p=0.037) were both significantly increased in the seed group compared to the insect group. Spirochaetae also increased over the course of captivity in the seed diet group (p=0.003, FDR=0.12). Cyanobacteria was significantly higher in the pre seed group compared to the post insect group (p=0.001). With regard to specific phyla interactions, there was no effect of sex, habitat or sample day on Firmicutes while adults tended to have lower Firmicutes than juveniles (p =0.07). Bacteroidetes increased after captivity (p =0.05) and no effect of sex, age or habitat was observed. No effect of age, sex, habitat, sample day, CORT 0 or CORT 30 was evident on Tenericutes abundance. Similarly, there was no effect of age, sex, habitat, sample day, CORT 0 or CORT 30 on Actinobacteria. There were no significant differences based on exploration at phylum level. None of the phylum-level taxa predicted exploration either at day 1 or day 13. Actinobacteria was significantly higher in birds from urban habitats (p=0.02). There was no significant effect of diet, habitat, sex, or age on Firmicutes or Tenericutes and there were no significant interactions between diet and habitat for all models.

Table 3: Dietary manipulation had a significant effect on microbial composition at phylum level. Statistical significance was accepted as p<0.05 and data is adjusted using False Discovery Rate (FDR).

Phylum	FDR-corrected p value	Comparison
Cyanobacteria	0.010209	Pre vs. Post Insect
Deinococcus Thermus	0.360228	Pre vs. Post Insect
Proteobacteria	0.360228	Pre vs. Post Insect
Bacteria WD272	0.360228	Pre vs. Post Insect

Spirochaetae	0.125123	Pre vs. Post Seed
Cyanobacteria	<0.001	Post Insect vs. Post Seed
Spirochaetae	0.048	Post Insect vs. Post Seed
No blast hit; Other	0.037	Post Insect vs. Post Seed
Cyanobacteria	0.001	Post Insect vs. Pre Seed

At genus level, insect and seed groups shared predominant genera pre- and post-dietary manipulation such as *Methylobacterium, Enterobacter* and *Pseudomonas*. The most prevalent genera in the pre insect diet group were *Methylobacterium* (12.4%), *Enterobacter* (9.9%), *Pseudomonas* (5.6%), *Cronobacter* (4.3%), *Serratia* and *Rahnella* (both 3.6%). The most common genera in the post insect diet group included *Methylobacterium* (43.3%), *Enterobacter* (3.7%), *Pseudomonas* (12.3%), *Clostridium sensu stricto 13* (5.3%) and *Lactobacillus* (2%). The pre seed group was dominated by *Enterobacter* (15.9%), *Methylobacterium* (11.9%), *Diplorickettsia* (5%), *Rickettsiella* (3.8%), *Pseudomonas* (2.9%), *Erwinia* (2.9%) and *Enterococcus* (2.7%). Finally, prominent genera in the post seed group consisted of *Enterobacter* (21.3%), *Methylobacterium* (20.4%), *Campylobacter* (5%), *Erwinia* (4.8%), *Lactobacillus* (3.7%), *Enterococcus* (2.6%) and *Pseudomonas* (2.4%). Figure 10 depicts relative abundance across dietary treatments at genus level.

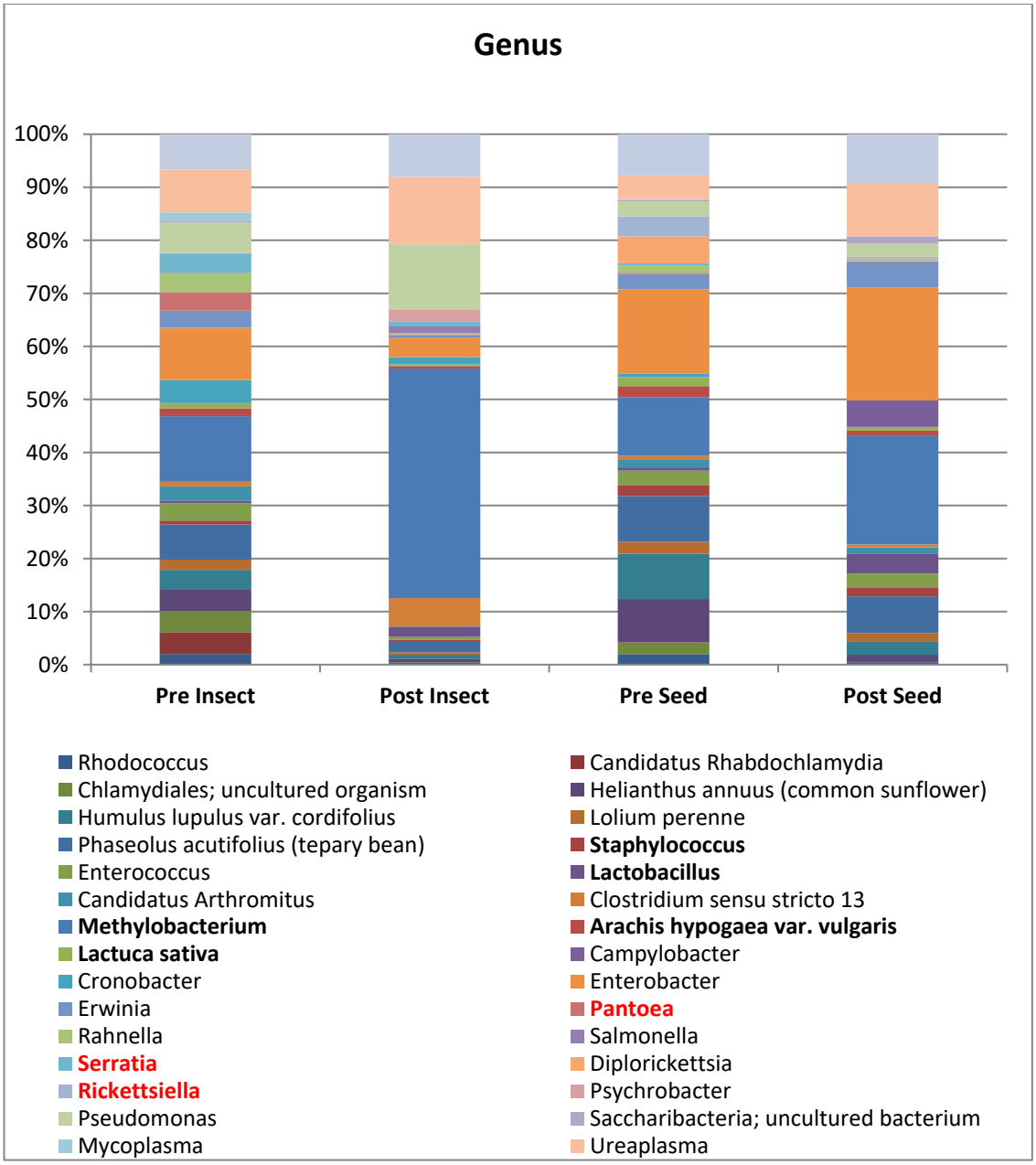


Figure 10: Relative abundances (%) distribution of bacterial genera in insect and seed groups pre versus post dietary manipulation. Statistically significant differences are highlighted in bold and red ($p<0.05$). Statistically significant differences before FDR correction are highlighted in bold. Represented only taxa >1% in at least one group. The Other category contains all other genera present at <1% of assignable reads at genus level.

At genus level, fast and slow exploration groups shared common genera. The most prevalent genera across all samples were *Methylobacterium, Enterobacter* and *Pseudomonas.* The most common genera in the fast group were *Methylobacterium* (18.3%), *Enterobacter* (10.4%), *Pseudomonas* (6.5%), *Clostridium sensu stricto 13* (3%), *Enterococcus* (2.4%), *Erwinia* (2.4%), and *Rhodococcus* (2%). The most prominent genera in the slow exploration group included *Methylobacterium* (25%), *Enterobacter* (12.1%), *Pseudomonas* (5.7%), *Erwinia* (3.2%), *Pantoea* (2.5%), *Cronobacter* (2.3%), and *Enterococcus* (2.2%). Figure 11 outlines relative abundance across exploration behaviours.

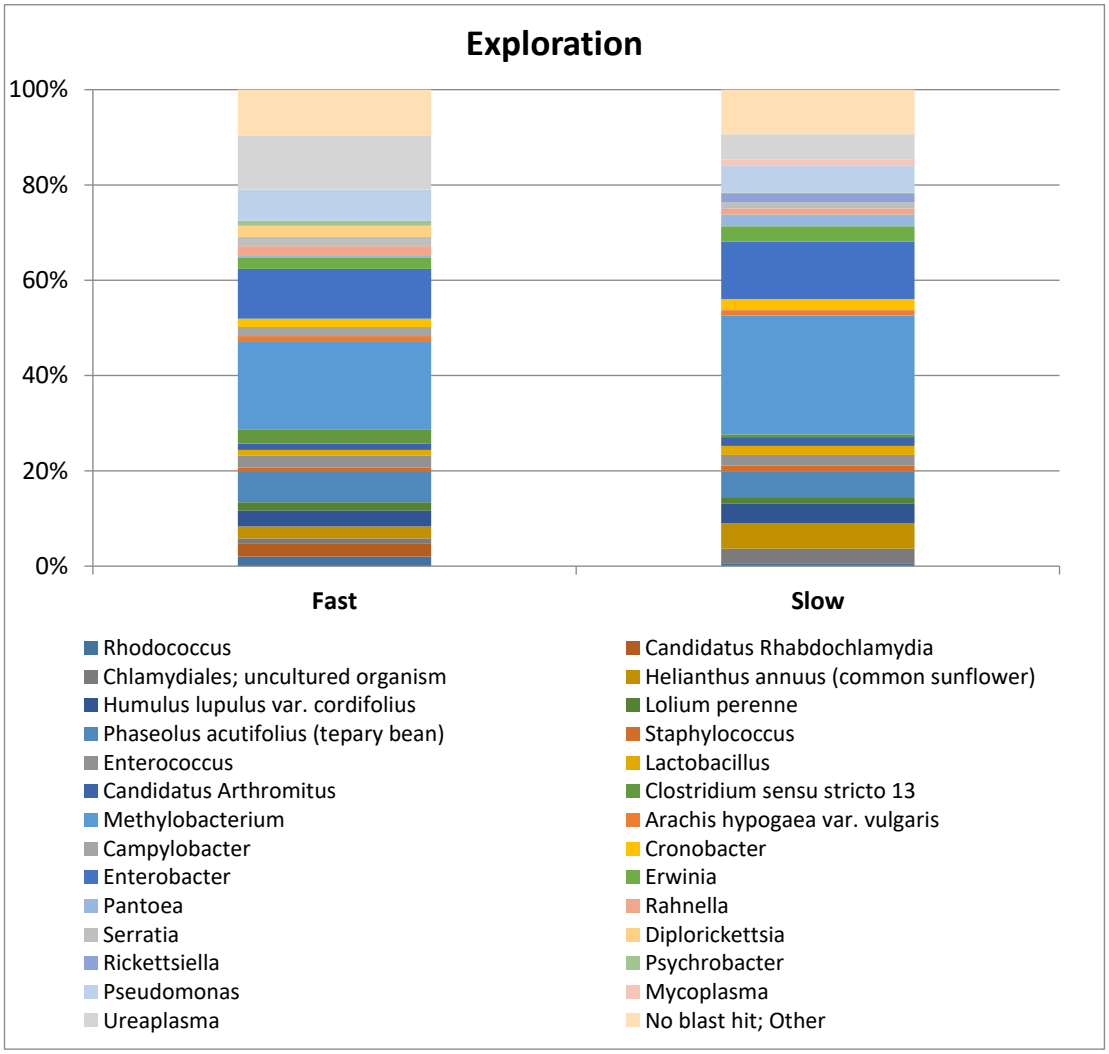


Figure 11: Relative abundances (%) distribution of bacterial genera in fast and slow exploration groups. Represented only taxa >1% in at least one group. The Other category contains all other genera present at <1% of assignable reads at genus level.

Significant differences in genus-level abundance attributed to dietary treatments were found for 43 genera. A complete summary of the significant differences between the post insect and post seed groups can be found in Table 4. There were 55 significant differences between the pre- and post-insect diet groups, however significance was lost after FDR correction. Similarly, 70 differentially abundant genera were observed between the pre- and post-seed diet groups, however significance was lost after FDR correction. These results are outlined in Supplementary Table 2. Birds given the insect diet showed a decrease in *Devosia, Rickettsiella, Sphingomonas, Pantoea, Arthrobacter, Brevibacterium, Brachybacterium, Clostridium and Carnobacterium*, and an increase in *Methylobacterium* and *Candidatus* species such as *Solibacter, Aquiluna* and *Protochlamydia*. Birds given the seed diet showed a decrease in *Cronobacter* and *Serratia*, and an increase in *Microbacterium*. Birds in both dietary groups showed a decrease in *Bradyrhizobium, Staphylococcus and Rahnella*, and an increase in *Lactobacillus, Bacillus, Ureaplasma, Delftia, Flavobacterium, Streptococcus and Rhodococcus*.

Table 4: Kruskal Wallis analysis was performed in R Studio on relative abundance data at genus level to determine if statistically significant differences occurred between insect and seed dietary manipulation. 43 genera were found to be significant between post insect and post seed diet groups. Statistical significance was accepted as $p<0.05$ and data is adjusted using False Discovery Rate (FDR).

		Pre-dietary Manipulation		Post-dietary Manipulation	
Genus	**p (FDR)**	**insect**	**seed**	**insect**	**seed**
Acidimicrobiaceae CL50029 marine group	0.022	0.002	0.003	0.000	0.002
Corynebacterium	0.037	0.008	0.017	0.002	0.009
Williamsia	0.022	0.012	0.042	0.006	0.013
Corynebacteriales uncultured	0.008	0.020	0.038	0.078	0.011
Geodermatophilaceae uncultured	0.009	0.001	0.000	0.000	0.002
Demetria	0.028	0.002	0.000	0.000	0.064
Intrasporangiaceae uncultured	0.001	0.012	0.020	0.004	0.052
Lysinimonas	0.004	0.020	0.096	0.005	0.011
Nesterenkonia	0.009	0.018	0.034	0.001	0.008
Aeromicrobium	0.016	0.008	0.010	0.002	0.009
Nocardioides	0.004	0.022	0.237	0.018	0.033
Pseudonocardia	0.025	0.010	0.017	0.001	0.007
Bacillus	0.025	0.038	0.015	0.025	0.025
Sinobaca	0.004	0.014	0.078	0.000	0.017
Listeria	0.048	0.005	0.007	0.003	0.006
Planctomycetaceae uncultured	0.01	0.021	0.092	0.001	0.006
Caulobacter	0.009	0.000	0.004	0.000	0.004
Caulobacteraceae uncultured	0.001	0.005	0.014	0.001	0.025
Aurantimonadaceae uncultured	0.009	0.004	0.047	0.000	0.002

Bartonella	0.028	0.007	0.009	0.000	0.002
Ochrobactrum	0.049	0.005	0.028	0.006	0.027
Paracocccus	0.02	0.001	0.006	0.236	0.787
Sulfitobacter	0.022	0.000	0.000	0.000	0.014
Rhodobacteraceae uncultured	0.021	0.001	0.001	0.002	0.021
Cellvibrio	0.039	0.003	0.005	0.001	0.006
Pantoea	0.009	3.491	0.240	0.122	0.185
Proteus	0.049	0.001	0.000	0.031	0.000
Serratia	0.002	3.623	0.475	0.867	0.075
Rickettsiella	0.004	0.072	3.738	0.025	0.308
CrystalBog021C3 Uncultured gamma proteobacterium	0.025	0.000	0.000	0.000	0.000
Rubritalea	0.047	0.000	0.000	0.004	0.047
Luteolibacter	0.009	0.004	0.021	0.000	0.001
Roseibacillus	0.021	0.000	0.000	0.002	0.043
Lactuca sativa	<0.001	0.992	1.695	0.325	0.695
Cronobacter	<0.001	4.319	0.700	1.370	0.008
Other	0.037	0.046	0.075	0.006	0.099

Significant differences in genus-level abundance attributed to exploration behaviours were found for seven genera, while four genera fell just outside of the threshold for significance (Table 5). *Corynebacterium, Flavihumibacter, Sphingobacterium, Geobacillus, Aerococcus, Finegoldia, Acetobacteraceae uncultured*, and *Chlorochromatium* were increased in the fast group while *Actinotalea, Ruminococcus 2*, and *Hyphomonadaceae uncultured* were increased in the slow group compared to the fast exploration group.

Table 5: Kruskal Wallis analysis was performed in R Studio on relative abundance data at genus level to determine if statistically significant differences occurred between fast and slow exploration behaviours. Seven genera were found to be significant fast and slow exploration groups. Statistical significance was accepted as $p<0.05$ and data is adjusted using False Discovery Rate (FDR).

Genus	P value
Corynebacterium 1	0.014
Actinotalea	0.015
Flavihumibacter	0.028
Sphingobacterium	0.053
Geobacillus	0.051
Aerococcus	0.028
Finegoldia	0.028
Ruminococcus 2	0.013
Hyphomonadaceae uncultured	0.026
Acetobacteraceae uncultured	0.053
Chlorochromatium	0.051

To determine whether there were bacteria present in the gut microbiota that could discriminate based on insect versus seed dietary intervention, a feature selection statistical analysis (LDA Effect Size (LEfSe)) was used. LEfSe identified 19 discriminating bacteria for the pre seed group, seven discriminating genera for the post seed group, and nine discriminating taxa for the post insect group (Figure 12).

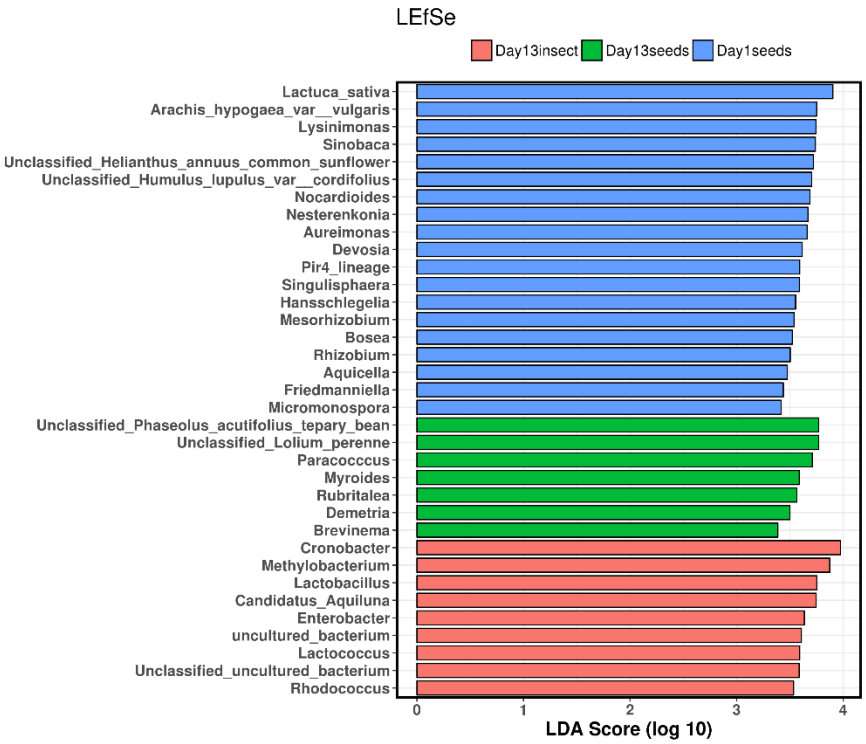
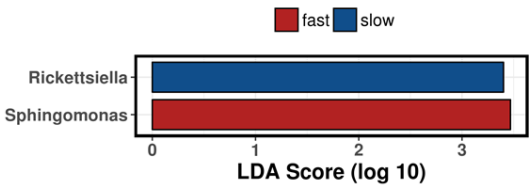


Figure 12: Feature selection statistical analysis (LDA Effect Size (LEfSe)) was used to determine the genera most likely to explain differences between groups. LEfSe identified 19 discriminating bacteria for the pre seed group (blue), seven discriminating genera for the post seed group (green), and nine discriminating taxa for the post insect group (red).

To determine whether there were bacteria present in the gut microbiota that could discriminate based on fast versus slow exploration behaviour, a feature selection statistical analysis (LEfSe) was used, which determined the genera most likely to explain differences between exploration groups (Figure 13). LEfSe identified one discriminating genus for the slow exploration group (*Rickettsiella*, LDA score = 3.4) and one discriminating genus for the fast exploration group (*Sphingomonas*, LDA score = 3.5).

A



B

Selected Taxa	LDA Score	Associated Group
Rickettsiella	3.402	slow
Sphingomonas	3.469	fast

Figure 13: Feature selection statistical analysis (LDA Effect Size (LEfSe)) was used to determine the genera most likely to explain differences between groups. LEfSe identified one discriminating bacteria for the slow exploration group (blue) and one discriminating genus for the fast exploration group (red).

Finally, hierarchical clustering of the 50 most abundant genera ($p < 0.05$; analysis of variance) were visualised using a heatmap to determine patterns in gut microbiota based on insect or seed dietary manipulation and based on exploration behaviour as well as other explanatory variables such as habitat, age, and sex (Figure 14). Rows represent explanatory variables, columns represent taxa, red represents a positive correlation and blue represents a negative correlation. Genera highlighted in the first, second and third rows were present at greater abundances in the pre dietary manipulation groups, while genera highlighted in the 12th row were present at greater abundances in the post seed group. Genera highlighted in red in the last row were present at greater abundances in the post insect group, while genera in the same row highlighted in blue were present at lower abundances post insect intervention.

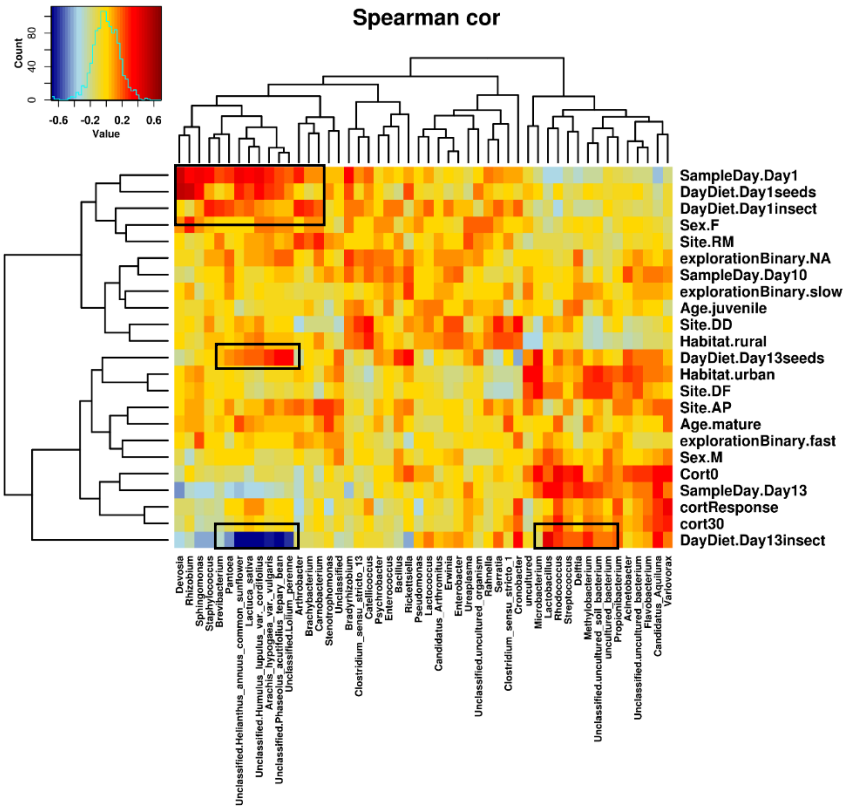


Figure 14: Microbiota associated with insect or seed dietary manipulation and with fast or slow exploration behaviour. Heatmap based on genus relative abundance data. Rows represent explanatory variables, columns represent taxa, red represents a positive correlation and blue represents a negative correlation. Black boxes highlight positive and negative correlations observed based on explanatory variables.

5.4.4 Stress Response

Dietary intervention had no significant impact on CORT response. There was a significant difference in baseline CORT concentrations (CORT 0) between pre- and post-insect groups ($p = 0.039$), indicating an effect of time rather than diet as there were no differences in CORT 0 between insect and seed groups. This is likely due to the effect of captivity which increased baseline CORT in the insect group but not the seed group, suggesting a possible role of the seed diet in protecting from the effects of captivity on stress. No significant differences in either CORT 30 or CORT response were observed based on diet (Figure 15). There was a significant effect of

habitat on CORT 0, with urban habitat birds having lower baseline CORT levels than deciduous birds (p = 0.06). There was no relationship between exploration and CORT 0, CORT 30 or CORT response, and no significant interactions between CORT and sample day. CORT 30 tended to be lower with higher Firmicutes (p = 0.086) when controlling for age and sample day. CORT 0 tended to increase with Bacteroidetes (p=0.09) when controlling for sample day, however, when sample day was not controlled for there was a significant effect on CORT (p = 0.03), further highlighting the influence of time, particularly in captivity on baseline CORT levels. Regarding alpha diversity interactions, CORT 0 was higher in birds with higher Chao1 (p = 0.04), and with more observed species (p = 0.02).

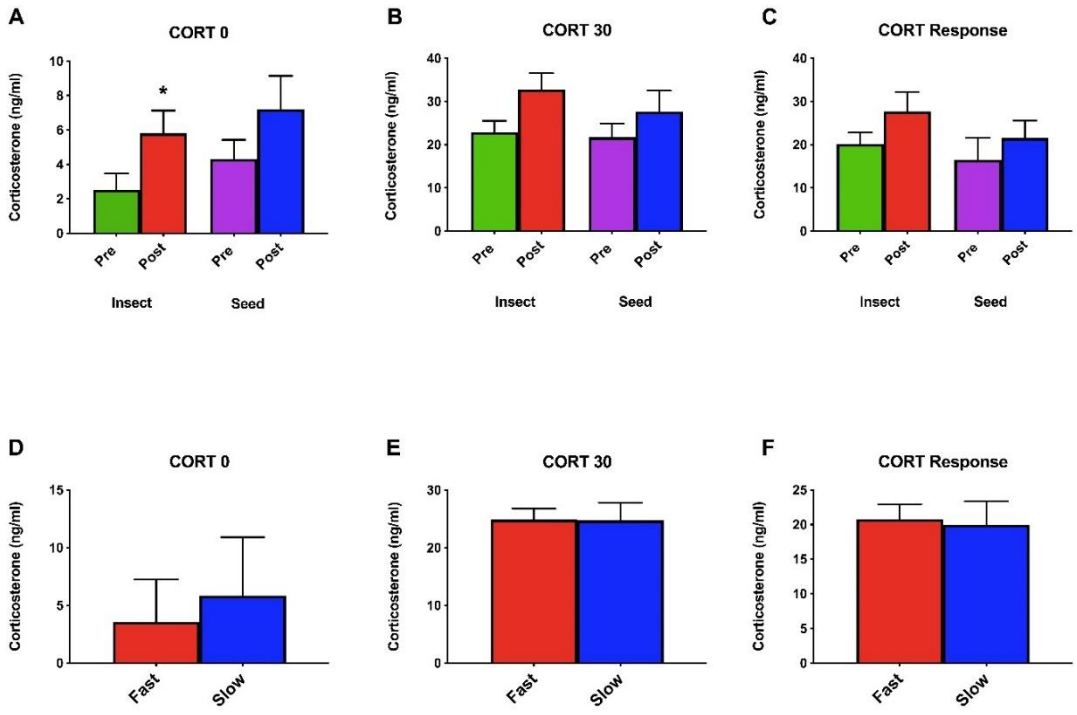


Figure 15: Blood corticosterone responses to acute stressor did not change after dietary intervention. Baseline CORT was sampled within three minutes of the first stressor before birds were held in a cloth bag until a second sample was taken 30 minutes from the initial stressor. There was also no difference in baseline CORT, CORT 30 or CORT response based on exploration behaviour.

Table 6: CORT p values based on diet, time point and exploration.

Group	CORT 0	CORT 30	CORT Response
Pre vs Post Insect	0.039	0.173	0.582
Pre vs Post Seed	0.395	0.634	0.678
Pre Insect vs Pre Seed	0.395	0.87	0.67
Post Insect vs Post Seed	0.906	0.315	0.582
Pre Insect vs Post Seed	0.131	0.634	0.8
Pre Seed vs Post Insect	0.395	0.173	0.582
Exploration	0.182	0.518	0.885

5.5 Discussion

This study demonstrates that an experimentally induced dietary change caused significant alterations to the gut microbiome diversity, and to phylum- and genus-level abundance in a wild bird species. This study provides experimental evidence in a non-model lab organism that microbial communities in the gut are influenced by diet. Furthermore, this study provides evidence of an interaction between microbiota composition and a behavioural trait, in this case exploration behaviour as alterations in gut microbiota composition at the genus level were observed based on fast versus slow exploration behaviour. We discuss these findings in the context of the microbiome-gut-brain axis and environment-behaviour interactions.

5.5.1 Diet and the microbiome

Gut microbial communities in wild animals are strongly influenced by seasonality and geographic location, and these differences have been attributed to changes in food availability (Amato *et al.*, 2015; Maurice *et al.*, 2015; Hicks *et al.*, 2018). Our findings support this hypothesis because we observed changes in microbial community composition and diversity following dietary intervention in a population of wild birds temporarily taken into captivity. These changes were independent of

other factors, such as hormonal differences (Escallón, Belden and Moore, 2019) which may change with seasonality and influence the gut microbiota, because the changes observed in this study were recorded under controlled conditions over a two week period (Clarke *et al.*, 2014; David *et al.*, 2014). We show that microbiota composition changes at phylum and genus level, as well as diversity changes, were attributed to dietary intervention, possibly due to differences in relative protein, fat, and fibre content. We observed higher abundance of Bacteroidetes present in subjects with lower fat, protein and fibre intake following administration of an insect diet, a finding in line with Clarke *et al.* (2014). In contrast to that study, however, we did not find a decrease in Firmicutes, and found an increase in Proteobacteria, possibly due to differences in the core microbiome between birds and mammals (Waite and Taylor, 2014), and the type of fat and/or fibre (reviewed in Singh *et al*., 2017). Birds in the insect diet group showed a decrease in alpha diversity compared to birds administered a seed diet, potentially because the insect diet itself was less diverse than the seed and nut diet. A trend towards significance was observed in beta diversity between birds post insect diet compared to birds post seed diet.

Several significant differences were also observed at both phylum and genus level, although most of these differences were between the post seed and post insect groups. LeFSe analysis revealed 19 discriminating taxa for the pre seed group, including *Nocardioides*, a p-nitrophenol degrading species which has been found to produce the novel antitumour antibiotic sandramycin, active against leukaemia in mice (Matson and Bush, 1989); *Devosia*, which has been shown to have protective effects on growth performance, serum chemistry, immunity function, antioxidant capacity and tissue residues in kidneys of mice (Zhao *et al.*, 2016) and in pigs (Li *et al.*, 2018) exposed to deoxynivalenol; and *Micromonospora* which produces the antibiotic gentamicin (Weinstein *et al.*, 1963). Seven discriminating taxa were identified for the post seed group including *Paracoccus* from which astaxanthin has been extracted and demonstrated to have anxiolytic-like effects (Nishioka *et al.*, 2011); and *Myroides* which has been identified as a potential probiotic against colitis after it successfully prohibited inflammatory markers and acted as a potent

probiotic (Islam *et al.*, 2014). Finally, nine discriminating taxa were revealed for the post insect group including *Lactococcus,* which has been demonstrated to have anti-inflammatory effects and consequently identified as a potential treatment for Irritable Bowel Disease (Luerce *et al.*, 2014), it has probiotic characteristics such as gastric acid resistance and survival in NaCl and bile salts, it has also been identified as a bacteriocin producer against *Listeria monocytogenes* and a producer of neuroactive bioamines such as dopamine, noradrenaline, serotonin (Stoyanova *et al.*, 2017), and GABA (Song *et al.*, 2017). Furthermore, several studies have demonstrated various strains of this genus improve depressive- and anxiety-like behaviour (Ramalho *et al.*, 2019; Colica *et al.*, 2017; Liu, Walsh and Sheehan, 2019).

While significant differences were observed at genus level abundance for birds in both the seed and insect diet groups, only birds given the insect diet showed significant changes following dietary intervention in both alpha and beta diversity. A possible explanation for this could be that the birds had already been taking seeds at the feeders used to lure them for capture, and because great tits consume a high proportion of plant-based foods in the winter (Vel'ký, Kaňuch and Krištín, 2011). The use of garden feeders in both urban and rural environments may also explain why there were no rural versus urban habitat differences in alpha diversity. There was, however, a significant difference in beta diversity as well as a higher proportion of Actinobacteria in urban birds compared to rural birds, similar metrics to those that have previously been shown to be related to urban environments in house sparrows (*Passer domesticus*) which indicates that the community composition at the OTU level differed between habitat types (Teyssier *et al.*, 2018). This study aimed to mimic variation in individual food consumption and in seasonal food availability by examining wild birds in captivity over a short time period. However, in order to confirm whether similar microbial diversity and taxa changes would occur in the wild under natural conditions, longitudinal sampling of individuals in the wild across seasons would be necessary, particularly given that the invertebrate species accessible in the wild would be different from those provided in this experiment. In conclusion, our manipulation showed clearly that diet modifies the microbiome in this model species of field ecology and suggests

that environmental drivers of variation in diet, such as seasonality and food availability, may hold a significant role in driving these effects.

5.5.2 Behaviour and the microbiome

The influence of the gut microbiome on behaviour via the microbiota-gut-brain axis may be attributed to the metabolic function of the microbial community which is largely dependent on the diet of the host (Roager and Dragsted, 2019). Studies have attempted to elucidate nutritional and microbial effects on behaviour through the use of germ free animals, by depleting the original host microbes and re-introducing specific bacterial organisms, or by transplanting gut microbiomes between hosts (Bruce-Keller *et al.*, 2015; Möhle *et al.*, 2016). While it was not possible to directly manipulate the microbiome in this study due to logistical and ethical constraints, the aim of our study was to test diet-microbiome-behaviour relationships within an ecologically relevant context that would translate to wild animals in their natural environment. Vitamin supplements were provided during this study to control for nutritional deficiencies that may have an impact on behaviour independent of microbiome, though other nutritional differences cannot be dismissed. Fat content and fibre content were five-fold and three-fold higher in the seed diet compared to the insect diet, respectively. Studies have shown that mice fed a high fat diet or given microbiome transplantations from obese donors show poorer cognitive performance than control mice (Magnusson *et al.*, 2015; Bruce-Keller *et al.*, 2015), however, a higher proportion of fibre present in the seed diet may have offset any negative effects of a high-fat diet. Non-digestible carbohydrates, which are fermented by gut microbes in the large intestine, promote the growth of microbial organisms and can have positive effects on cognition and behaviour in humans, rodents and pigs (Cryan *et al.*, 2019).

Exploration behaviour was also investigated as part of this study; however, few differences were observed in microbial composition or diversity between fast and slow exploration groups, apart from seven differences at genus level, suggesting that the microbiome may not have a strong influence on exploration behaviour in

this model. Similarly, dietary intervention had no significant impact on CORT response. There was a significant difference in baseline CORT concentrations (CORT 0) between pre- and post-insect groups, indicating an effect of time rather than diet as there were no differences in CORT 0 between insect and seed groups. This is likely due to the effect of captivity which increased baseline CORT in the insect group but not the seed group, suggesting a possible role of the seed diet in protecting from the effects of captivity on stress.

5.5.3 Future studies

Future studies should employ metabolomics profiling to provide a functional assessment of microbial products such as short chain fatty acids involved in gut-brain axis communication (Stilling *et al.*, 2016; Heintz-Buschart and Wilmes, 2018; Cryan *et al.*, 2019) which could help to further elucidate the mechanisms underlying phenotypic plasticity in innovative behaviour. Furthermore, in order to effectively examine the evolutionary and ecological patterns in vertebrate microbiomes, broader sampling is needed. Exponential advances have been made in next generation sequencing technologies in recent years so that sampling many different vertebrate species (broad sampling) or many individuals within a species (deep sampling) has now become both more cost and time effective. However, sample collection, particularly from the field, remains a limiting factor. Therefore, coordination between scientists in different fields is likely to be essential, with field zoologists and ecologists collecting samples for laboratory-based microbiologists, and bioinformatics specialists working with the subsequent data (Colston and Jackson, 2016). Collecting samples for microbiome analysis when in the field carrying out phylogenetic/phylogeographical studies would vastly increase our knowledge of the microbiome of animals in the wild and the factors that influence its composition and diversity.

Most next generation sequencing studies of the animal microbiome have focused solely on community composition using 16S rRNA sequencing. However, a metagenomic approach is being increasingly incorporated in human studies and

would be a useful addition to animal studies by providing an insight into the functional genes present in the microbiome, which could further elucidate the influence of diet, geography, and phylogeny on the host microbiome. This will aid in studying multiple potential mechanisms, which is necessary to uncover the interactions between cognition, behaviour, diet and the microbiome, and will help to clarify the environmental and genetic effects determining individual differences in cognition and behaviour (Davidson *et al.*, 2018). It would be useful for future studies examining the link between animal personality and stress to also examine other components of the HPA cascade, and the targets of CORT (Baugh *et al.*, 2012). For example, it is unclear whether slow birds have an increased sensitivity to the perception of stressors, secreting more corticosterone releasing factor in response to the stressor, resulting in an earlier onset of the stress response or rather whether they exhibit a dampened negative feedback control of CORT, causing them to achieve higher stress-induced CORT levels. It has also been suggested that researchers working with wild taxa would benefit from examining both the HPA and serotonin systems simultaneously (Koolhaas *et al.*, 2007).

5.6 Conclusion

To conclude, our findings lend support to the hypothesis that the gut microbiome and diet are interlinked, and more generally that the microbiome may be an important source of variation in ecologically important behaviours, such as those linked to diet and foraging. The experimentally induced dietary change employed in this study resulted in multiple alterations to the gut microbiome diversity, as well as to phylum- and genus-level abundance in a non-laboratory model wild bird species whereby birds in the insect diet group showed a decrease in alpha diversity compared to birds administered a seed diet; a trend towards significance was observed in beta diversity between birds post insect diet compared to birds post seed diet; and several significant differences were observed at both phylum and genus level, predominantly between the post seed and post insect groups. We hypothesise that these microbiota composition and diversity changes were largely

attributed to differences in relative protein, fat, and fibre content in the different dietary interventions in line with other studies. Furthermore, this study also investigated the interaction between microbiota composition and exploration behaviour, however few differences were observed in microbial composition or diversity between fast and slow exploration groups, suggesting that the microbiome may not have a strong influence on exploration behaviour in this model. Finally, dietary intervention had no significant impact on CORT response, apart from a significant difference in CORT 0 between pre- and post-insect groups, indicating an effect of time rather than diet and suggesting a possible role of the seed diet in protecting from the effects of captivity on stress.

However, future studies involving longitudinal sampling of individuals in the wild across seasons will be necessary to confirm whether similar microbial diversity and taxa changes would occur in the wild under natural conditions. It would also be useful to employ metabolomics profiling in future studies to provide a functional assessment of microbial products such as short chain fatty acids involved in gut-brain axis communication (Stilling *et al.*, 2016; Heintz-Buschart and Wilmes, 2018; Cryan *et al.*, 2019) as well as a metagenomic approach to provide insight into the functional genes present in the microbiome, all of which will aid in studying potential mechanisms of the link between diet, the microbiome, behaviour and the stress response. In conclusion, our manipulation showed clearly that diet modifies the microbiome in this model species of field ecology and suggests that environmental drivers of variation in diet, such as seasonality and food availability, may play a significant role in driving these effects.

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Authors contributions

G. L. Davidson, A. C. Cooke and J. L. Quinn designed the experiment with input from C. Stanton and R. P. Ross. G. L. Davidson and A. C. Cooke ran the experiment with assistance from M. S. Reichert, I. G. Kulachi, I. D. Hera and J. M.S. Crane. N. Wiley, G. L. Davidson and C. N. Johnson carried out DNA extraction and library prep. F. Fouhy carried out bioinformatics analysis. N. Wiley and G. L. Davidson analysed data. N. Wiley wrote the chapter.

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5.8 Supplementary Material

Table S1: Estimated nutritional content of food types provided in dietary treatments. Percentages of protein, fat and fibre content attained from online resources.

Food	Protein	Fat	Fibre
Mealworm larvae	18.70%	13.40%	2.50%
Waxworm larvae	14.10%	24.90%	3.40%
Peanuts	25.80%	49.20%	8.50%
Sunflower hearts	18.33%	46.67%	10%
Beef suet	<1%	>95%	0

Table S2: Wilcox analysis was performed in R Studio on relative abundance data at genus level to determine if statistically significant differences occurred between pre insect and post insect diet and between pre seed and post seed dietary manipulation. Statistical significance was accepted as $p<0.05$ and data is adjusted using False Discovery Rate (FDR).

Genus	P value	FDR	Comparison
Blastocatella	0.0137	0.363859	Pre vs Post Insect
Subgroup 6 uncultured bacterium	0.037919	0.535017	Pre vs Post Insect
Acidimicrobiaceae CL500 29 marine group	0.017625	0.406471	Pre vs Post Insect
Nocardiaceae Williamsia	0.039051	0.535017	Pre vs Post Insect
Corynebacteriales uncultured bacterium	0.017753	0.406471	Pre vs Post Insect
Sporichthyaceae uncultured	0.037919	0.535017	Pre vs Post Insect
Kineosporiaceae uncultured bacterium	0.037919	0.535017	Pre vs Post Insect
Intrasporangiaceae uncultured	0.001116	0.123493	Pre vs Post Insect
Candidatus Aquiluna	0.021606	0.448326	Pre vs Post Insect
Lysinimonas	0.005125	0.291468	Pre vs Post Insect

Rhodoglobus	0.009858	0.311714	Pre vs Post Insect
Nesterenkonia	0.002685	0.222854	Pre vs Post Insect
Isoptericola	0.028524	0.526996	Pre vs Post Insect
Aeromicrobium	0.050569	0.549625	Pre vs Post Insect
Marmoricola	0.007901	0.291468	Pre vs Post Insect
Nocardioides	0.020239	0.433509	Pre vs Post Insect
Friedmanniella	0.013234	0.363859	Pre vs Post Insect
Pseudonocardia	0.005315	0.291468	Pre vs Post Insect
Nitriliruptor	0.037919	0.535017	Pre vs Post Insect
Solirubrobacterales 480.2 uncultured bacterium	0.043187	0.549625	Pre vs Post Insect
Patulibacter	0.011451	0.345619	Pre vs Post Insect
Solirubrobacter	0.049615	0.549625	Pre vs Post Insect
Sphingobacteriales ST12K33 uncultured bacterium	0.046721	0.549625	Pre vs Post Insect
Thermus	0.007901	0.291468	Pre vs Post Insect
Sinobaca	0.009524	0.311714	Pre vs Post Insect
Staphylococcus	0.031025	0.535017	Pre vs Post Insect
Lactobacillus	0.039482	0.535017	Pre vs Post Insect
Singulisphaera	0.013234	0.363859	Pre vs Post Insect
Planctomycetaceae uncultured	0.01914	0.423624	Pre vs Post Insect
Rhizobiales 1174.901.12 uncultured bacterium	0.006106	0.291468	Pre vs Post Insect
Aureimonas	0.000625	0.085818	Pre vs Post Insect
Aurantimonadaceae uncultured	0.007901	0.291468	Pre vs Post Insect
Bartonella	0.023494	0.472727	Pre vs Post Insect
Methylocella	0.027461	0.526996	Pre vs Post Insect
Beijerinckiaceae uncultured	0.007901	0.291468	Pre vs Post Insect
Rhizobiales Bradyrhizobiaceae Bosea	0.005315	0.291468	Pre vs Post Insect
Methylobacterium	0.044369	0.549625	Pre vs Post Insect
Agaricicola	0.037919	0.535017	Pre vs Post Insect

Variibacter	0.008508	0.29732	Pre vs Post Insect
Rubellimicrobium	0.017625	0.406471	Pre vs Post Insect
Arachis hypogaea var vulgaris	0.005407	0.291468	Pre vs Post Insect
Lactuca sativa	0.000242	0.080381	Pre vs Post Insect
Alcaligenes	0.037919	0.535017	Pre vs Post Insect
Massilia	0.039051	0.535017	Pre vs Post Insect
Methylophilaceae OM43clade	0.037919	0.535017	Pre vs Post Insect
Pantoea	0.002234	0.211929	Pre vs Post Insect
Proteus	0.037919	0.535017	Pre vs Post Insect
Luteolibacter	0.017625	0.406471	Pre vs Post Insect
Bacteria WD272.D 2 uncultured bacterium	0.037919	0.535017	Pre vs Post Insect
Chloroplast Arachis duranensis	0.028572	0.526996	Pre vs Post Insect
Chloroplast Eimeria praecox	0.054834	0.549625	Pre vs Post Insect
Common sunflower	0.000421	0.085818	Pre vs Post Insect
Chloroplast Humulus lupulus var cordifolius	0.000213	0.080381	Pre vs Post Insect
Lolium perenne	0.007866	0.291468	Pre vs Post Insect
Tepary bean	0.000646	0.085818	Pre vs Post Insect
Acidobacteria Subgroup 6 uncultured bacterium	0.03591	0.383406	Pre vs Post Seed
Acidimicrobiaceae uncultured	0.002548	0.196498	Pre vs Post Seed
Candidatus Microthrix	0.032642	0.383406	Pre vs Post Seed
Acidimicrobiales uncultured actinobacterium	0.03591	0.383406	Pre vs Post Seed
Mycobacterium	0.014351	0.331481	Pre vs Post Seed
Nocardia	0.021286	0.383406	Pre vs Post Seed
Geodermatophilaceae uncultured	0.044654	0.449147	Pre vs Post Seed
Nakamurella	0.03591	0.383406	Pre vs Post Seed
Cellulomonas	0.002548	0.196498	Pre vs Post Seed
Frondihabitans	0.03591	0.383406	Pre vs Post Seed
Lysinimonas	0.023308	0.383406	Pre vs Post Seed

Rathayibacter	0.020584	0.383406	Pre vs Post Seed
Rhodoglobus	0.012797	0.331481	Pre vs Post Seed
Actinoplanes	0.026185	0.383406	Pre vs Post Seed
Micromonospora	0.005118	0.227192	Pre vs Post Seed
Marmoricola	0.002548	0.196498	Pre vs Post Seed
Actinomycetospora	0.047119	0.467148	Pre vs Post Seed
Actinobacteria MBA2108 uncultured bacterium	0.015762	0.331481	Pre vs Post Seed
Solirubrobacterales 480.2 uncultured bacterium	0.005005	0.227192	Pre vs Post Seed
Prevotella 9	0.023892	0.383406	Pre vs Post Seed
Dyadobacter	0.03591	0.383406	Pre vs Post Seed
Fluviicola	0.041322	0.42802	Pre vs Post Seed
Myroides	0.002548	0.196498	Pre vs Post Seed
Sediminibacterium	0.0184	0.375575	Pre vs Post Seed
Neochlamydia	0.03591	0.383406	Pre vs Post Seed
Caldilineaceae uncultured	0.032029	0.383406	Pre vs Post Seed
Clostridium sensu stricto 18	0.03591	0.383406	Pre vs Post Seed
Gemmata	0.024747	0.383406	Pre vs Post Seed
Isosphaera	0.021286	0.383406	Pre vs Post Seed
Planctomycetaceae Pir4 lineage	0.004285	0.227192	Pre vs Post Seed
Planctomycetaceae uncultured	0.040638	0.427311	Pre vs Post Seed
Aureimonas	0.007871	0.231127	Pre vs Post Seed
Aurantimonadaceae uncultured	0.00712	0.231127	Pre vs Post Seed
Methylocella	0.031452	0.383406	Pre vs Post Seed
Beijerinckiaceae uncultured	0.032029	0.383406	Pre vs Post Seed
Bradyrhizobiaceae Bosea	0.005765	0.227192	Pre vs Post Seed
Rhodopseudomonas	0.029532	0.383406	Pre vs Post Seed
Devosia	0.001631	0.196498	Pre vs Post Seed
Rhodoplanes	0.006101	0.227192	Pre vs Post Seed
Hyphomicrobiaceae uncultured	0.002548	0.196498	Pre vs Post Seed

Methylobacteriaceae uncultured	0.002548	0.196498	Pre vs Post Seed
Hansschlegelia	0.004016	0.227192	Pre vs Post Seed
Mesorhizobium	0.007993	0.231127	Pre vs Post Seed
Rhizobium	0.005803	0.227192	Pre vs Post Seed
Agaricicola	0.03591	0.383406	Pre vs Post Seed
Bauldia	0.03591	0.383406	Pre vs Post Seed
Nordella	0.03591	0.383406	Pre vs Post Seed
Pseudolabrys	0.006101	0.227192	Pre vs Post Seed
Variibacter	0.005069	0.227192	Pre vs Post Seed
Amaricoccus	0.015051	0.331481	Pre vs Post Seed
Palleronia	0.03591	0.383406	Pre vs Post Seed
Paracocccus	0.014351	0.331481	Pre vs Post Seed
Rubellimicrobium	0.015762	0.331481	Pre vs Post Seed
Sulfitobacter	0.03591	0.383406	Pre vs Post Seed
Roseomonas	0.032642	0.383406	Pre vs Post Seed
Erythrobacter	0.033808	0.383406	Pre vs Post Seed
Advenella	0.03591	0.383406	Pre vs Post Seed
Aquicella	0.009426	0.261665	Pre vs Post Seed
Legionella	0.000383	0.196498	Pre vs Post Seed
Vibrio	0.03591	0.383406	Pre vs Post Seed
Lysobacter	0.006547	0.227192	Pre vs Post Seed
Saccharibacteria soil bacterium WF55	0.03591	0.383406	Pre vs Post Seed
Saccharibacteria uncultured soil bacterium	0.002548	0.196498	Pre vs Post Seed
Spiroplasma	0.044656	0.449147	Pre vs Post Seed
Chthoniobacterales 01D2Z36 uncultured cyanobacterium	0.03591	0.383406	Pre vs Post Seed
Candidatus Xiphinematobacter	0.015762	0.331481	Pre vs Post Seed
Rubritalea	0.015762	0.331481	Pre vs Post Seed
Akkermansia	0.007673	0.231127	Pre vs Post Seed
Roseibacillus	0.006547	0.227192	Pre vs Post Seed
Candidatus Saccharibacteria bacterium	0.03591	0.383406	Pre vs Post Seed

6. Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome

Elaine Patterson[1,2#], Paul M. Ryan[1,2#], Niamh Wiley[1,2], Ilaria Carafa[1,2,3], Eoin Sherwin[1], Gerard Moloney[1], Elena Franciosi[3], Rupasri Mandal[4], David S. Wishart[4,5,6], Kieran Tuohy[3], Paul Ross[1,7], John F. Cryan[1,8], Timothy G. Dinan[1,9] and Catherine Stanton[1,2,*]

[1] APC Microbiome Ireland, University College Cork

[2] Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland

[3] Department of Food Quality and Nutrition, Research and Innovation Centre-Fondazione Edmund Mach, San Michele all'Adige, Trento, Italy

[4] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

[5] Department of Computing Science, University of Alberta, Edmonton, Alberta, Canada

[6] National Institute for Nanotechnology, Edmonton, Alberta, Canada

[7] College of Science, Engineering and Food Science, University College Cork, Cork, Ireland

[8] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[9] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[#]Authors contributed equally to this work.

***Corresponding Author**:

Prof Catherine Stanton

Email: Catherine.Stanton@teagasc.ie

Telephone: +353 (0) 25 42606

6.1 Abstract

Metabolic and neuroactive metabolite production represents one of the mechanisms through which the gut microbiota can impact health. One such metabolite, gamma-aminobutyric acid (GABA), can modulate glucose homeostasis and alter behavioural patterns in the host. We previously demonstrated that oral administration of GABA-producing *Lactobacillus brevis* DPC6108 has the potential to increase levels of circulating insulin in healthy rats. Therefore, the objective of this study was to assess the efficacy of endogenous microbial GABA production in improving metabolic and behavioural outcomes in a mouse model of metabolic dysfunction. Diet-induced obese and metabolically dysfunctional mice received one of two GABA-producing strains, *L. brevis* DPC6108 or *L. brevis* DSM32386, daily for 12 weeks. After 8 and 10 weeks of intervention, the behavioural and metabolic profiles of the mice were respectively assessed. Intervention with both *L. brevis* strains attenuated several abnormalities associated with metabolic dysfunction, causing a reduction in the accumulation of mesenteric adipose tissue, increased insulin secretion following glucose challenge, improved plasma cholesterol clearance and reduced despair-like behaviour and basal corticosterone production during the forced swim test. Taken together, this exploratory dataset indicates that intervention with GABA-producing lactobacilli has the potential to improve metabolic and depressive-like behavioural abnormalities associated with metabolic syndrome in mice.

6.2 Introduction

The obesity and metabolic dysfunction pandemic currently underway represents one of the most serious global health challenges of the 21st century. According to the World Health Organization (WHO) in 2016, more than 1.9 billion adults were overweight, and of these, over 650 million were obese (World Health Organization, 2018). Attempts to curb this significant public health issue by creating efficacious primary and secondary prevention strategies have been largely unsuccessful due to the multifaceted nature of this disease. Failure in compliance with lifestyle

interventions, coupled with novel environmental stressors, has rendered the global population susceptible to this pandemic. As a result, metabolic surgery is currently the most effective therapy for morbid obesity and metabolic disturbances (Basso *et al.*, 2016). This has driven the demand for researchers to investigate novel, non-invasive therapies for obesity and its associated sequelae. While significant inroads have been made in unravelling the biochemical and endocrinological underpinnings of obesity and its related comorbidities, derived translational therapeutic interventions have been sparse or are often limited by their side-effects (Paravattil, Wilby and Turgeon, 2016).

The composition, diversity and functionality of the gut microbiota are substantially discordant between healthy and obese subjects (Le Chatelier *et al.*, 2013; Ley *et al.*, 2006; Turnbaugh *et al.*, 2009). Thus, the gut microbiota has become a popular target for novel therapies directed towards obesity and diabetes, one which by-passes the need for significant lifestyle changes or surgery. Indeed, consumption of a high-fat (HF) diet and increased energy intake predisposes mice (Cani *et al.*, 2008) and humans (Amar *et al.*, 2008) to a low-grade inflammation, insulin resistance and considerable shifts in the microbiota. In turn, circulating bacterial particles such as lipopolysaccharide (LPS) (Amar *et al.*, 2011; Denou *et al.*, 2015) are increased and trigger the host inflammatory immune response in tissues such as the liver and adipose tissue (Cani *et al.*, 2008; Cani *et al.*, 2007a). Furthermore, mice subcutaneously infused with LPS display increased susceptibility to weight gain and inflammation (Cani *et al.*, 2007a; Cani *et al.*, 2007b). It is therefore plausible that changes in the composition of the gut microbiota may be intrinsically linked to the pathogenesis of obesity and related metabolic dysfunction.

Certain microbial therapeutics have proven efficacious in improving several physiological abnormalities associated with obesity and metabolic dysfunction in both preclinical (Stenman *et al.*, 2014; Udayappan *et al.*, 2016; Ryan *et al.*, 2017; Hänninen *et al.*, 2018; Plovier *et al.*, 2017) and clinical studies (Depommier *et al.*, 2019; Stenman *et al.*, 2016). As such, specific bacterial strains and their metabolites may be considered adjunct therapies for certain metabolic disorders. Microbial metabolites, such as short chain fatty acids and neuroactives, may be central to this

effect. One such neurotransmitter produced by certain bacteria is gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system. Additionally, evidence suggests that this neurotransmitter may have a therapeutic role in treating type-1-diabetes (T1D) and long-standing type-2-diabetes (T2D) in which the pancreatic β-cells become fatigued. Activation of the $GABA_A$ and $GABA_B$ receptors expressed on β-cells has been shown to stimulate insulin secretion and induce β-cell proliferation (Soltani *et al.*, 2011; Tian, Dang and Kaufman, 2011; Tian *et al.*, 2013). Moreover, the $GABA_A$ receptor is widely expressed on CD4+ T cells and several studies have indicated a putative immunomodulatory role of this neurotransmitter, suggesting an alternative mechanism through which it may impact upon systemic inflammation and the autoimmunity of T1D (Tian *et al.*, 2004; Jin, Mendu and Birnir, 2013).

Peripheral inflammation associated with obesity and metabolic dysfunction enhances the risk for neuropsychiatric and neurological disorders (for reviews see (Castanon, Luheshi and Layé, 2015; Farooqui *et al.*, 2012). It has been shown that obese and T2D (*db/db*) mice display enhanced anxiety-like behaviour and impaired spatial recognition memory (de Cossío *et al.*, 2017; Dinel *et al.*, 2011), and we now understand that the gut microbiota plays a central role in gut-brain signalling which can impact brain physiology, function and behaviour (Dinan *et al.*, 2015). In line with this, convincing preclinical evidence has demonstrated that enteric microbial GABA production can mediate physiological and behavioural effects via the vagus nerve (Bravo *et al.*, 2011). However, little probing has been done into the behaviour-modifying attributes of GABA-secreting microbes in the context of obesity.

We previously isolated *L. brevis* DPC6108 from an infant faecal sample and *L. brevis* DSM32386 from a traditional Italian cheese, and demonstrated their ability to produce GABA *in vitro* (Barrett *et al.*, 2012; Carafa *et al.*, 2015). We also explored the effects of intervention with *L. brevis* DPC6108 on the metabolic profile of two different rodent models; healthy and streptozotocin-induced T1D rats (Marques *et al.*, 2016). The aim of the present study was to assess whether microbial GABA production could attenuate the metabolic dysfunction observed in diet-induced

obese mice. In addition, we aimed to determine whether microbial GABA production could attenuate impairments in the neuropsychiatric behavioural profile observed in these mice and associated with metabolic dysfunction.

6.3 Materials and Methods

6.3.1 Animals and treatment

All experimental procedures were performed in accordance with the protocols approved by the University College Cork Ethics Committee, under a license issued from the Health Products Regulatory Authority (AE19130/P026). Male C57BL/6J mice, three weeks of age, were obtained from Envigo (Blackthorn, UK) and housed under barrier-maintained conditions at the Biological Services Unit, UCC. All mice acclimatised to their environment for five weeks prior to administration of diets. Mice were randomly divided into four groups (*n* 14 per group) and housed in groups of three to four mice per cage at standard conditions (room temperature of 21°C, with a 12-h light–dark cycle, lights on at 07:00).

The groups were assigned as follows:

Dietary intervention: a low fat (LF) group fed *ad libitum* with Open Source Diets (D15072701 – 10% kcal from fat; (Research Diets Inc., NJ 08901 USA)) and three high fat (HF) groups fed *ad libitum* with Open Source Diets (D12492 – 60% kcal from fat; Research Diets Inc.) were allowed free access to food and water for 12 weeks. The compositions of the diets are outlined in Supplementary Table S1. After 12 weeks of either LF or HF feeding, two control groups were maintained on either the LF diet (LFC; *n* 14) or HF diet (HFC; *n* 14) for a further 12 weeks and the remaining two HF dietary groups were subdivided into intervention groups for a further 12 weeks.

Continued dietary intervention + microbial intervention: The HF intervention groups were as follows; (1) HF + *L. brevis* DPC6108 (1 x 10^{10} CFU/day; DPC6108) and (2) HF + *L. brevis* DSM32386 (1 x 10^{10} CFU/day; DSM32386). All interventions were administered daily in drinking water. Water containing either *L. brevis* DPC6108 or *L. brevis* DSM32386 was the only water supplied to the mice in these groups for the

12 week intervention period and bottles were replaced daily. Bodyweight and food intake were measured weekly for all groups. Following eight weeks of intervention, all mice were subjected to the behavioural test battery to measure anxiety- and depressive-like behaviour and cognitive function. Mice were then allowed to recover for one week and after 10 weeks of intervention, all mice underwent the metabolic profiling battery. Following 12 weeks of intervention, mice were fasted overnight, and body mass was measured using a Minispec mq benchtop NMR spectrometer (Bruker Instruments, Germany). The mice were subsequently sacrificed by cervical dislocation. All dissected tissue samples were flash frozen immediately in liquid nitrogen. Individual blood samples were collected in microtainer^{TM} collection tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Microtainer Plasma Separator Tubes, BD Diagnostics, Oxford, UK), thoroughly mixed in the tube and stored on ice until for 10 minutes at 2,000*g* under refrigerated conditions to isolate the plasma. Isolated plasma was immediately transferred to a clean eppendorf tube following centrifugation.

6.3.2 Preparation and administration of L. brevis DPC6108 and DSM32386

L. brevis DPC6108 and *L. brevis* DSM32386 are efficient GABA producers, with maximum conversion *in vitro* when grown on MRS broth supplemented with 3% (w/v) monosodium glutamate (MSG) (Barrett *et al.*, 2012). Rifampicin-resistant variants of *L. brevis* DSM32386 and *L. brevis* DPC6108 were isolated by spread-plating ~10^9 CFU from an overnight culture (1% inoculum) onto MRS agar (Difco Laboratories) containing 500 µg rifampicin/mL (Sigma-Aldrich Ireland Ltd. Arklow, Ireland) and stocked at -80°C. Before freeze-drying, frozen stocks were plated on MRS agar and single colonies were isolated for inoculation in 10 mL fresh MRS broth supplemented with 30 mg/mL MSG. The cultures were incubated overnight at 37°C under anaerobic conditions and then inoculated into 1 L MRS broth containing 3% (w/v) MSG and allowed to grow overnight at 37°C, under anaerobic conditions. The overnight culture aliquots were then inoculated into large volumes of MRS containing MSG and allowed to grow overnight at 37°C under anaerobic conditions. The overnight cultures were washed twice in phosphate buffered saline (Sigma-

Aldrich Ireland Ltd.) and the pellets re-suspended in 15% (w/v) trehalose (Sigma-Aldrich Ireland Ltd.) in dH2O. One-millilitre aliquots of bacterial cultures were freeze-dried by using a 24-hour programme (freeze temperature, -40°C; condenser set point, -60°C; vacuum set point, 600 mTorr). Freeze-dried aliquots were prepared every two weeks, continuously underwent quality control checks, and were stored at 4°C until use. Vials of freeze-dried microbial powder were found to contain 2 x 10^{10} CFU following lyophilisation. With an average calculated fluid intake of ~5 ml/mouse/day, a dose of ~1 x 10^{9} CFU/day was achieved by suspending a single vial in 100 ml of distilled water. This dilution delivered a concentration of 2 x 10^{8} CFU/ml. During re-suspension, 100mL of sterile water containing the freeze-dried powder was vortexed extensively to ensure solubility and even dispersion of the powder in water. Fresh water containing the live microorganisms was administered to mice each day.

6.3.3 Culture dependent microbial analysis

To confirm that *L. brevis* DPC6108 and *L. brevis* DSM32386 tolerated freeze-drying conditions, the strains were plated on MRS agar supplemented with rifampicin before and after freeze-drying. Fresh faecal samples were taken for microbial analysis to verify strain survival following gastric transit. A detailed description of the methods for culture dependent microbial analysis following gastric transit can be found in Supplementary Methods.

6.3.4 Metabolic profiling battery

After 12 weeks of feeding, an intraperitoneal-glucose tolerance test (IP-GTT) and an intraperitoneal-insulin tolerance test (IP-ITT) was performed in the LFC (*n* 7) and HFC (*n* 7) groups. Following confirmation of metabolic disturbance in the HFC group, the IP-GTT and IP-ITT were performed in individual mice from groups LFC, HFC, DPC6108 and DSM32386 after 10 weeks of intervention. The insulin resistance index was also calculated from the plasma insulin concentrations that were

measured in plasma collected from tail blood during the IP-GTT and a mixed-meal tolerance test was performed, also after 10 weeks of intervention from individual mice in the LFC, HFC, DPC6108 and DSM32386 groups. A detailed description of the methods for the glucose and insulin tolerance tests, determination of insulin resistance index and the mixed meal tolerance test can be found in Supplementary Methods.

6.3.5 GABA assay

Frozen samples of epididymal adipose tissue and small intestinal content were diluted (10%, (w/v)) in tissue lysis solution (0.01 N HCl, 1mM EDTA, 4 mM sodium metabisulphite (Sigma-Aldrich Ireland Ltd)) and homogenised. A commercially available enzyme immunoassay was then used for the quantitative determination of GABA by ELISA (ImmuSmol, Pessac, France). Quantification of unknown samples was achieved by comparing their absorbance with a standard curve prepared with known standards and results were standardised to individual sample weights.

6.3.6 Plasma Metabolome – direct flow injection and LC-MS/MS

Plasma was isolated from whole blood, as previously described and analysed using the Biocrates AbsoluteIDQ p180 Kit (BIOCRATES Life Sciences AG, Austria), using methods previously described (Walsh *et al.*, 2012). Following extraction and derivatisation, all analytes present in the samples were detected and quantified on an ABI 4000 Q-Trap mass spectrometer (MDS Sciex) run in conjunction with a reverse-phase HPLC-column. The analysis revealed levels of a range of specific amino acids, biogenic amines (BA), acylcarnitines (AC), lysophosphotidylcholines (lysoPC), phosphotidylcholines (PC), sphingomyelins (SM) and hexoses.

6.3.7 Microbial DNA extraction, 16S rRNA amplification and Illumina Miseq sequencing

Caecal contents were collected from individual mice following 12 weeks of dietary intervention. Total metagenomic DNA was extracted from caecal contents with the QIamp® PowerFecal® DNA Kit (Qiagen, Milano, Italy) where an additional bead beating step was incorporated into the protocol. Extracted DNA was quantified using the NanoDropTM 8000 Spectrophotometer (Thermo Fisher Scientific). Total genomic DNA was then subjected to PCR amplification by targeting a 464-bp fragment of the 16S rRNA variable region V3-V4 (Baker, Smith and Cowan, 2003; Claesson *et al.*, 2010) using the specific bacterial primer set 341F (5'-CCTACGGGNGGCWGCAG -3') and 806R (5'- GACTACNVGGGTWTCTAATCC -3') with overhang Illumina adapters. Unique barcodes were attached to the forward primer for facilitating the differentiation of samples. Amplicons were cleaned with the Agencourt AMPure kit (Beckman Coulter) following the manufacturer's instructions, and DNA was quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen). Amplicons were mixed and combined in equimolar ratios, and the quality and purity of the library was checked with the High Sensitivity DNA Kit (Agilent, Palo Alto, CA, USA) by the Bioanalyser 2100 (Agilent). The library was sequenced on an Illumina MiSeq platform at CIBIO (Center of Integrative Biology) – University of Trento, Italy. The bioinformatic analysis that was used to analyse the 16S rRNA Illumina Miseq sequencing data can be found in the Supplementary Methods.

6.3.8 Behaviour test battery

Following 20 weeks of high fat feeding and eight weeks of *L. brevis* intervention, mice from all groups underwent a behavioural test battery. A detailed description of the methods for each behaviour test and stress-induced corticosterone production can be found in Supplementary Methods.

6.3.9 Statistical analyses

All data are expressed as mean ± SEM. Data were analysed using the appropriate unpaired student t-test and one-way analysis of variance (ANOVA). Bodyweight

data were analysed using the mixed model ANOVA and Tukey's post-hoc test was applied. Data were deemed significant when $p < 0.05$. Levenes test for homogeneity of variances was used and where homogeneity was not found, Welch's robust test of equality of means was applied. Mauchly's test of sphericity was used and if values were significant, then Greenhouse Geisser was applied. All student t-tests, one-way ANOVA, and post hoc analyses were performed using PASW Statistics 22. The correlation matrix of microbiota profile data and metabolic parameters was performed using *Pearson's Product-Moment Correlation.* Graphs were generated using Graphpad Prism 7. Metabolomic data was log normalised prior to ANOVA and multivariate analysis (sPLS-DA) was also performed on metabolomic data. For metabolomic data, a false discovery rate (FDR) q value less than 0.05 was considered statistically significant.

6.4 Results

6.4.1 Survival of L. brevis DPC6108 and DSM32386 following gastrointestinal transit

Quantification of the number of colony forming units (CFUs) of administered rifampicin-resistant *L. brevis* DPC6108 and DSM32386 in the faeces of mice confirmed survival following gastrointestinal transit. Faecal recovery of *L. brevis* DPC6108 was ~ 2 x 10^9 and *L. brevis* DSM32386 was ~ 1 x 10^9 CFU/g faeces after two weeks and remained at similar numbers following four weeks of intervention. Colonies isolated from the plates were tested for GABA production to assess if gastrointestinal transit affected the ability of *L. brevis* DPC6108 and DSM32386 to produce GABA *in vitro*. Pooled fresh faecal samples collected from each cage of mice in DPC6108 and DSM32386 (four cages per group) were analysed in duplicate following two and five weeks of intervention. At two and five weeks of intervention, bioconversion of MSG to GABA by DPC6108 was 68% and 83%, respectively (MRS containing 1% (w/v) MSG). At two and five weeks of intervention, bioconversion of MSG to GABA by DSM32386 was 66% and 78%, respectively. All colonies tested which were isolated from faecal samples had a similar percentage conversion of

MSG to GABA. The average bioconversion of the wild-type DPC6108 and DSM32386 strains were 77% and 56% respectively. However, these averages are based on triplicates taken at one time-point only during the study and previous experimental analyses comparing the GABA-producing capabilities of these strain revealed no major differences between the wild-type strains. No significant differences were observed in the GABA-producing capabilities between the strains which had passed gastrointestinal transit compared to the wild-type strains.

6.4.2 Intervention with GABA-producing microbes affected adipose deposition following HF diet

Following 12 weeks of HF feeding, there were no significant differences in bodyweight gain over the intervention period between the HF groups (Figure 1B). *L. brevis* DPC6108 ($p<0.01$) and DSM32386 ($p<0.05$) were associated with reduced quantity of mesenteric adipose tissue (MAT), compared with the high fat control (HFC; Figure 1C). Both DPC6108 ($p<0.001$) and DSM32386 ($p<0.05$) reduced percentage body fat, compared with the HFC (Figure 1D). There were no differences in final bodyweight observed between the groups consuming the HF diet ($F_{2,38} = 0.108$, $p>0.05$; Figure 1E). As expected, there was a significant difference in final bodyweight between the low fat control (LFC) and the HFC ($p<0.001$; Figure 1E).

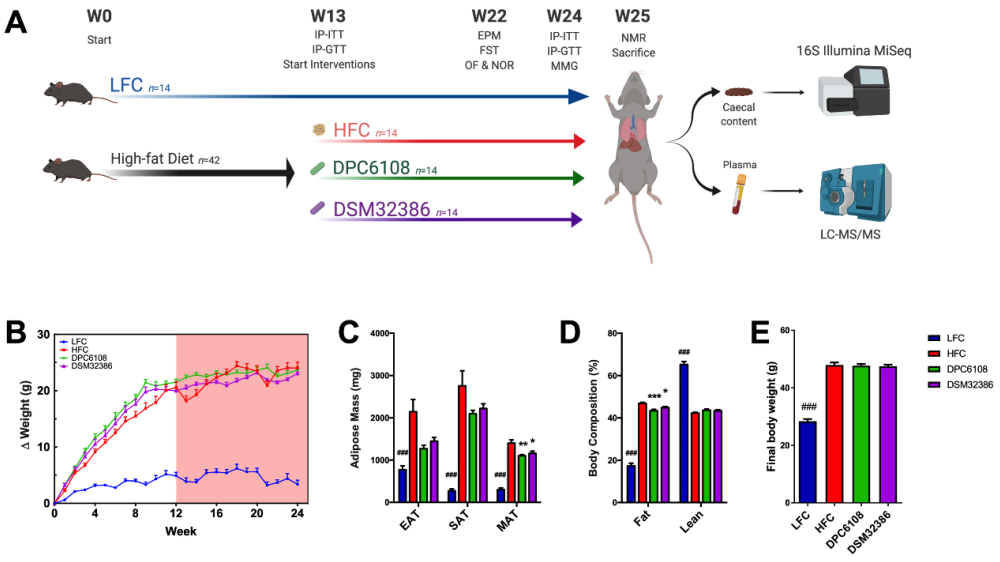


Figure 1: *L. brevis* reduced fat mass and distribution of adiposity after 12 weeks of intervention. (A) Schematic figure representing mouse trial design and timeline for procedures, with high-fat diet represented by the black arrow and treatments in a 12-week intervention represented by coloured arrows. (B) Bodyweight gain throughout the pre-feeding (white background) and intervention (red background) for n=14 mice per group. (C) Adipose tissue mass and distribution across EAT, SAT and MAT and (D) Fat and lean body composition. (E) Final body weight after 24-week feeding (HFC; n=13 and LFC; n=10) and 12-week intervention with DPC6108 (n=14) and DSM32386 (n=14). Data are expressed as mean ± SEM. Bodyweight data were analysed using the mixed model ANOVA. Tukey's post-hoc test was applied. All other data were analysed using the appropriate unpaired student t-test (HFC vs. LFC) and one-way analysis of variance (ANOVA). ### $p < 0.001$ HFC vs. LFC, * $p < 0.05$ treatment vs. HFC, ** $p < 0.01$ treatment vs. HFC and *** $p < 0.001$ treatment vs. HFC. HFC: n=14; LFC: n=14; DPC6108: n=14; DSM32386: n=14. IP-GTT: intraperitoneal glucose tolerance test, IP-ITT: intraperitoneal insulin tolerance test, EPM: elevated plus maze, FST: forced swim test, OF: open field, NOR: novel object recognition, MMG: mixed meal gavage, NMR: nuclear magnetic resonance, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC:

low fat control, EAT: epididymal adipose tissue, SAT: subcutaenous adipose tissue and MAT: mesenteric adipose tissue. Figure 1A was created with BioRender.com.

6.4.3 L. brevis DSM32386 improved HF diet-induced glucose intolerance and impaired insulin sensitivity

After 12 weeks of HF feeding and prior to intervention with *L. brevis* (Figure 1A), diet-induced hyperglycaemia and insulin insensitivity was confirmed in a subset of mice from the HFC, compared with a subset of mice from the LFC (Figures 2A-D). After 12 weeks of HF feeding, the intraperitoneal glucose tolerance test (IP-GTT) revealed that HF-fed mice had higher blood glucose levels at 15 ($t_{6.804}$ = 4.432 p<0.01), 30 ($t_{5.520}$ = 9.949, p<0.001), 60 ($t_{6.580}$ = 13.1, p<0.001), 90 (t_{10} = 9.854, p<0.001) and 120 ($t_{5.692}$ = 7.326, p<0.001) ; Figure 2A) minutes, and a higher area under the curve (AUC) for glucose ($t_{6.226}$ = 10.976, p<0.001; Figure 2B) compared with the LFC, indicating the impaired ability of HF-fed mice to respond to the glucose challenge. The intraperitoneal insulin tolerance test (IP-ITT) revealed that HF-fed mice were insensitive to exogenous insulin compared with the LFC ($t_{7.364}$ = 3.783, p<0.01; Figure 2D). Therefore, 12 weeks of feeding with the HF diet was deemed sufficient to induce glucose intolerance (Figures 2A and 2B) and insulin insensitivity (Figures 2C and 2D), prior to microbial interventions.

Following an additional 10 weeks HF feeding in combination with *L. brevis* DPC6108 and *L. brevis* DSM32386, all mice underwent IP-GTT (Figures 2E-2F) and IP-ITT (Figures 2G-2H). After 22 weeks HF feeding, repeat IP-GTT revealed that mice in the HFC had higher fasting blood glucose levels at 0 (t_{12} = 3.077 p=0.01), 15 ($t_{7.046}$ = 10.358 p<0.001), 45 (t_{12} = 8.101 p<0.001), 90 ($t_{7.856}$ = 3.173 p<0.05) and 120 (t_{12} = 3.179 p<0.01) minutes post-glucose load, compared with the LFC (Figure 2E). The HFC also had a higher AUC for glucose ($t_{6.579}$ = 7.137 p<0.001), compared with the LFC (Figure 2F). Somewhat in line with the results for the IP-GTT when comparing between HFC and LFC, the IP-ITT revealed a significant effect of HF diet on blood glucose levels at 0 (t_{12} = 2.930 p<0.05) and 15 (t_{12} = 5.550 p<0.001) minutes post-insulin challenge, compared with the LFC (Figure 2G). There was only a marginally

significant effect on the AUC for glucose between the HFC and LFC (t_{12} = 2.160 p = 0.052; Figure 2H).

The microbial interventions had a significant effect overall on blood glucose levels at 15 ($F_{3,32}$ = 7.390, p<0.01) and 45 ($F_{3,32}$ = 6.072, p<0.01) minutes post-glucose load, compared with the HFC (Figure 2E). DSM32386 mice displayed lower blood glucose levels at 15 (p<0.05) and 45 (p<0.01) minutes post-glucose load, compared with the HFC (Figure 2E). There was a significant effect overall of the interventions on the AUC for the IP-GTT ($F_{2,32}$ = 6.016, p<0.01; Figure 2F) and it was revealed that DSM32386 was the only intervention to significantly improve the ability to clear blood glucose following glucose challenge (p=0.01; Figure 2F). In line with this, the microbial interventions had a significant effect overall on blood glucose levels at 0 ($F_{3,32}$ = 11.452, p<0.001), 15 ($F_{3,32}$ = 13.146, p<0.001), 45 ($F_{3,32}$ = 16.118, p<0.001) and 120 ($F_{3,32}$ = 8.776, p=0.001) minutes post-insulin load during the IP-ITT, compared with the HFC (Figure 2G). DSM32386 had lower blood glucose levels at 0 (p<0.01), 15 (p<0.001) and 120 (p = 0.001) minutes post-insulin load, compared with the HFC (Figure 2G). DPC6108 had lower blood glucose levels at 0 (p<0.001) and 45 (p<0.05) minutes post-insulin load, compared with the HFC (Figure 2G). Overall, intervention with both *L. brevis* strains had a significant effect on total AUC during the IP-ITT ($F_{2,32}$ = 14.408, p<0.001; Figure 2H) and DSM32386 improved insulin sensitivity (p = 0.001 ; Figure 2H).

Plasma was collected during T0 and T15 of the IP-GTT to measure insulin and, subsequently, insulin resistance index (Figures 2I and 2J). At 0 minutes, before glucose load, there was an overall effect of *L. brevis* on plasma insulin ($F_{2,30}$ = 5.46, p = 0.01; Figure 2I). Intervention with DSM32386 increased resting plasma insulin at 0 minutes (p<0.01), compared with the HFC (Figure 2I). Insulin production 15 minutes post glucose load was higher in both microbial interventions (DPC6108; p<0.001 and DSM32386 (p<0.001), compared with the HFC (Figure 2I). No differences in the insulin resistance index were observed following microbial intervention, compared with the HFC (Figure 2J).

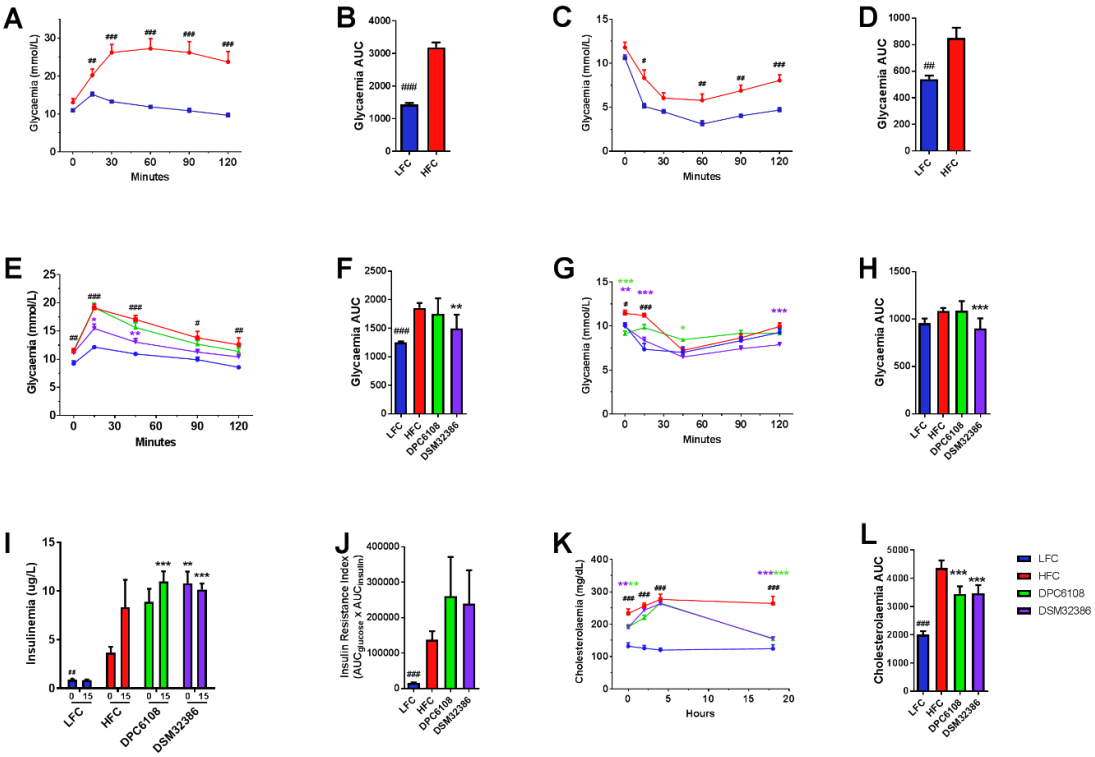


Figure 2: *L. brevis* DSM32386 improved glucose clearance in the IP-GTT while both strains improved glucose-dependent insulin secretion following glucose challenge in the IP-ITT. HFC (n=7) and LFC (n=7) mice were assessed for 1g/Kg glucose (IP-GTT; A and B) and 0.75 IU/Kg insulin (IP-TT; C and D) glycaemic responses following 12 weeks of feeding, prior to commencement of treatments at week 13. At week 24 of feeding and after 10 weeks of intervention, LFC (n=7), HFC (n=7), DPC6108 (n=14), and DSM32386 (n=14) mice were assessed for glycaemic responses to glucose (E and F) and insulin (G and H) challenges. Plasma insulin concentration as determined from samples collected at T=0 and T=15 during glucose challenge for LFC (n=7), HFC (n=5), DPC6108 (n=14) and DSM32386 (n=12) (I) and insulin resistance index determined by multiplying the area under the curve of both the blood glucose (0 to 120 min) and the plasma insulin (0 to 15 min) obtained following glucose challenge (J). At week 24 of feeding and after 10 weeks of intervention LFC (n=7), HFC (n=7), DPC6108 (n=14), DSM32386 (n=14) mice were assessed for cholesterol metabolism by oral gavage with a complete meal, Ensure Plus, Abbott Nutrition. Total plasma cholesterol was measured at T=0, T=2, T=4 and

T18 hr post-gavage (K). Total AUC for cholesterol metabolism was calculated from individual time points for all groups (L). Data are expressed as mean ± SEM. All data was analysed using the appropriate unpaired student t-test (HFC vs. LFC) and one-way analysis of variance (ANOVA). # $p < 0.001$ HFC vs. LFC, ## $p < 0.001$ HFC vs. LFC and ### $p < 0.001$ HFC vs. LFC, * $p < 0.05$ treatment vs. HFC, ** $p < 0.01$ treatment vs. HFC and *** $p < 0.001$ treatment vs. HFC. AUC: area under curve, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.

6.4.4 GABA-producing microbes improved post-prandial cholesterol metabolism

Assessment of the plasma at T0, prior to the mixed meal gavage (MMG) challenge indicated that microbial intervention had a significant impact on fasted plasma cholesterol levels ($F_{2,32}$ = 6.931, p<0.01), compared with the HFC (Figure 2K). Post-hoc analysis revealed DPC6108 (p<0.01) and DSM32386 (p<0.01) reduced cholesterol levels at T0, compared with the HFC (Figure 2K). No significant impact of intervention on plasma cholesterol was identified at two or four hours post-MMG; however, at 18 hours DPC6108 (p<0.001) and DSM32386 (p<0.001) both displayed reduced cholesterol levels, compared with the HFC (Figure 2K). There was a significant effect overall of microbial intervention on total AUC for plasma cholesterol throughout the MMG ($F_{2,13.573}$ = 14.547, p<0.001, Figure 2L). DPC6108 (p<0.001) and DSM32386 (p<0.001) significantly reduced plasma cholesterol levels, compared with the HFC (Figure 2L).

6.4.5 GABA-producing microbes increased luminal GABA levels in the small intestinal content

Diet had no impact on either adipose tissue ($t_{15.738}$ = 1.522) or small intestinal content (t_{20} = -0.898) of GABA, between the HFC and LFC groups (Figures 3A and 3B). This was unsurprising as both diets were matched for amino acid profiles. *L. brevis* did have a significant impact on small intestinal content of GABA ($F_{2,20.551}$ =

6.906, p<0.001), compared with the HFC (Figure 3B). While intervention with DPC6108 had a tendency to increase luminal contents of GABA (p=0.067), DSM32386 significantly increased GABA (p<0.01) in the small intestine, compared with the HFC (Figure 3B).

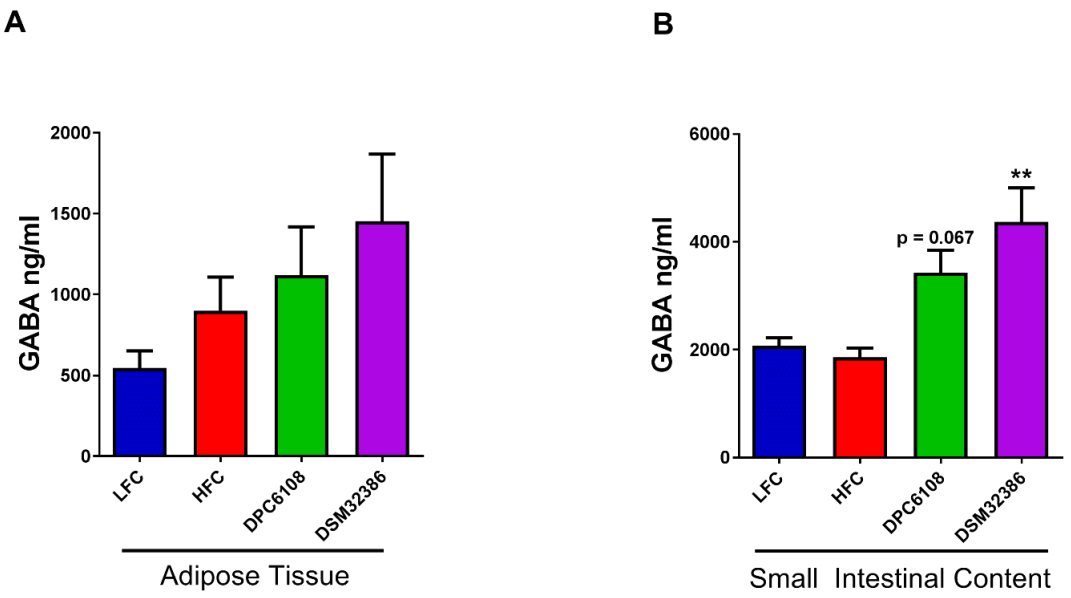


Figure 3: *L. brevis* increased GABA levels in small intestinal content. GABA levels were determined from tissue samples collected at the end of the 12-week intervention from LFC (n=8), HFC (n=12), DPC6108 (n=14), DSM32386 (n=11) from epididymal adipose tissue (A) and from LFC (n=10), HFC (n=12), DPC6108 (n=14), DSM32386 (n=14) in total small intestinal content (B). Data are expressed as mean ± SEM. All data was analysed using the appropriate unpaired student t-test (HFC vs. LFC) and one-way analysis of variance (ANOVA). ** p < 0.01 treatment vs. HFC. AUC: area under curve, GABA: gamma-aminobutyric acid, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.

6.4.6 GABA-producing microbes had little effect on host plasma metabolome

Targeted analysis of 140 metabolites from the plasma metabolome taken from fasted mice unveiled modest metabolic alterations as a result of microbial intervention. Figure 4A depicts a sparse partial least square discriminant analysis (sPLS-DA) plot, with the metabolites most important to the model displayed in the loadings plot (Figure 4B). DPC6108 had little impact on the plasma metabolome, solely increasing the biogenic amine ornithine when compared with the HFC (q<0.05; Figure 4C). DSM32386 also increased ornithine compared with the HFC (q<0.05; Figure 4C), while decreasing arginine compared to both the HFC and DPC6108 (q<0.01; Figure 4D).

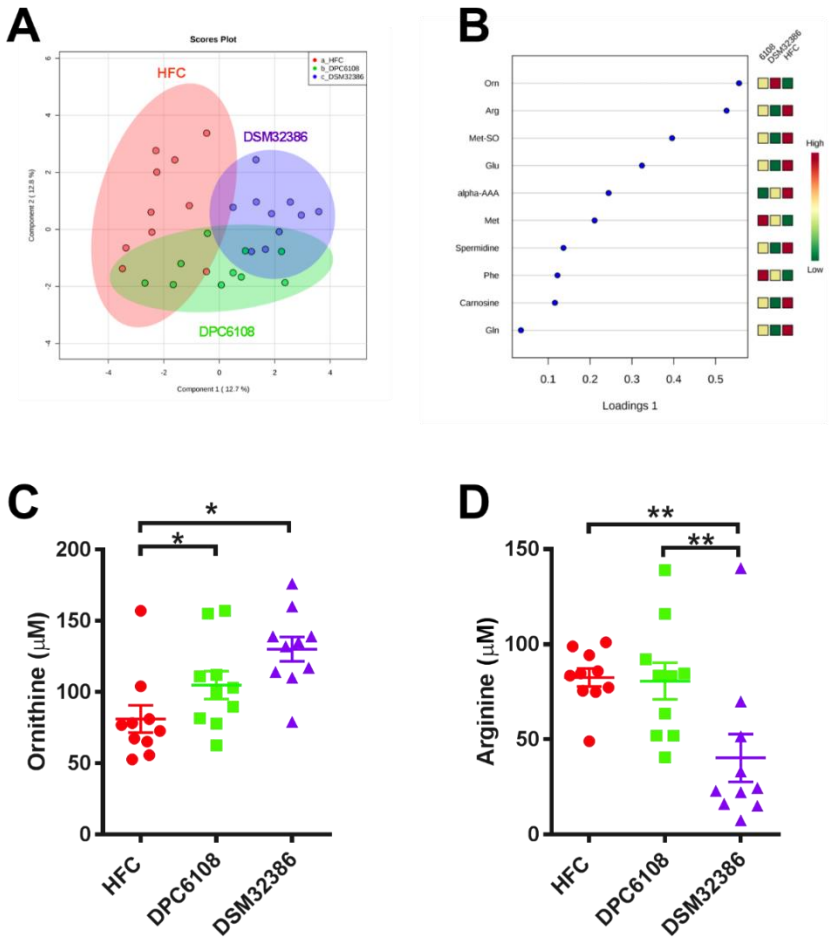


Figure 4: *L. brevis* altered plasma ornithine and arginine. (A) Sparse partial least square discriminant analysis (sPLS-DA) plot displays HFC (n=10; red), DPC6108 (n=10; green) and DSM32386 (n=10; purple) samples. (B) Plot metabolite loadings graph displaying key metabolite differences between groups. Quantitative data is displayed for the metabolites which were significantly altered by DPC6108 or DSM32386; this included the biogenic amine ornithine **(C)** and amino acid arginine (D). Orn:Ornithine, Arg:Arginine, Met-SO:Methioninesulfoxide, Glu:Glutamate, alpha AAA:alpha Aminoadipic Acid, Met:Methionine, Phe:Phenylalanine, Gln:Glutamine, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386. * $q < 0.05$ treatment vs. HFC, ** $q < 0.01$ treatment vs. HFC. Plots depict individual replicates with mean and SEM.

6.4.7 Diet and GABA-producing microbes had a significant impact on gut microbial composition and diversity

A total of 51 faecal samples were subjected to 16S metataxanomic analysis. Illumina sequencing generated a total of 2,560,853 high quality sequences. After rarefying the sequencing depth, Chao1 diversity index and observed operational taxonomy units (OTU) were calculated (Supplementary Table S2). Chao1 index showed a higher diversity profile in the LFC (mean value = 4,397.0, $p<0.05$) and DPC6108 (mean value = 4,138.5, $p<0.05$), compared with the HFC. Moreover, DPC6108 showed higher diversity, compared with the HFC. The data shows that there was a significant difference in the richness, with reduced numbers of OTUs in all HF diet-fed groups, compared with the LFC; although the number of OTUs was increased in DPC6108, compared with the HFC. To assess beta-diversity, Bray-Curtis distance matrix was calculated, and differences were identified between LFC ($p<0.001$) and DSM32386 ($p<0.05$), compared with the HFC (Supplementary Table S2).

At phylum level, all groups demonstrated comparable microbial compositions (Figure 5A). In LFC, Firmicutes (43.8%) and Bacteroidetes (44.3%) were dominant (ratio Firmicutes/Bacteroides = 0.98). The phylum Deferribacteres (6.1%) was

significantly higher in LFC, compared with all other HF diet-fed groups. A reduction of Deferribacteres (1.7%, p<0.001) and increase of Firmicutes (52.3%, p <0.05) was observed in the HFC, compared with the LFC. HFC, DSM32386 and DPC6108 mice had similar relative abundance of Firmicutes and Bacteroidetes (ratio Firmicutes/Bacteroides HFC = 1.28, DSM32386 = 1.22, DPC6108 = 1.30), while the relative abundance of Deferribacteres was higher in DSM32386 (2.7%, p=0.09) and DPC6108 (2.9%, p=0.08), compared with the HFC (1.7%).

At the genus level (Figure 5B), *Akkermansia* was higher in the LFC (0.02%), compared with the HFC (0.01%; p<0.0001), but absent in both DSM32386 and DPC6108. A higher abundance of *Odoribacter* (6.02%) and a lower abundance of *Lachnoclostridium* (3.57%) were detected in the LFC, compared with the HFC (*Odoribacter* p<0.001, *Lachnoclostridium* p=0.01), DSM32386 (*Odoribacter* p<0.05, *Lachnoclostridium* p=0.05) and DPC6108 (*Odoribacter* p<0.001). *Oscillospira* and *Clostridiales_other* were higher in the HFC (*Oscillospira* p=0.01, *Clostridiales_other* p<0.01), DSM32386 (*Oscillospira* p<0.01, *Clostridiales_other* p=0.001) and DPC6108 (*Oscillospira* p<0.05, *Clostridiales_other* p<0.001), compared with the LFC (1.17%, 9.07%, 2.43%, respectively). All sequences associated to the genus *Lactobacillus* were blasted manually on the NCBI Basic Local Alignment Search Tool and the species *L. brevis* was detected only in DSM32386 and DPC6108 groups.

6.4.8 Bifidobacterium, Erysipelotrichaceae, Muribaculum *and* Odoribacter *were related to reduced glycaemia and cholesterolaemia*

The principal component analysis (PCA) plot (Figure 5C) and correlation matrix (Figure 5D) were generated based on the relative microbial composition and metabolic parameters. Correlations between microbiota and biochemical analyses revealed that the increase of certain microbial taxa corresponded to the improvement or deterioration of glycaemia (after IP-GTT or IP-ITT), insulin or cholesterol levels (Supplementary Table S3). Indeed, diet appeared to be the primary determinant of these microbiota-biochemistry associations, as both are known to be substantially impacted by such factors. The PCA scores clearly

separated the LFC from the HF groups, with DSM32386 and DPC6108 clustering together away from the HFC (Figure 5C). PCA1 and PCA2 explained the 21.42% and 15.0% of variance between groups, respectively. The variance associated to the LFC was related to *Erysipelotrichaceae, Muribaculum* and *Bifidobacterium* on PCA1 (|loadings|≥ 0.52), and *Bifidobacterium* on PCA2 (|loadings|≥ 0.52). The variance associated to DSM32386 and DPC6108 was related to *Odoribacter,* cholesterol (T0, T2 and T4), glycaemia under IP-GGT (at T0, T15, T45, T120) on PCA1 (|loadings |≥ 0.50) and *Alistipes*, other *Bacteroidetes*, and resting insulin on PCA2 (|loadings |≥ 0.64), apart from four samples belonging to DPC6108 (Figure 5D).

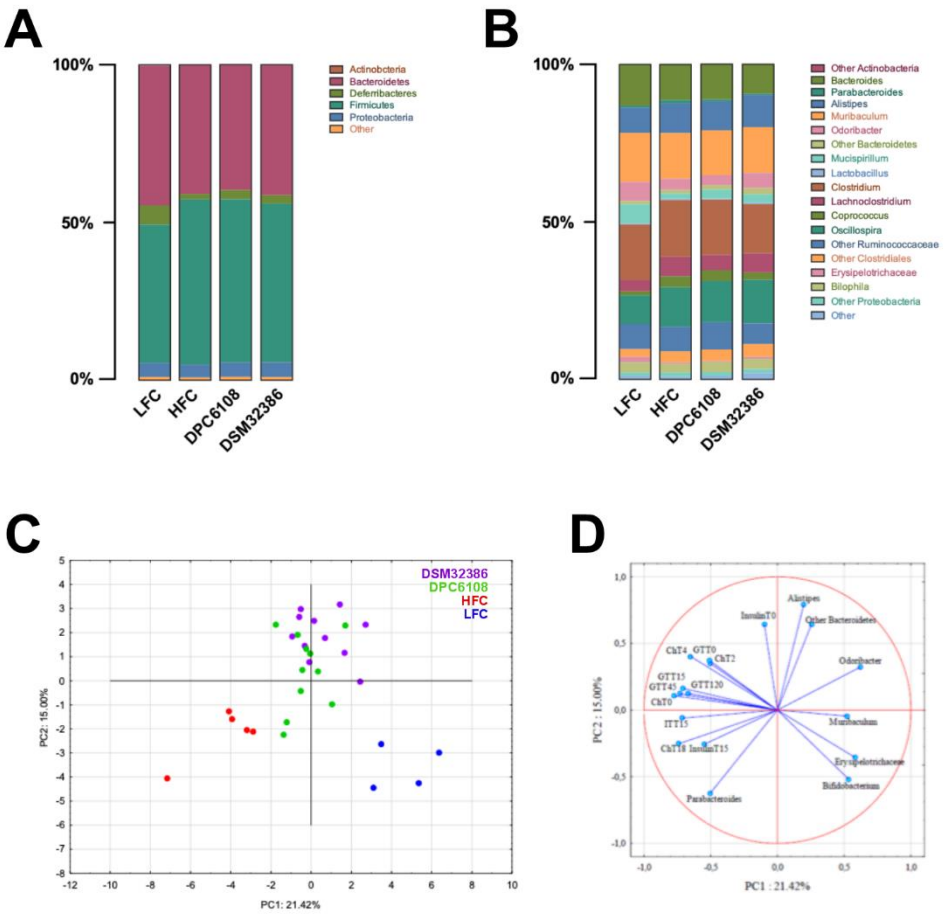


Figure 5: Both diet and *L. brevis* had a significant impact on gut microbiota composition. Relative abundance of microbial taxa at (A) phylum and (B) genus level. Phyla and genera representing <0.1% abundance in any sample were

considered as Other. The PCA plot based on OTU relative abundances of the bacterial community and the metabolic parameters measured in mice (C). The first two principal coordinates (PC1 and PC2) are plotted for each sample (D), with the variables related to the variance between groups. PCA: Principal Component Analysis, OTU; Operational Taxonomy Unit.

6.4.9 GABA-producing microbes improved depression-like behaviour and basal corticosterone levels during the forced swim test (FST)

Diet and bodyweight impacted immobility time during the FST. Mice in the HFC group spent more time immobile, compared with the LFC (t_{21} = 4.245, p<0.001; Figure 6A). While it was hypothesised that obesity itself impaired mobility in the HFC, data from the NOR open field test would suggest that bodyweight had no significant effect on mobility (Figure S2D). Therefore, depression-like behaviour was induced through consumption of the HF diet. *L. brevis* had an overall impact on immobility time during the FST ($F_{2,36}$ = 19.474, p<0.001), compared with the HFC (Figure 6A). Post-hoc analysis revealed that DPC6108 (p<0.001) and DSM32386 (p<0.001) reduced the duration of time spent immobile during the FST, compared with the HFC (Figure 6A). In addition, mice in the HFC group produced more faecal pellets during the FST (t_{24} = 3.021, p<0.05), compared with the LFC, indicative of enhanced anxiety during this test (Figure 6B). Finally, DSM32386 produced significantly fewer faecal pellets during the FST (p<0.01), compared with the HFC (Figure 6B).

Plasma was isolated from individual mice at 0 minutes pre FST, then at 15, 45, 90 and 120 minutes post FST to assess the impact of diet and microbial intervention on the hypothalamic-pituitary-adrenal (HPA) axis response to stress. Basal corticosterone levels were increased in the HFC, compared with the LFC (t_{23} = 3.619, p<0.01; Figure 6C). Interestingly, *L. brevis* restored basal corticosterone to levels comparable with the LFC and prevented the apparent HF dietary stimulation of the HPA axis and the stress response ($F_{2,36}$ =16.110, p<0.001; Figure 6C). DPC6108

(p<0.001) and DSM32386 (p<0.001) reduced basal corticosterone levels, compared with the HFC (Figure 6C).

Following the FST, the HFC had higher levels of corticosterone at 15 (t_{21} = 4.104, p<0.001) and 45 (t_{22} = 6.692, p<0.001) minutes, compared with the LFC (Figure 6D). In addition, DPC6108 at 45 (p=0.001), and DSM32386 at 15 (p<0.01) and 45 (p<0.01) minutes post-FST displayed higher corticosterone levels, compared with the HFC (Figure 6D).

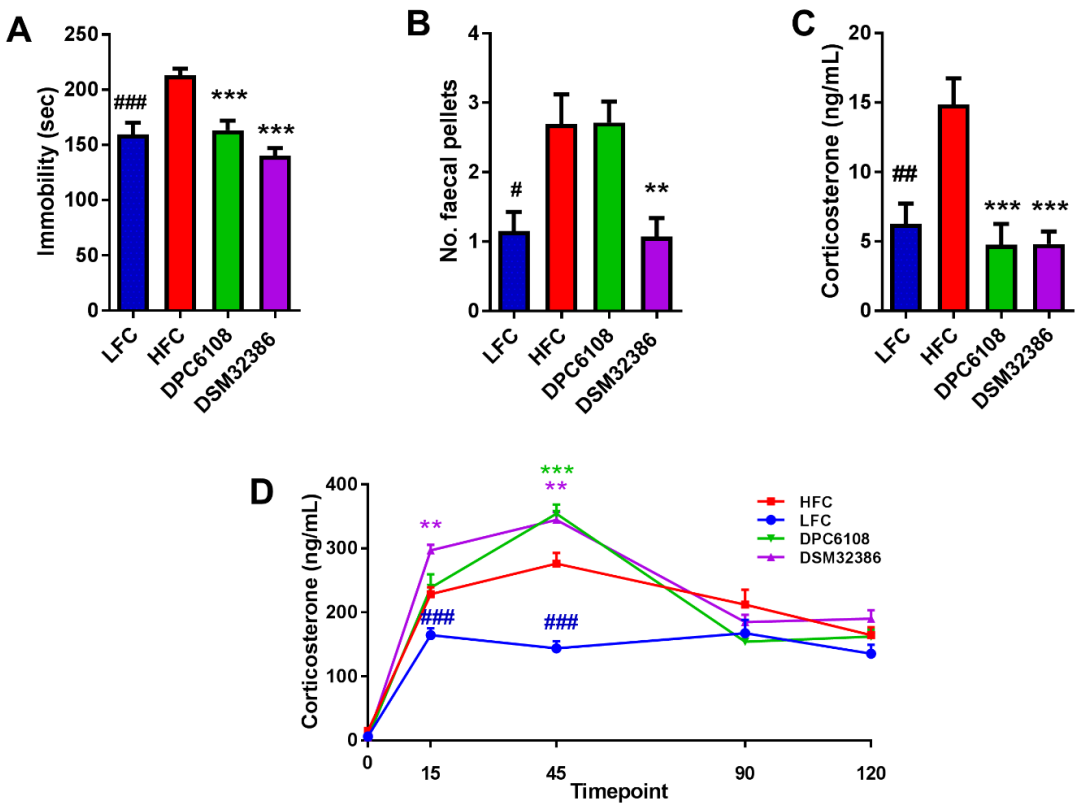


Figure 6: *L. brevis* improved symptoms of depression-like behaviour in the FST and resting corticosterone levels. The effect of HF-diet feeding and *L. brevis* intervention on depression-like behaviour and the HPA axis response to stress was assessed after 21 weeks of feeding and after eight weeks of intervention. Despair behaviour was assessed thorough immobility time (A) by mice throughout the test and anxiety-like behaviour was assessed through the number of faecal pellets produced during the test (B). Plasma isolated at T=0, just prior to the FST was used

to measure corticosterone production (C) and subsequent plasma samples collected at T=15, T=45, T=90 and T=120 post-FST were used to calculate corticosterone as a measure of the HPA axis response to stress (D). Samples were collected from mice in LFC (n=13), HFC (n=12), DPC6108 (n=11), DSM32386 (n=14). All data was analysed using the appropriate unpaired student t-test (HFC vs LFC) and one-way analysis of variance (ANOVA). # $p < 0.05$ HFC vs. LFC, ## $p < 0.01$ HFC vs. LFC and ### $p < 0.001$ HFC vs. LFC, ** $p < 0.01$ treatment vs. HFC. FST: forced swim test, HF: high fat, HFC: high fat control, HPA: hypothalamic pituitary adrenal, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.

6.4.10 Diet and bodyweight affected mobility, while GABA-producing microbes had no effect on anxious behaviour

Diet and bodyweight had a significant impact on mobility, represented by total distance travelled during the aversive open-field (OF) test (Figure S1A). Mice in the HFC group travelled significantly less in the open arena, compared with the LFC (t_{24} = 3.678, p<0.01; Supplementary Figure S1A). *L. brevis* had no impact on the total distance travelled, compared with the HFC (Supplementary Figure S1A). There was no effect of diet (HFC vs LFC) on duration of time spent in inner (Supplementary Figure S1B) or outer (Supplementary Figure S1C) zones of the aversive OF. In addition, *L. brevis* had no effect on the duration of time spent in inner (Supplementary Figure S1B) and outer zones (Supplementary Figure S1C) of the OF. There was no effect of diet (HFC vs LFC) on faecal pellet production in the aversive OF test ($t_{(24)}$ = 0.5527, p=0.5856). In addition, *L. brevis* had no effect on faecal pellet production (F (2, 40) = 0.923, p=0.406).

In contrast, diet and bodyweight did not impact mobility, represented by total distance travelled during the OF test conducted during the habituation phase (Day 1) of the novel object recognition (NOR) test. In this open arena, there was no difference in mobility between the HFC and LFC (Supplementary Figure S1D). *L. brevis* had a significant impact on distance travelled during this test ($F_{2,38}$ = 8.911, p<0.001; Supplementary Figure S1D) and it was revealed that DPC6108 increased

total distance travelled, compared to the HFC (p<0.001; Supplementary Figure S1D). There was no effect of diet on duration of time spent in inner (Supplementary Figure S1E) or outer (Supplementary Figure S1F) zones of the NOR open field arena, between the HFC and LFC. Similarly, *L. brevis* had no effect on time spent in inner (Supplementary Figure S1E) or outer (Supplementary Figure S1F) zones of the NOR open field arena.

6.4.11 L. brevis DSM32386 had a modest effect on cognitive function during the NOR test

During day two of the NOR test, all groups spent equal proportions of time investigating the two identical objects (A and B) within the open arena (Supplementary Figure S2A). During day three, following introduction of a novel object in the space which was previously allocated to object B, while all mice increased the interaction time spent with the novel object, the effect was only significant following intervention with DSM32386 ($t_{(26)}$ = 3.317, p<0.01; Supplementary Figure S2B). There was no impact of diet or *L. brevis* on the ability of mice to discriminate between the novel and familiar objects, as reflected in the discrimination index ($F_{(2,40)}$ = 1.514, p= 0.233) (Supplementary Figure S2C).

6.4.12 GABA-producing microbes had no impact on anxiety-like behaviour during the elevated plus maze (EPM) test

In all aspects on the EPM test, there was no impact of diet or bodyweight identified with respect to the number of open (Supplementary Figure S3A) (t_{24} = 1.353, p=0.1887) and closed (Supplementary Figure S3B) ($t_{(24)}$ = 0.0647, p=0.9468) arm entries for the HFC, compared with the LFC. Non-parametric analysis on the % of time spent in open (Mann-Whitney U value: 78.50; Wilcoxon W: 169.50 p=0.758) and closed arms (t_{24}=0.3962, p=0.6955), did not reveal any impact of diet on anxiety-like behaviour (Supplementary Figures S3C and S3D). Similarly, *L. brevis* had no effect on anxiety-like behaviour as measured by the % of time spent in open (Chi

squared= 1.772; df= 2; p=0.0412 [Kruskall-Wallis test]) and closed arms ($F_{2,40}$ = 0.085, *p*=0.918; Supplementary Figures S3C and S3D).

6.5 Discussion

This exploratory study demonstrates that daily administration of GABA-producing lactobacilli has a moderate efficacy in improving insulin sensitivity and glucose tolerance in metabolically dysfunctional mice. Two GABA-producing *L. brevis* strains of different origins were selected to compare their therapeutic potential in a mouse model of diet-induced obesity. The ability of certain lactic acid bacteria to biosynthesise GABA is a strain-dependent attribute, genetically governed by the genes *gadB* and *gadC*, whereby the GABA-production efficiencies differ between strains (Tajabadi *et al.*, 2015; Nomura *et al.*, 1999). The GABA-production capability of *L. brevis* DSM32386 was comparable to that of *L. brevis* DPC6108 *in vitro*. However, DSM32386 appeared to have a greater impact, albeit modest, in improving some of the metabolic and behavioural outcomes associated with metabolic dysfunction in this mouse model.

GABA is synthesised from glutamate by glutamic acid decarboxylase (GAD) and is produced by pancreatic β-cells (Adeghate and Ponery, 2002). Once released, GABA binds the $GABA_A$ receptor (ligand-gated Cl^- ion channel) on pancreatic α-cells, causing Cl^- influx following membrane hyperpolarisation, in turn suppressing glucagon secretion (Rorsman *et al.*, 1989; Xu *et al.*, 2006). It has been suggested in diabetic subjects that an impaired insulin-Akt-$GABA_A$ receptor-glucagon secretory pathway in the islet may be an underlying mechanism for unsuppressed glucagon secretion, despite hyperglycaemia (Xu *et al.*, 2006; Tsuchiyama *et al.*, 2007). Unlike in α-cells, $GABA_A$ receptor signalling in β-cells induces membrane depolarisation, which opens voltage-dependent calcium channels, stimulating insulin secretion. Therefore, microbial intervention with GABA-producing strains has the potential to agonise $GABA_A$ receptors in the pancreas, thereby preventing unsuppressed glucagon secretion and stimulating insulin secretion.

In this study, 12 weeks of HF feeding was sufficient to induce glucose intolerance and insulin insensitivity. The results indicate that microbial intervention, particularly with *L. brevis* DSM32386, improved glucose homeostasis by attenuating diet-induced obesity associated hyperglycaemia and insulin insensitivity. During the glucose tolerance test, intervention with both *L. brevis* strains significantly enhanced insulin secretion in response to glucose load. This could represent an increase in the sensitivity of the endocrine response to promote glucose clearance following GABA-producing *L. brevis* intervention.

Although there were no differences in the final bodyweight gain between the HF diet fed groups, *L. brevis* had a significant effect on the distribution of adiposity. Intervention with both DSM32386 and DPC6108 resulted in less MAT accumulation, compared with the HFC. This is important as the oxidative phosphorylation potential of MAT is known to be far greater than that of SAT in humans (Kraunsøe *et al.*, 2010). Moreover, MAT mass is known to correlate well with the development of insulin resistance and T2D, a characteristic which SAT does not possess (Wajchenberg, 2000). Therefore, it appears that GABA-secreting microbial interventions altered fat distribution in a manner which contributes beneficially to metabolic fitness. Interestingly, both DSM32386 and DPC6108 also reduced percentage body fat mass, compared with the HFC. While these outcomes are intriguing, the mechanisms involved are not entirely clear, although certain probiotic bacteria have been shown to attenuate HF diet-associated weight gain and enhance insulin sensitivity in previous studies (Kim *et al.*, 2013; Schneeberger *et al.*, 2015). For example, Schneeberger *et al.* (2015) have demonstrated that *Akkermansia muciniphila* was negatively associated with HF feeding, correlating strongly with changes in the expression of lipid metabolism-related genes and inflammation markers in adipose tissue. Modification of immune cells in MAT has also been associated with beneficial effects on diabetes (Kitamura *et al.*, 2013). While the underlying mechanisms through which *L. brevis* reduces MAT in the host remain unknown, there is evidence to suggest that the therapeutic effects could be mediated by re-distribution of fat in the host, but this remains to be explored. Equally as intriguing is the impact of *L. brevis* on host lipid metabolism, as both

strains were associated with reductions in fasting circulating cholesterol levels and an overall improvement in post-prandial cholesterolaemia. The cholesterol-lowering effect of certain probiotic bacteria have previously been linked with microbial bile acid modification (Joyce *et al.*, 2014) and exopolysaccharide production (London *et al.*, 2014; Ryan *et al.*, 2019). However, this exploratory study is the first to demonstrate a potential role of microbial GABA-production in addressing diet-induced hypercholesterolaemia.

We hypothesised that HF feeding would significantly affect the gut microbiota composition of the mice in this study. Taking a metataxanomic approach, we aimed to determine whether improvements in the metabolic profile following microbial intervention could be attributed to a beneficial impact on the gut microbiota. Several studies have characterised the gut microbiota composition of diet-induced obese mice using next generation sequencing methods (Everard *et al.*, 2011; Lee and Ko, 2014; Ravussin *et al.*, 2012; Wang *et al.*, 2015). Interestingly, there appears to be a characteristic shift in the relative abundances of the two most abundant phyla following obesity, whereby there is an increase in Firmicutes and a decrease in Bacteroidetes. Our data demonstrated that the HF diet did indeed induce alterations in the intestinal microbial composition, significantly increasing Firmicutes, with a stable abundance of Bacteroidetes in the HFC and in the groups exposed to *L. brevis* and HF diet. As a consequence, the ratio of Firmicutes to Bacteroidetes was increased following HF diet consumption; however, *L. brevis* did not mitigate this shift. Lower Firmicutes to Bacteroidetes ratios are associated with reduced blood glucose levels or increased glucose tolerance (Everard *et al.*, 2011; Rabot *et al.*, 2016). Indeed, our data reflected this by showing that the LFC mice had a lower ratio Firmicutes to Bacteroidetes and concordant glucose tolerance when compared with the HFC. Conversely, despite the fact that the ratio of Firmicutes to Bacteroidetes was similar to the HFC, intervention with DSM32386 significantly increased glucose tolerance. Actinobacteria were decreased in all groups fed the HF diet irrespective of microbial intervention. Deferribacteres decreased in all groups fed the HF diet compared with the LFC and again, microbial intervention did not significantly reverse that change. A high inter-group variation

was observed in terms of *Odoribacter* abundance, although DSM32386 intervention trended towards a reversal. Members of the *Odoribacter* spp. can cause health problems in humans and animals, but it has been reported that they may be beneficial in the prevention of hypertension (Göker *et al.*, 2011; Hardham *et al.*, 2008) and modulating host blood glucose levels (Manfredi *et al.*, 2015; Salomäki-Myftari *et al.*, 2016). However, *L. brevis* did not modulate the genus *Akkermansia* and thus does not explain the improved metabolic profile. Despite the low relative abundance of *Lactobacillus*, the OTUs belonging to this genus were manually blasted, and the species *L. brevis* was only found in the DSM32386 and DPC6108 groups.

A further objective was to identify whether changes in specific microbial genera were related to any metabolic parameters measured during the study. Considering both microbial and metabolic factors, the LFC and *L. brevis* groups clearly separated from the HFC on PCA. *Parabacteroides* appeared to be related to the increase of glycaemia after IP-ITT, the reduction of resting insulin levels and the increase of cholesterolaemia. *Bifidobacterium* and *Muribaculum* were related to reduced glycaemia (upon IP-GTT and/or IP-ITT) and the reduction of cholesterolaemia. Finally, *Erysipelotrichaceae* was related to reduced cholesterolaemia, and *Odoribacter* appeared to be related to reduced glycaemia (upon IP-GTT and/or IP-ITT). Since those genera tended to be increased in the LFC and *L. brevis* groups, respectively, they could be involved in the metabolic response of metabolically dysfunctional mice, although until proven, this point remains hypothetical. Nevertheless, if the gut microbiota changes would be the only correlation with the changes identified in the metabolic parameters, then intervention with DSM32386, which had improved metabolic parameters, would have had a different microbial profile than DPC6108. Thus, we hypothesise that the changes in the metabolic parameters observed in DSM32386 may be related to the higher luminal content of GABA in the small intestine and endogenous microbial GABA production. Therefore, alterations to the gut microbiota composition following *L. brevis* intervention do not solely explain the improvements to metabolic function.

The targeted analysis of >140 acylcarnitines, phosphatidylcholines, amino acids and biogenic amines revealed just two significantly altered metabolites. We observed an increase in ornithine for both microbial intervention groups and a decrease in arginine for DSM32386 alone. Both arginine and ornithine represent important amino acid metabolites in the arginine-proline metabolism pathway, which ultimately enters into the butanoate metabolism pathway through which GABA is synthesised in both *Mus musculus* and model bacterium *E. coli* K-12 MG1655. However, perhaps of greater relevance is the fact that ornithine is directly exchanged for arginine in lactobacilli in order to sustain the arginine deaminase pathway which contributes to GABA production (Laroute *et al.*, 2016). In line with this, cultures of such bacteria will invariably produce ornithine and deplete arginine from their environment. Although this may indeed be an important contributor to the observed result, the implications to the host remain unclear.

Mounting literature describes the role of the gut microbiota and microbial intervention in the bidirectional communication system of the microbiome-gut-brain axis (Dinan *et al.*, 2015; Luczynski *et al.*, 2016). For example, intervention with specific bacterial strains has proven effective in modulating behaviour and brain function in animal models ranging from metabolic syndrome (Davari, Talaei and Alaei, 2013; Beilharz *et al.*, 2017) and depression (Moya-Perez *et al.*, 2017), to neurodevelopmental disorders such as autism (Hsiao *et al.*, 2013). Bacteria can produce a range of signalling molecules, or metabolites, including neuroactive amines and amino acids such as GABA, within the intestine which may signal to central and peripheral neural networks (Bravo *et al.*, 2011). Obesity and stress-related psychiatric disorders are frequently comorbid. Clinical data from patients with metabolic syndrome and pre-clinical data from animal models of obesity have demonstrated an increased risk for neuropsychiatric disorders (Castanon, Luheshi and Layé, 2015; Farooqui *et al.*, 2012). In addition, maternal obesity carries significant risks for offspring in terms of metabolic disturbances and behavioural deficits that manifest later in life (Sasaki *et al.*, 2013; Desbonnet *et al.*, 2010). Considering the associated risk of behavioural abnormalities associated with obesity, we aimed to identify whether microbial GABA production in the intestine

could relieve any behavioural abnormalities associated with obesity and metabolic dysfunction. Previously, *L. rhamnosus* JB-1 induced region-dependent alterations in $GABA_{B1\beta}$ and $GABA_{A\alpha2}$ mRNA expression in a number of brain regions which correlated with an improvement in anxiety- and depression-related behaviour in mice (Bravo *et al.*, 2011). Moreover, a recent clinical study investigating 16S microbiome sequencing and MRI brain scans of 23 patients suffering from major depressive disorder demonstrated a strong inverse correlation between relative abundances of a GABA-producing taxa, *Bacteroides,* and a key imaging feature of the disorder (Strandwitz *et al.*, 2018). Neither HF diet nor *L. brevis* intervention had any impact on anxiety-related behaviour as measured by the OF and EPM tests. While mice in the DSM32386 group produced less faecal pellets during the FST, compared to the HFC, the method of counting faecal pellets produced during the FST is a particularly crude measure of anxiety-related behaviour and is not typically used as a robust output from this test which has been traditionally used to assess antidepressant-like activity preclinically (Cryan, Markou and Lucki, 2002). Furthermore, there was no effect of *L. brevis* on faecal pellet production in the aversive OF test and we cannot conclude that either strain had any effect on anxiety-like behaviour. Intervention with DSM32386 had a modest effect towards improving recognition memory in the NOR test. This is particularly interesting since hyperglycaemia is a risk-factor for cognitive impairment (Davari, Talaei and Alaei, 2013; Biessels *et al.*, 1999; Selvarajah *et al.*, 2011). One study previously demonstrated that impaired spatial memory in streptozotocin-induced diabetic rats was considerably improved following probiotic intervention (Davari, Talaei and Alaei, 2013). Finally, *L. brevis* improved HF diet-induced behavioural despair as observed in the FST and basal corticosterone levels taken before the induction of FST-induced stress were lower following *L. brevis* intervention, compared with the HFC. Interestingly, the HF diet increased basal corticosterone in the HFC, compared to the LFC. This effect of HF feeding alone has been observed previously for both inducing despair behaviour and increasing corticosterone, compared to control diet (Aslani *et al.*, 2015). The observation that both DPC6108 and DSM32386 reduced both despair behaviour and basal corticosterone levels compared to the HFC to levels which were comparable to the LFC is intriguing and opens the possibility that

these strains could modulate brain behaviour and signalling, mediated through microbial GABA production.

6.6 Conclusion

The data presented herein outline a potential role of metabolic- and neuroactive-microbial metabolite production in the modulation of diet-induced metabolic dysfunction, including abnormal behaviour. Supplementation with GABA-producing *L. brevis* increases endogenous GABA concentration in the small intestine with an impact on host health. Microbial modulation of the gut microbiota is a safe and effective means to modulate and improve health outcomes in this rodent model of obesity and metabolic dysfunction. This dataset describes that increased microbial GABA production can impact host metabolism and behaviour. Further substantiation of these exploratory health benefits in mice need to be confirmed before moving towards the clinic. This is the first study to our knowledge identifying a potential role of microbial GABA production in alleviating some symptoms of metabolic dysfunction. Future experiments should utilise this dataset to focus on the potential mechanism of action of *L. brevis* DPC6108 and DSM32386 on the outcomes observed in this study. Furthermore, the potential for other metabolites to have a causative effect on the outcomes observed in this study should be explored.

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Authors' Contributions

P. Ross, J.F. Cryan, T.G. Dinan and C. Stanton designed research; E. Patterson, P.M. Ryan, N. Wiley conducted research, analysed data and drafted the manuscript; I. Carafa, E. Sherwin and G. Moloney performed laboratory work, analysed data and contributed to the writing of the manuscript. E. Franciosi, R. Mandal, D.S. Wishart and K. Tuohy provided resources and intellectual input that supported the study. C. Stanton & P. Ross had primary responsibility for final content.

Data Sharing Statement

The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. PRJNA414526.

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Competing Interests Statement

The authors declare no competing interests.

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Supplementary Material

6.8 Supplementary Methods

6.8.1 Culture dependent microbial analysis

Pooled fresh faecal samples collected from each cage of mice were analysed in duplicate following two and five weeks of intervention. Microbial analysis involved enumeration of *L. brevis* DSM32386 and *L. brevis* DPC6108 strains after plating serial dilutions on MRS agar supplemented with 100 µg rifampicin/mL (Sigma-Aldrich Ireland Ltd.) and incubating anaerobically for 48 hours at 37°C. In addition, isolated colonies were tested for GABA production *in vitro*, as described previously (Barrett *et al.*, 2012). Briefly, isolated colonies were grown anaerobically in MRS containing 3% (w/v) and 1% (w/v) MSG at 37°C for 55 hours. Samples were then deproteinised by mixing equal volumes of 24% (w/v) trichloroacetic acid (TCA) and culture, allowed to stand for 10 minutes and centrifuged at 14,000*g* for 10 minutes. Supernatants were removed and diluted with 0.2 mol/L sodium citrate buffer, pH 2.2 to yield 250 nmol of each amino acid residue. Samples were then diluted with the internal standard, norleucine, to yield a final concentration of 125 nm/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd, Garden City, Herts, UK) fitted with a Jeol Na^+ high-performance cation exchange column. To calculate the % bioconversion of 1% MSG to GABA the following calculation was used:

Glutamate in MRS (nmol/mL) – Glutamate in sample (nmol/mL) = nmol/mL of MSG consumed

(GABA in sample (nmol/mL) / nmol/mL of MSG consumed) *100

6.8.2 Glucose and insulin tolerance tests

After 12 weeks of feeding, an intraperitoneal-glucose tolerance test (IP-GTT) and an intraperitoneal-insulin tolerance test (IP-ITT) was performed in the LFC (*n* 7) and HFC (*n* 7) groups. After 10 weeks of intervention, the IP-GTT and IP-ITT were

performed on individual mice in the LFC, HFC, DPC6108 and DSM32386 groups. For the IP-GTT, mice were injected with a glucose load (1g/Kg body weight) directly into the peritoneal cavity, following a 6 hour fast. Blood glucose levels were measured before and 15, 30, 60, 90 and 120 minutes after glucose load. For the IP-ITT, mice were injected with an insulin load (0.75IU/g bodyweight) directly into the peritoneal cavity, following a six hour fast. Blood glucose levels were measured before and 15, 30, 60, 90 and 120 minutes after insulin load. The concentration of blood glucose during the IP-GTT and IP-ITT was determined using a glucose meter (Accu-Chek Aviva, Roche Diabetes Care Ltd., West Sussex, UK) on blood samples collected from the tip of the tail vein.

6.8.3 Insulin resistance index

The plasma insulin concentrations were measured in plasma collected from tail blood during the IP-GTT, after 10 weeks of intervention, using a Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden), according to the manufacturer's instructions. The insulin resistance index was determined by multiplying the area under the curve of both the blood glucose (0 to 120 minutes) and the plasma insulin (0 to 15 minutes) obtained from the IP-GTT.

6.8.4 Mixed-meal tolerance test

A mixed-meal tolerance test was performed after 10 weeks of intervention. Mice were fasted for six hours and a baseline blood sample was taken from the tail following tail incision and collected into EDTA tubes (BD Diagnostics). Mice were then administered 200 µl of Ensure Plus liquid diet (1.5kcal/mL, 29.5% fat; Abbott Nutrition, Dublin, Ireland) by intragastric gavage. Blood was collected 2, 4 and 18 hours post-gavage. Individual blood samples were collected in microtainer^{TM} collection tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Microtainer Plasma Separator Tubes, BD Diagnostics), thoroughly mixed in the tube and stored on ice until centrifugation for 10 minutes at 2,000*g* to isolate the plasma. Isolated plasma was immediately transferred to a clean eppendorf tube following centrifugation. The plasma was then analysed for cholesterol concentration at time

points T0, T2, T4 and T18 hours (EnzyChrom colorimetric assay; Cambridge Biosciences, UK).

6.8.5 Bioinformatic analysis by QIIME

Sequences obtained from Illumina sequencing were processed using Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9 (Caporaso *et al.*, 2010). The paired-end reads were associated to the corresponding sample through the unique barcode and joined. Reads were further processed with the inclusion of quality filtering based on a quality score of > 20 followed by subsequent removal of sequences below length threshold (Caporaso *et al.*, 2010). UCLUST was then used for clustering the reads left into operational taxonomic units (OTUs) at 97% identity (Edgar, 2010). PyNAST (Caporaso *et al.*, 2009) was used to align OTUs with a minimum alignment of 150 bp and 80% of minimum identity, and taxonomy was assigned by using Ribosomal Database Project (RDP) classifier 2.0.1 (Maidak *et al.*, 2001). QIIME was used to generate alpha (Chao1, observed OTUs) and beta diversities (Bray Curtis) distance matrices, and principal coordinate analysis (PCoA) plots were generated based on the beta diversity distance matrices. The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. PRJNA414526.

6.8.6 Behaviour test battery

For all behavioural tests, mice were habituated to the testing room by placing home-cages in the test room for at least 30 minutes prior to testing. The same mice were assessed across all behavioural tests. The behaviour tests were completed over two weeks. All apparatus was cleaned with 70% (v/v) ethanol between mice in each test. A researcher remained in the testing room during each behavioural measure. All outputs were measured by an experimenter blinded to the experimental groups.

6.8.7 Aversive open field test

Following eight weeks of dietary intervention, mice were tested in the open field (OF) for anxiety-related behaviour and locomotor activity. The apparatus was a grey plastic open arena without any bedding (40 cm × 30 cm × 25 cm, *L* × *W* × *H*). At the beginning of each trial, mice were placed in the centre of the brightly illuminated (1,000 lux) open field arena. Mice were allowed 10 minutes free exploration in the box. During this time, behaviour was recorded using a video camera and the number of faecal pellets in the arena were counted as an index of anxiety. At the end of each trial, mice were returned to their home cages with littermates. Total activity and time spent in inner zone were analysed using a tracking system from recorded material (Ethovision, Noldus, Wageningen, The Netherlands).

6.8.8 Novel object recognition

Following eight weeks of dietary intervention, the novel object recognition (NOR) test was used to evaluate cognition (memory and learning) and was conducted as previously described (Burokas *et al.*, 2012; Pusceddu *et al.*, 2015). Day 1, the habituation phase, was performed as the OF test (as described above) where no objects were placed in the grey plastic open arena (40 cm × 30 cm × 25 cm, *L* × *W* × *H*) under low light conditions (60 lux). Day 2, 24 hours following the habituation/open field test, mice were reintroduced to the arena containing two identical objects placed in adjacent corners of the arena, approximately 5 cm from each wall. Mice were again allowed 10 minutes free exploration in the arena, during such time, behaviour was recorded using a video camera as above. Day 3, 24 hours after day 2, mice were once again reintroduced to the arena, this time containing one familiar and one novel object, and again, mice were allowed free exploration of the arena for 10 minutes and during this time, behaviour was recorded using a video camera. After each phase mice were returned to their home cages with littermates. The arena and objects were cleaned with 70% (v/v) ethanol between trials. Object exploration was defined as the time when the animal's nose comes within a 2 cm radius of the object. Memory was defined by the

discrimination index for the novel object (DI) as the difference of time mice spent investigating between the novel and the familiar object divided by the total time exploring both objects. [Discrimination Index, DI = (Novel Object Exploration time - Familiar Object Exploration time) / (Novel Object Exploration time + Familiar Object Exploration time)].

6.8.9 Elevated plus maze

Following nine weeks of dietary intervention, mice were tested in the elevated plus maze (EPM) test to assess anxiety-like behaviour. The apparatus used was an elevated (1 m from the floor) cross plastic maze, comprising two closed 'safe' arms and two open 'fearful' arms (50 cm × 5 cm × 15 cm walls). Mice were individually placed into the centre of the maze facing an open arm to avoid direct entrance into a closed arm and left to explore for five minutes. Both the time spent in each arm, as well as the number of entries was scored manually (entrance in one arm being defined as all four paws inside the arm). At the end of each trial, mice were returned to their home cages with littermates.

6.8.10 Forced swim test

Following nine weeks of dietary intervention, depressive-like behaviour and stress responsiveness were assessed using the forced swim test (FST), as previously described (Cryan *et al.*, 2001). Mice were individually placed in a transparent plexi-glass cylinder (24 cm x 21 cm, *H* × *D*), containing 15 cm-depth water maintained at room temperature (22 ± 1°C) for a single six-minute trial. Water was renewed between each trial. The total time of immobility was scored in the last four minutes (Cryan, Markou and Lucki, 2002). Immobility was defined as the total absence of movement, except slight motions to maintain the head above water. After the trial, mice were gently dried and single-housed for two hours of recovery, before being placed back to their home cages with littermates.

6.8.11 Stress-induced corticosterone production

To assess stress-responsiveness, blood samples were taken in response to an acute stress (FST). First, a blood sample was collected from the tail following tail incision, five minutes before the test. After the acute stress, mice were singly housed following removal from the FST, and blood samples were collected at 15, 45, 90 and 120 minutes after the test.

Bleeding was performed in a separate room to the FST. Blood samples (50-70 µl) were taken from the tail and collected in heparin coated capillary tubes. The blood was then transferred to a microtainer^{TM} collection tubes containing EDTA (BD Diagnostics), thoroughly mixed in the tube and stored on ice until centrifugation for 10 minutes at 2,000g to isolate the plasma. Isolated plasma was immediately transferred to a clean eppindorf tube following centrifugation. Isolated plasma was stored at -80°C for later corticosterone quantification. Corticosterone was quantified using a commercially available ELISA kit (Enzo Life Sciences (UK) Ltd., Exeter, UK) according to the manufacturer's protocol.

6.9 Supplementary Tables and Figures

Supplementary Table S1: Mice were fed *ad libitum* with either a low fat diet (Open Source Diets (D15072701 – 10% kcal from fat and equal parts corn starch and sucrose; (Research Diets Inc., NJ 08901 USA)) or a high fat diet (Open Source Diets (D12492 – 60% kcal from fat; Research Diets Inc.)) and were allowed free access to food and water, for 24 weeks.

Product #	**D15072701**			**D12492**	
	gm	*Kcal (%)*		gm	*Kcal (%)*
Protein	19	*20*		26	*20*
Carbohydrate	67	*70*		26	*20*
Fat	4	*10*		35	*60*
Total		*100*			*100*
kcal/gm	3.8			5.2	
Ingredient	**gm**	***kcal***		**gm**	***kcal***
Casein	200	*800*		200	*800*
L-Cysteine	3	*12*		3	*12*
Corn Starch	280	*1120*		0	*0*
Maltodextrin 10	140	*560*		125	*500*
Sucrose	280	*1120*		68.8	*275*
Cellulose, BW200	50	*0*		50	*0*
Soybean Oil	25	*225*		25	*225*
Lard	20	*180*		245	*2205*

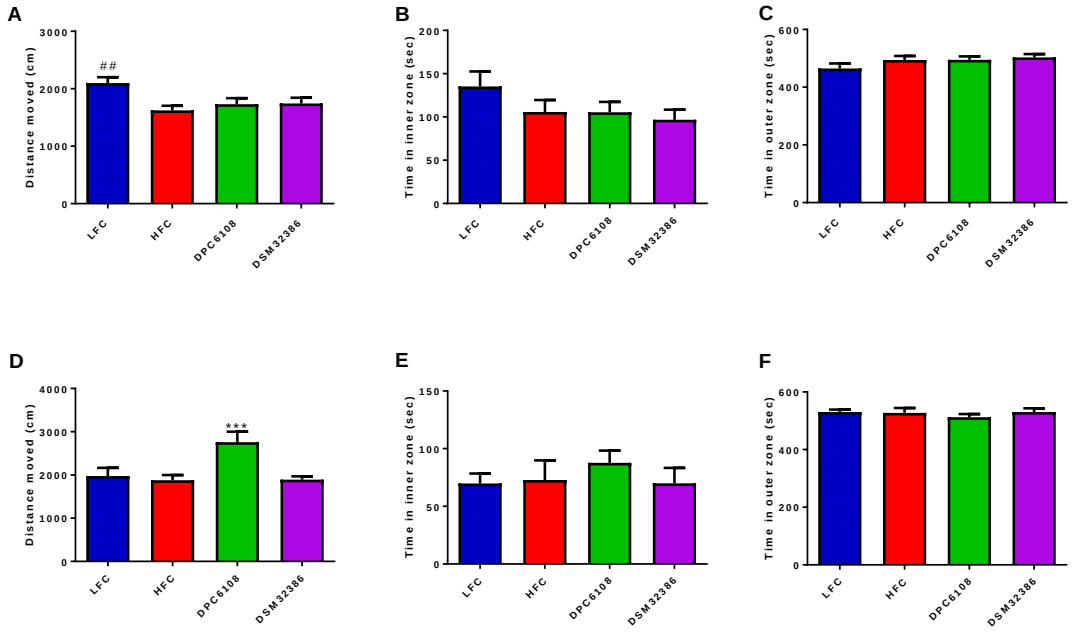
Mineral Mix S10026	10	*0*		10	*0*
DiCalcium Phosphate	13	*0*		13	*0*
Calcium Carbonate	5.5	*0*		5.5	*0*
Potassium Citrate, 1 H20	16.5	*0*		16.5	*0*
Vitamin Mix V10001	10	*40*		10	*40*
Choline Bitartrate	2	*0*		2	*0*
FD&C Yellow Dye #5	0	*0*		0	*0*
FD&C Red Dye #40	0.025	*0*		0	*0*
FD&C Blue Dye #1	0.025	*0*		0.05	*0*
Total	**1055.05**	***4057***		**773.85**	***4057***

Supplementary Table S2: Alpha (Chao1, numbers of observed OTUs) and Beta (Bray-Curtis) diversity indexes are shown as mean values ± standard deviation. Different letters indicate a significant difference (p <0.05).

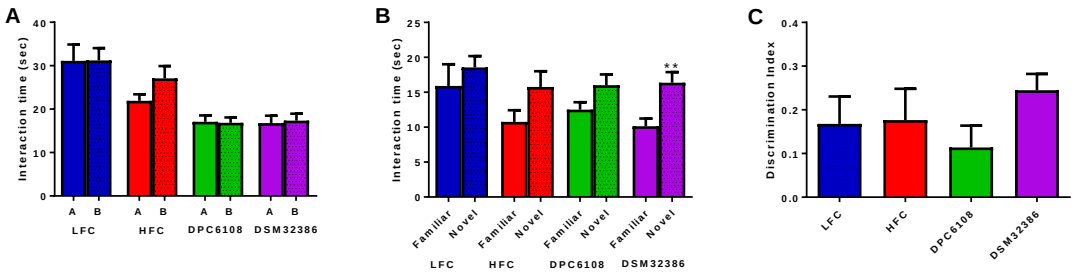
	Chao1	**OTUs (n)**	**Bray-Curtis**
LFC	4397 ± 621^{a}	2501 ± 383^{a}	0.36 ± 0.06^{a}
HFC	3599 ± 576^{b}	1943 ± 330^{b}	0.41 ± 0.06^{b}
DPC6108	4138 ± 720^{a}	2238 ± 494^{a,b}	0.42 ± 0.09^{b,d}
DSM32386	3375 ± 487^{b}	1857 ± 328^{b}	0.44 ± 0.08^{c,d}

Supplementary Table S3: Diet appeared to be the main determinant of microbiota-biochemistry associations. Correlation between the faecal microbiota and glycaemia during IP-GTT, glycaemia during IP-ITT, insulin or cholesterol levels in the blood. Green arrows indicate a negative correlation, while red arrows indicate a positive correlation (p <0.05). The dash indicates no significant correlation (p >0.05).

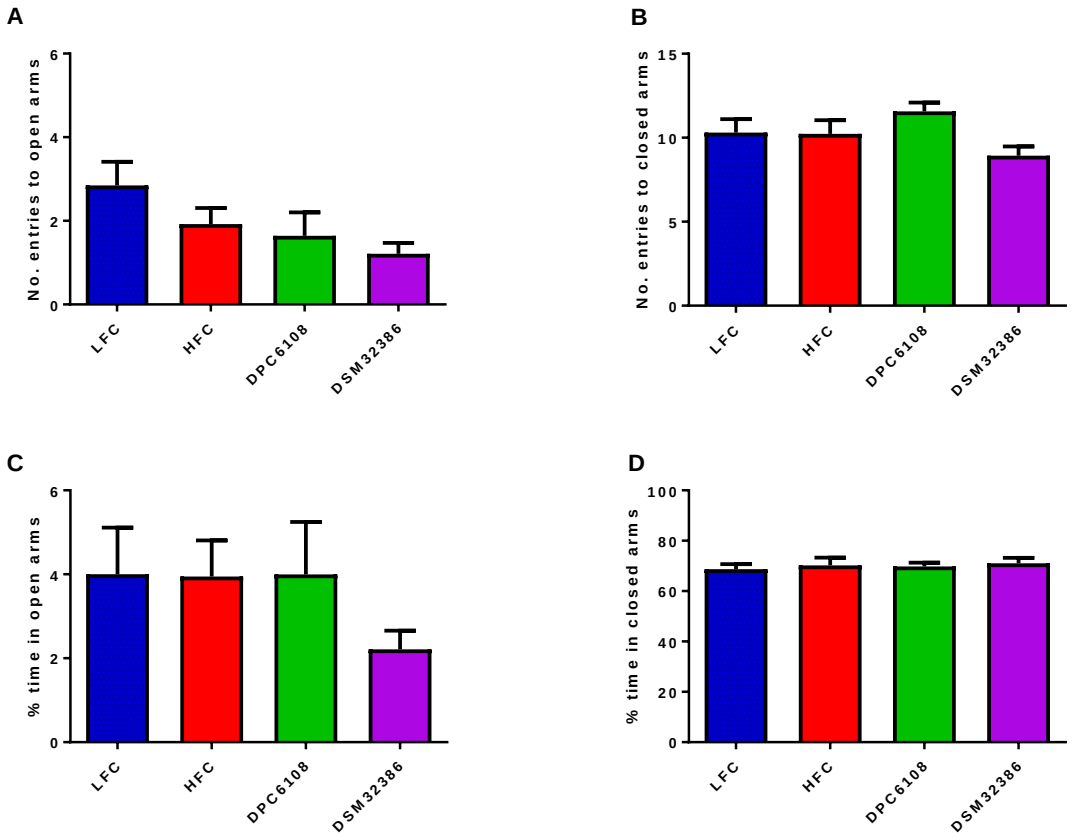
	Glycaemia_GTT	**Glycaemia_ITT**	**Insulin**	**Cholesterolemia**
Bifidobacteria	↓	↓	-	↓
Parabacteroides	-	↑	↓	↑
Muribaculum	↓	↓	-	↓
Odoribacter	↓	↓	-	-
Bacteroidetes_other	-	-	-	↑
Erysipelotrichaceae	↓	-	-	↓



Supplementary Figure S1: *L. brevis* had no effect on anxiety-like behaviour in the aversive and NOR open field test. The effect of HF-diet feeding and probiotic interventions on anxiety-like behaviour was assessed after 21 weeks of feeding and after eight weeks of intervention. Total distance moved (A), time spent in the inner zone (B) and time spent in the outer zone (C) was measured in the aversive OF test and the same outcomes were again measured in the NOR OF test (D, E and F, respectively) for LFC (n=13), HFC (n=13), DPC6108 (n=14), DSM32386 (n=14). Data are expressed as mean ± SEM. All data was analysed using the appropriate unpaired student t-test (HFC vs. LFC) and one-way analysis of variance (ANOVA). ## $p < 0.01$ HFC vs. LFC, * $p < 0.05$ treatment vs. HFC. HF: high fat, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.



Supplementary Figure S2: *L. brevis* DSM32386 had a modest effect on object recognition behaviour / cognitive function in the NOR test. The effect of HF diet feeding and microbial interventions on cognitive function was assessed after 21 weeks of feeding and eight weeks of intervention. On day one of the test, mice were allowed to familiarise themselves with two identical objects (A). On day two, one of the familiar objects was replaced by a novel object (B) and the discrimination index represents how the mice could identify the change between the familiar and novel object during day two (C). The NOR test was performed on LFC (n=13), HFC (n=13), DPC6108 (n=14), DSM32386 (n=14). Data are expressed as mean ± SEM. All data was analysed using the appropriate unpaired student t-test (HFC vs. LFC) and one-way analysis of variance (ANOVA). ** $p < 0.01$ novel vs. familiar. NOR: novel object recognition, HF: high fat, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.



Supplementary Figure S3: *L. brevis* had no effect on anxiety-like behaviour in the EPM test. The effect of HF-diet feeding and microbial interventions on anxiety-like behaviour was assessed after 21 weeks of feeding and after eight weeks of intervention. The number of entries to the open (A) and closed (B) arms of the maze was assessed and the percentage of time spent in the open (C) and closed (D) arms was also calculated for LFC (n=13), HFC (n=13), DPC6108 (n=14), DSM32386 (n=14). Data are expressed as mean ± SEM. All data was analysed using the appropriate unpaired student t-test (HFC vs. LFC) and one-way analysis of variance (ANOVA). EPM: elevated plus maze, HF: high fat, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.

7. A specific dietary fibre supplementation improves cognitive performance – an exploratory randomised, placebo-controlled, crossover study

Kirsten Berding[1], Caitriona M. Long-Smith[1], Carina Cabria[1,*], Thomaz F.S. Bastiaanssen[1*], Marcel van den Wouw[1,*], Niamh Wiley[1,2,3,*], Conall Strain[2], Fiona Fouhy[1,2], Catherine Stanton[1,2,], John F. Cryan[1,4], Timothy G. Dinan[1,3]

* Authors contributed equally to this work

[1]APC Microbiome Ireland, University College Cork, Cork, Ireland

[2]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[3]Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[4]Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[5]School of Microbiology, University College Cork, Cork, Ireland

7.1 Abstract

The impact of the microbiota on numerous aspects of physiology as well as the gut-brain axis is increasingly recognised, resulting in considerable interest in investigating methods of modulating the gut microbiota using a variety of therapeutic tools. While an expanding body of research demonstrates that prebiotic intervention can manipulate the microbiota and affect host health, the influence on cognition and acute stress response is less well understood. Therefore, the objective of this study was to investigate the efficacy of a prebiotic, polydextrose, in improving cognitive performance and acute stress responses through manipulation of the gut microbiota in a healthy population. Furthermore, we aimed to assess the effects of this prebiotic on microbial composition, diversity and SCFA production. In this double-blind, randomised, placebo-controlled, cross-over design study, 18 healthy female participants received 12.5 g of Litesse®Ultra (>90% polydextrose polymer) or placebo (maltodextrin) for four weeks. Stool samples were collected pre- and post-intervention for microbiota compositional analysis, SCFA analysis, and culture analysis. Cognitive performance, mood and acute stress responses were also assessed. PDX supplementation resulted in a modest improvement in cognitive flexibility as evidenced by the decrease in number of errors in the intra-extra dimensional set shift task. An increased ability for sustained attention was also observed through higher number of correct responses and rejections in the rapid visual processing task. Although there was no change in microbial diversity, abundance of *Ruminiclostridium* 5 significantly increased after PDX supplementation compared to placebo. Therefore, the results indicate that PDX could benefit gut-to-brain communication and modulate behavioural responses.

Keywords (9)

Microbiota, short chain fatty acids, prebiotic, polydextrose, cognition, stress

7.2 Introduction

Considerable interest in modulating gut microbiota composition and metabolic function has emerged in recent years as the essential role it plays in health has become more evident. Recent research regarding the gut microbiota suggests that the symbiotic relationship between microbes and their host significantly impacts host health. Gut microbiota are vital for physiological functioning, influencing nearly every aspect of health and disease from the degradation of complex carbohydrates to provide energy to the host, to immune system development, to modulating behaviour (Lyte, 2014). Furthermore, production of metabolites such as fatty acids, secondary bile acids, and certain vitamins are microbiota dependent. The microbiota-gut-brain axis communication system is vital for maintaining homeostasis and integrates neural, hormonal and immunological signalling (Collins, Surette and Bercik, 2012). Mechanisms for signalling from the brain to the gut include physical communication between the microbiota and the gut epithelium via the immune or circulatory system; as well as biochemical communication through hypothalamic-pituitary-adrenal (HPA) axis and via the autonomic nervous system including the vagus nerve. Bacteria can produce biochemical signalling molecules such as short chain fatty acids (SCFAs) and bile acids and can promote gut peptide and hormone production. Importantly, many bacteria can produce neuroactive agents and neurotransmitters, including serotonin, GABA, catecholamines and acetylcholine. The brain can communicate with the gut microbiota either directly, by releasing signalling molecules into the gut lumen, or indirectly by altering gastric motility, secretion, and intestinal permeability (Rhee, Pothoulakis and Mayer, 2009).

Several disease states have now been identified as having an altered gut microbiota profile, usually a decreased abundance of beneficial bacteria or increased abundance of pathogenic bacteria. Such disruption to the microbiota can consist of reductions in diversity, SCFA producers and/or bacteria with anti-inflammatory properties as well as bloom of pathogenic bacteria (Walker and Lawley, 2013). Such alterations have been described in inflammatory bowel disease (Sheehan, Moran and Shanahan, 2015), AIDS (Zilberman-Schapira *et al.*, 2016), cancers such as bowel

cancer (Yamamoto and Matsumoto, 2016), obesity (Martinez, Leone and Chang, 2017), cardiovascular disease, hypertension and chronic kidney disease (Nallu *et al.*, 2017), amongst others. Modulation of gut microbiota is thus emerging as an exciting potential therapy both in the clinic, and in the increasingly health-conscious population. With regard to brain-related disorders, many neurological and psychiatric diseases have been implicated as having altered gut microbiota status, including Parkinson's disease (Scheperjans *et al.*, 2015; Keshavarzian *et al.*, 2015), autism (Parracho *et al.*, 2005), depression (Kelly *et al.*, 2016) and brain amyloidosis (Cattaneo *et al.*, 2017). Additionally, an ever- increasing number of preclinical studies have shown that gut microbiota composition impacts stress, anxiety, memory, cognition and neuroinflammation (Rea, Dinan and Cryan, 2016; Sandhu *et al.*, 2017; Sherwin *et al.*, 2016). Currently, the impact of stress and inflammation on our daily lives has a substantial negative impact on brain health. One in five Irish people suffer from a brain disorder, costing the State over €7.5bn per annum (Olesen *et al.*, 2012), and over the next 20 years, the number of people suffering from brain diseases and mental health disorders is estimated to grow by as much as 20% (www.europeanbraincouncil.org). Thus, research focused on promoting brain health by decreasing stress and anxiety and by improving mood and cognition with nutraceuticals could have significant health benefit on the general population in the longer term.

The concept of targeting the gut microbiota to improve health was proposed over a century ago (Metchnikoff, 1907). Much focus has been directed towards attempting to directly modulate the gut microbiota using a variety of therapeutic tools from transplanting an entire faecal microbiota, to the introduction of living microorganisms, probiotics, to the system. However, this approach presents many pragmatic and technical difficulties. These include ensuring the survival of the probiotic transiting through the hostile environment of altered pH levels from the acidity of the stomach through to the alkali bile salt environment through to the location in the intestines where it can exert its potential therapeutic effects. Issues in continuity and consistency in batch production also need to be considered as well as the inability of probiotics to colonise the environment in the long-term and

the selection of appropriate strains. Alternatively, targeting beneficial host microbiota and enabling these bacteria to thrive by providing growth substrates, inducing compositional or metabolic changes is now presenting an exciting possibility. Prebiotic fibres that selectively improve the growth of beneficial gut microbes have this effect, and are characterised as non-digestible food ingredients, or carbohydrates, which pass undigested through the upper part of the GIT and are selectively fermented by the bacteria in the large intestine, stimulating the growth and/or activity of health promoting bacteria (Bindels *et al.*, 2015). This definition has been adjusted several times since it was first introduced in 1995 (Gibson and Roberfroid, 1995), and has recently been expanded to include additional criteria such as resistance to gastric acid, mammalian enzymatic hydrolysis and absorption (Bindels *et al.*, 2015). However a general consensus agrees that prebiotics have to be 'specific' or 'selective' for health-promoting beneficial metabolic activities or taxonomic groups (Rastall and Gibson, 2015). Such health-promoting bacteria are generally restricted to the genera *Lactobacillus* and *Bifidobacterium*, while *Roseburia* and *Eubacterium* are sometimes considered beneficial (Roberfroid *et al.*, 2010; Gibson and Roberfroid, 1995; Gibson *et al.*, 2010). However, *Bacteroides* and Clostridia are often considered detrimental because they perform proteolytic fermentation processes resulting in toxic metabolites (Roberfroid *et al.*, 2010; Nyangale, Mottram and Gibson, 2012). Interestingly, Clostridia have now been shown to be beneficial in models of colitis and allergy (Roberfroid *et al.*, 2010; Atarashi *et al.*, 2013), highlighting that a consensus has not been reached on which microorganisms should be classified as beneficial/detrimental. Therefore, it is important to consider that specific factors such as host state, genotype, lifestyle and diet also influence the overall effect of the gut microbiota on the host and that beneficial microorganisms have the potential to become detrimental when conditions change (Flint *et al.*, 2015; Moran *et al.*, 2009).

Sources of prebiotics include fruits and vegetables such as asparagus, leek, banana, chicory, as well as grains such as oats and wheat. Consequently, prebiotic intake has become increasingly lacking as consumption of Western-style diets which lack non-digestible carbohydrates has increased, and this has correlated with the rise in the

incidence of anxiety and stress disorders, inflammatory diseases, obesity, metabolic syndrome, and other 'lifestyle' disorders which can negatively impact on the brain. Furthermore, several health benefits have been associated with the metabolic end-products resulting from bacterial fermentation in the gut, such as SCFAs (Bindels *et al.*, 2015), which are considered to represent a key mechanism by which prebiotics exert their effects. SCFAs reduce intestinal pH, which impairs pathogen growth, have antimicrobial activity and have a variety of beneficial metabolic, physiological and immunological effects (Gibson and Roberfroid, 1995; Bindels, Dewulf and Delzenne, 2013). Lactate and acetate are produced by lactobacilli and bifidobacteria (Fukuda *et al.*, 2011), while butyrate and propionate are produced by members of the *Clostridium* clusters XIVa and IV (Reichardt *et al.*, 2014; Louis *et al.*, 2010). Butyrate is considered a particularly beneficial SCFA, providing nutrition for colonocytes, enhancing the integrity of the colonic mucosa and promoting appropriate cell differentiation (Hamer *et al.*, 2008). Conversely, acetate acts more systemically, influencing fatty acid and cholesterol synthesis in the liver, and propionate may impact satiety by regulating gastrointestinal-derived hormones (Hosseini *et al.*, 2011). Prebiotics may include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), resistant starch and other soluble dietary fibres (though not all dietary fibres are prebiotic, and modify selective gut microbiota, e.g. non-fermentable insoluble fibre which acts as roughage in the diet). Early culture dependent techniques indicated that inulin, FOS and GOS affect the human gut microbiota in a highly selective manner, increasing levels of lactobacilli and bifidobacteria while decreasing *Bacteroides*, Clostridia and Fusobacteria numbers (Gibson *et al.*, 1995). However, next generation sequencing and microarray approaches demonstrated that FOS and/or inulin administration alters the abundance of 102 taxa in genetically obese mice (Everard *et al.*, 2011), while GOS tends to be more selectively bifidogenic (Davis *et al.*, 2011). Prebiotics are increasingly included in the manufacture of infant milk formula and the potential of prebiotics to enhance gut health and cognition in a positive manner during periods of brain stress provides an exciting and novel prospective use of such prebiotics in the nutraceutical market.

The prebiotic employed in the current study, Litesse® Ultra polydextrose (PDX), is a randomly cross-linked glucose polymer with a highly branched and complex structure. Due to this complex structure, PDX is not hydrolysed by mammalian digestive enzymes in the small intestine and its fermentation in the large intestine, which is gradual and incomplete, requires a consortium of microbes. This gradual fermentation of polydextrose gives rise to moderate amounts of fermentation products, such as SCFAs and gas, and this slow and sustained fermentation most likely explains the good tolerance of PDX observed in human intervention studies (Roytio and Ouwehand, 2014). Evidence from multiple epidemiological studies indicate an inverse association between fibre intake and several colonic and systemic illnesses, including certain cancers and cardiovascular disease (Divisi *et al.*, 2006), possibly due to fermentation of fibre into SCFA, especially butyrate (Hamer *et al.*, 2008), as well as reducing production of other types of metabolites. Such beneficial effects of PDX are mediated either through the metabolites produced and/or altered microbiota composition. PDX fermentation metabolites have been shown to suppress the expression of colorectal cancer markers, for example administration of metabolites obtained from PDX fermentation reduced expression levels of the COX-2 gene (Makivuokko *et al.*, 2005), which is commonly overexpressed in colon cancer. Additionally, PDX has also been shown to modify the expression of genes in colon cancer cells that are related to cell cycle, apoptosis and energy metabolism (Putaala *et al.*, 2011).

Furthermore, diets with PDX as soluble fibre have been shown to increase urinary excretion of environmental carcinogens such as polychlorinated biphenyls compared to diets with water-insoluble fibre in rats (Kimura, Nagata and Buddington, 2004). Its administration has been reported to induce satiation, reduce LDL cholesterol, and ameliorate the glycaemic response to glucose load in humans, indicating potential benefits for weight maintenance and metabolic health. A study by Stenman *et al.* (2015) found that this prebiotic substantially improved the efficacy of antidiabetic drugs in mice whereby its administration significantly reduced glycaemia of metabolically dysfunctional mice in combination with the drug sitagliptin (SITA). This is possibly due to its ability to increase portal vein GLP-1

concentration together with SITA. Compared to SITA alone, polydextrose further decreased the oral glucose tolerance test response. In addition, fasting glucose was not affected by SITA, whereas polydextrose, *Bifidobacterium animalis ssp. lactis* 420 (B420) and their combination all induced a significant decrease, although the polydextrose group showed the most significant improvement. In addition to describing the benefits of polydextrose prebiotic, this study presented a benefit for combining probiotics and/or prebiotics with antidiabetic drugs. A further randomised, double-blind, placebo-controlled clinical intervention trial by Stenman et al. (2015) using the above probiotic and prebiotic combinations in overweight and obese adults presented preliminary clinical evidence that the probiotic and symbiotic interventions reduced body fat mass, waist circumference, energy intake and body weight compared to placebo. Furthermore, B420 and polydextrose appeared to have synergistic effects in increasing lean body mass. Therefore, this study demonstrated synergy between a probiotic and a prebiotic fibre supplement in promoting lean body mass accumulation. They also presented results on biomarkers associated with gut barrier function, reduced inflammatory tone and intestinal microbiota metabolism that suggests a potential mechanism of action for B420 as well as polydextrose + B420 in reducing body fat mass (Stenman *et al.*, 2016).

In the present study, we investigated the potential of a prebiotic substance, Litesse®Ultra (>90% PDX polymer) to positively enhance stress, mood, memory, and cognition. Furthermore, we investigated the effectiveness of the prebiotic as an anxiolytic agent in a situation of acute stress by employing the socially evaluated cold pressor test (Schwabe, Haddad and Schachinger, 2008), which is a well-established technique (Allen *et al.*, 2016; Kelly *et al.*, 2017) to acutely increase cortisol-response in individuals. This combination of a psychological and physiological stressor has previously been shown to be unaffected by repeat exposure (Minkley *et al.*, 2014), and so provided a useful tool to induce a stress response at different time-points throughout the study. Cognition, mood, and memory were analysed using a multi-modal approach, including the Cambridge Neuropsychological Test Automated Battery (CANTAB) (Allen *et al.*, 2016; Kelly *et*

al., 2017), in addition to validated paper-based tests. CANTAB offers within its remit 22 tests covering visual memory; working memory; executive function; attention; verbal memory; decision making and response control. CANTAB is a computerised neuropsychological test battery which has been extensively used for the assessment of cognitive function in a wide variety of neurological and psychiatric disorders (Fowler *et al.*, 2002; Blackwell *et al.*, 2004; Roiser *et al.*, 2009; Castaneda *et al.*, 2008) and functional GI disorders (Kennedy *et al.*, 2014). The particular utility of CANTAB is that the tests incorporated in the battery are adaptations of well documented paradigms developed on animals with damage to specific brain areas; mainly temporal and frontal lobes (Lowe and Rabbitt, 1998). Furthermore, we aimed to assess the effects of this prebiotic on microbial diversity and SCFA production. Stool samples were collected for microbiota compositional analysis, analysis of SCFA, and culture of strains. Determination of mode of birth, which greatly impacts on the colonisation of the gut by the microbiota, provided additional information on baseline stress levels and responsiveness to an acute stressor. Additionally, participants were screened for psychiatric history and completed a range of self-report measures regarding early life traumatic experiences, depression, anxiety, personality, sociability, sleep quality, physical activity as well as dietary intake, demographic data and medical history. We hypothesised that this intervention would have a positive effect on cognitive performance, as measured by CANTAB tasks; and attenuate the acute psychosocial stress response, potentially via reductions in subjective stress, reduction in HPA axis activity, normalisation of inflammatory profile, altered tryptophan and kynurenine metabolites, and altered heart rate variability. Results from this study are poised to benefit society to protect the brain from the negative impact of stress and lifestyle factors, possibly via positive modulation of gut microbiota through prebiotic supplementation. Successful completion of the project will advance the current state-of-the-art and will provide a functional platform for assessing use of similar novel prebiotic products in the brain in the future.

7.3 Materials and Methods

This study forms part of the SFI-funded APC Microbiome Ireland remit into research on the Brain-Gut Axis. A portion of this study is part of a Seventh FrameWork Programme (FP7), European Commission funded project entitled "Microbiome Influence on Energy Balance and Brain Development – Function Put into Action to Tackle Diet-related Diseases and Behaviour" (http://goo.gl/Brln6U, acronym: MyNewGut). This research study was conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. The protocol was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Protocol Number APC076). Informed consent was obtained from all participants prior to enrolment into the study.

7.3.1 Study Design

This study was designed as double-blind, randomised, and placebo-controlled, using a repeated measure cross-over design. Due to the higher prevalence of stress-related conditions such as depression in females, female participants between the ages of 18 and 40 were recruited in the Cork area and completed the study between March and December 2018. Study participation involved six study visits over a period of 16 weeks (Figure 1). Eligible participants were randomised to take 12.5 g of polydextrose Litesse®Ultra (>90% PDX polymer, Danisco USA Inc., Terre Haute, IN, USA) or placebo (maltodextrin; matched for taste, colour, odour) for four weeks, followed by a four-week washout period and a further four-week period of prebiotic or placebo. There was also a follow-up at two weeks post study completion to assess any ongoing effects. The dose of 12.5 g per day of Litesse®Ultra was selected for this study as it closely resembles the efficacious doses used in previous studies for metabolic health (Stenman *et al.,* 2016). Participants were instructed to consume one sachet of blinded study product every morning mixed in a hot drink, refrain from introducing other probiotic or nutrition supplements during the course of the intervention and keep dietary intake and physical activity consistent throughout the study. Regarding randomisation, the

prebiotic supplier generated a list of which participant number is randomly assigned to which group – one group took placebo in the first intervention period, and took the prebiotic supplement during the second period, while the second group took the opposite. Placebo and prebiotic supplement were coded. The list of which participants are assigned to which conditions was created using block randomisation (block of four). To balance group assignment, an allocation ration to group/treatment of 1:1 PDX:placebo was used. Remaining study product was collected from participants to check for compliance following visits three and five. Final breaking of blinding only occurred after all data had been collected. We utilised g*Power for power calculations. We employed an α-error probability (false positive) of 0.05 and a power of 80%. Effect sizes of 0.25 will be able to be differentiated using n=18 per group (ANOVA repeated measures).

Volunteers were screened according to the selection criteria. Participants were excluded from the study if they had any significant acute or chronic illness, including GI, immunological, or psychiatric disorders; were taking medication which would interfere with the objectives of the study; were peri-menopausal, menopausal, or post-menopausal; were pregnant, or lactating; were colour blind; had dyslexia or dyscalculia; were vegan or a smoker. A washout period of four weeks was applied for participants taking pre-, pro- or antibiotics. Volunteers who were poor attendees or unlikely for any reason to be able to comply with the trial were also excluded. Finally, subjects receiving treatment involving experimental drugs, or participation in an experimental trial not less than 30 days prior to this study also resulted in exclusion. A full synopsis on inclusion and exclusion criteria employed for this study is outlined in Supplementary Materials and Methods. At the initial screening visit, participants were screened for any psychiatric disorders using the MINI International Neuropsychiatric Interview (MINI) and demographic data were collected. The MINI is a brief, structured interview for making diagnoses of DSM-IV Axis I disorders (Sheehan *et al.*, 1998). It has been shown to have validity and reliability scores which are acceptably high but does not take as long to administer as interviews such as the structured clinical interview for DSM-IV-TR-Axis I disorders (SCID- I). The MINI was used to screen for major depressive

episodes, dysthymia, hypo/manic episodes, panic disorder, agoraphobia, social phobia, and generalised anxiety disorder. Additionally, a battery of self-reported questionnaires to obtain a baseline psychological profile was completed. Participants also completed the National Adult Reading Test (NART) (Nelson and Willison, 1991), a widely used and brief measure of verbal IQ (Nelson and Willison, 1991). Cognitive assessment and the acute stressor procedure as well as collection of biological samples and questionnaire data (as described below) were completed at visits 2, 3, 4 and 5, separated by four weeks each. All study visits were performed in the HRB Clinical Research Facility at University College Cork. Subjects, study facilitators, nurses and research analysts were kept blinded until all data analysis was completed. The block randomisation (block of four) of treatment schedules was carried out by DuPont. Remaining study product was collected from participants to check for compliance following visit three and five.

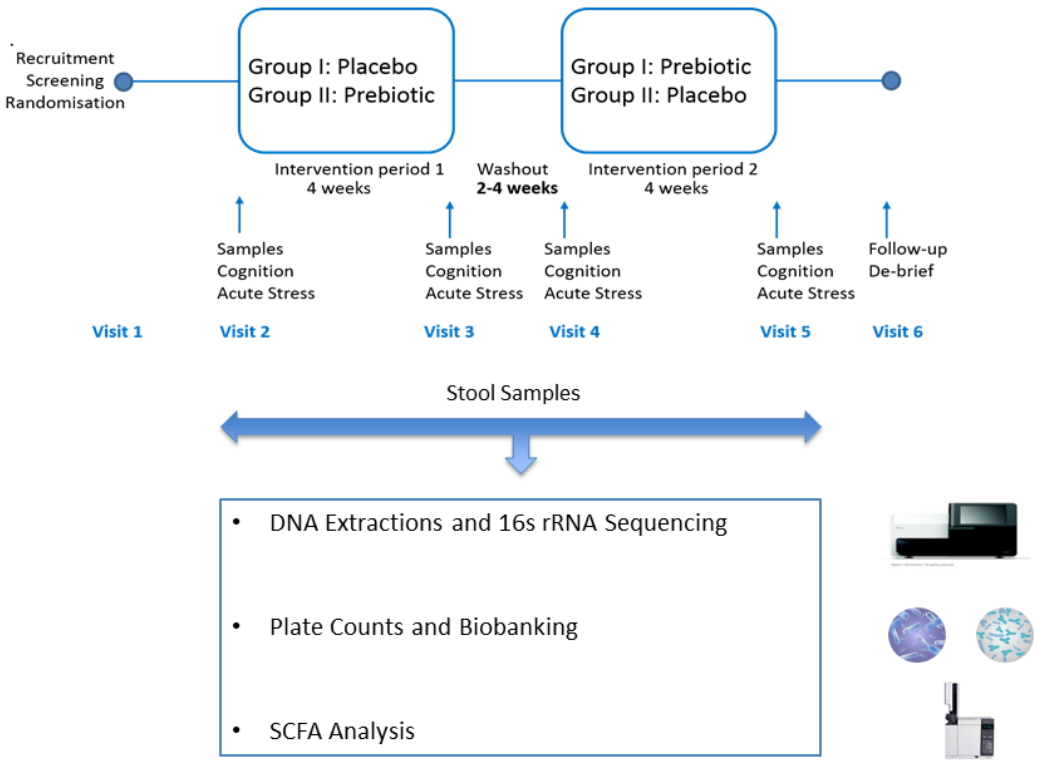


Figure 1: Study overview schematic illustrates study design and timeline for visits and procedures over the 12-week study period. The volunteers underwent a four-

week intervention with either prebiotic or placebo (maltodextrin), followed by a four-week washout period, followed by a four-week intervention with placebo or prebiotic to employ a repeated measures cross-over design. Biological samples (stool, saliva, and blood), cognitive performance (CANTAB), and acute stressor (SECPT) were collected and assessed pre- and post- intervention periods.

7.3.2 Stool Collection and Processing

Freshly voided faecal samples were collected from study participants into plastic containers containing an Anaerogen sachet (Oxoid AGS Anaerogen Compact, Fischer Scientific, Dublin). Participants were instructed to collect the faecal sample as close to the study visit as possible and to keep the sample in a cool place until delivery to the study site. Once transfered to the laboratory, they were processed fresh and assessed using the Bristol Stool Chart. 0.1 g of each sample was aliquoted into 2 ml tubes for serial dilutions and plating from fresh, while 0.25 g was weighed into 2 ml screw cap tubes containing zirconia beads (0.125 g of 0.1 mm and 0.125 g of 1.5 mm with a single 2.5 mm diameter bead, BioSpec Products) and frozen at −80°C for downstream DNA extraction. The range of times between samples produced by the participant and freezing in the laboratory vaired between four to six hours. The remainder of each sample was aliquoted into faecal tubes for further downstream analysis before being placed at −80°C.

7.3.3 Plating and Culturing

0.1 g of each sample was aliquoted into tubes and serially diluted with MRD from 10^{-1} to 10^{-8} before being plated on selective media to enumerate the relevant bacteria. 100 µl of each sample from dilutions 10^{-4} to 10^{-8} were spread plated on the following types of media: WCA containing sheep's blood (total anaerobes), BHI (total aerobes), modified MMRS containing mupirocin (total bifidobacteria), and LBS (total lactobacilli). All agar plates were incubated anaerobically at 37°C for five days, apart from BHI which was incubated aerobically at 37°C. The plates were then

checked for growth and colonies were counted on all media types which had between 30 and 300 colonies to assess CFU/ml of each sample. Further culturing work was carried out on MMRS and LBS plates to isolate bifidobacteria and lactobacilli, respectively, in order to develop a biobank of potential probiotics for stress. Five to ten single colonies from the MMRS and LBS plates were picked with sterile inoculation loops and inoculated in 10 ml of MRS broth for 24-48 hours. When the cultures had sufficient growth, they were checked under the microscope and any cultures which consisted of cocci morphology were discounted. The cultures which appeared pure and had rod morphology were stocked in glycerol and stored at −80°C for further downstream characterisation tests.

7.3.4 DNA Extractions

DNA was extracted from 250 mg faecal samples using a previously described modified protocol, which combined the repeat bead beating method with the QIAmp Fast DNA Stool Mini Kit (Qiagen, Germany). Briefly, 1 ml of lysis buffer was added to each tube and homogenised for three minutes at maximum speed on a Mini-Beadbeater (BioSpec Products) before being incubated at 70°C for 15 minutes with gentle shaking by hand every five minutes. The samples were then centrifuged at 4°C for five minutes at 15000 rpm and the supernatant was transferred to fresh 2 ml tubes. 300 µl of fresh lysis buffer was added to the lysis tube and the previous steps were repeated before the supernatant was pooled. Precipitation of nucleic acids was achieved by adding 260 µl 10M ammonium acetate (Sigma Aldrich, Germany) to each lysate tube and incubating on ice for 15 minutes. The lysates were mixed and centrifuged before the supernatant was transferred to two separate 1.5 ml tubes. One volume of isopropanol (Sigma Aldrich, Germany) was added to each tube, mixed, and incubated on ice for 30 minutes. Following this incubation step, the samples were centrifuged and the supernatant was removed by aspiration. The nucleic acids pellets were washed with 200 µl 70% ethanol before being allowed to dry. When the ethanol had evaporated completely, the nucleic acid pellets were dissolved in 100 µl of Tris-EDTA buffer (Sigma Aldrich,

Germany) and pooled, taking care to pipette gently to avoid shearing. The samples then underwent a series of purification steps to remove RNA and proteins. 2 µl of DNase-free RNase (Thermo Scientific) was added and incubated at 37°C for 15 minutes. 15 µl of proteinase K and 200 µl of Buffer AL (from the QIAamp Fast DNA Stool Mini Kit) were added to the samples, mixed well, and incubated at 70°C for 10 minutes. Subsequent removal of RNA, protein and purification of the DNA was completed according to the manufacturer's instructions. DNA was quantified using the QubitTM 3.0 Fluorometer (Bio-Sciences Dublin) and the Qubit® dsDNA HS Assay Kit (Bio-Sciences Dublin, Ireland), along with being run on a 1.5% agarose gel to check the DNA quality. Extracted DNA was then stored at -20°C until prepared for 16S rRNA sequencing.

7.3.5 16S rRNA Microbiome Sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was amplified from the DNA extracts and prepared for sequencing using the Illumina 16S Metagenomic Sequencing Library Protocol (http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor. Briefly, the first PCR was performed using forward primer (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3').
Each 50 µl PCR reaction contained 5 ng/µl microbial genomic DNA, 10 µl of each primer (1 µM) and 25 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK or KAPA Biosystems, South Africa). The PCR conditions follow as: initial denaturation at 95°C x 3 min; 30 cycles of 95°C x 30 s, 55°C x 30 s, 72°C x 30 s; and 72°C x 5 min for final extension and held at 4°C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V) after each PCR reaction. Successful PCR products were cleaned using AMPure XP magnetic bead-based

purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5 µl) to index each sample, allowing samples to be pooled for sequencing on a single flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2X Kapa HiFi Hot Start Ready mix, and 10 µl PCR grade water. PCRs were completed as described above, but eight amplification cycles were completed instead of 30. Following this, a second clean-up step with the Agencourt AMPure XP system was carried out. PCR products were quantified, normalised, and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Bio-Sciences Dublin, Ireland). The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. The sample pool was prepared following Illumina guidelines. 2 × 300 (bp) paired-end sequencing was performed on the Illumina MiSeq platform at the Teagasc Sequencing Centre, using standard Illumina sequencing protocols.

7.3.6 Bioinformatic Sequence Analysis

Three hundred base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Alpha and beta diversities were generated in QIIME and calculated based on weighted and unweighted Unifrac distance matrices.

7.3.7 SCFA Analysis

Standard Curve and internal standard: Acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid, and 2-ethyl-butyric acid (internal standard, IS) were purchased from Sigma Aldrich. Acidic Milli-Q water was prepared (50 ml of Milli-Q water with 50 µl hydrochloric acid 25% (Merck, Germany). A 100 mM stock solution of 2-ethylbutyric acid in formic acid was diluted to 10 mM in Milli-Q water and was stored at -20°C. An external standard solution of acetate (20 mM), propionate (20 mM), iso-butyrate (2 mM), butyrate (20 Mm), iso-valerate (2 mM) and valerate (20 mM) in water was generated and stored at -20°C. A seven point standard curve was produced (SCFA 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM & 10 mM; BCFA 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM & 1 mM) in acidic Milli-Q water with 1 mM IS.

Extraction: Faecal water was prepared by homogenising the faecal samples (approximately 200 mg) with 1.5 ml of acidic Milli-Q water in 2 ml screw cap tubes containing one large glass bead (diameter 3.5 mm) using a vortex mixer for 15 minutes before centrifugation at 15,000 rpm for 20 minutes. The centrifugation time was extended from the standard protocol in order to obtain a clear supernatant that would not clog the filters. The supernatants were syringe filtered with 0.22 µM filters (Corning) and duplicate aliquots of 270 µl of filtrate were mixed with 30 µl of 10 mM internal standard. The samples were homogenised briefly before a further centrifugation step of 15,000 rpm for three minutes. The supernatant was transferred into 250 µl (Agilent) inserts placed in amber glass 2 ml GC vials (Agilent) sealed with silicone/PTFE screw caps (Agilent), taking care not to transfer any of the pellet.

GC-FID analysis: Standards and samples were analysed by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system, fitted with a Zebron ZB-FFAP column (30 m L x 0.32 mm ID x 0.25 µm df; Phenomenex) and a flame ionisation detector with a CP-8400 autosampler. Helium was employed as the carrier gas at an initial flow rate of 1.3 ml/min. The initial oven temperature was 50°C, and was maintained for 30 sec, raised to 140°C at 10°C/min and held for 30

sec, before being increased to 240°C at 20°C/min, and held for 5 min (total run time 20 mins). The temperatures of the detector and the injection port were 300°C and 240°C, respectively. A splitless injection of 0.2 µL was carried out for each sample or standard using a 10 µL syringe (Aglient) installed to a CP-8400 autosampler (Varian). Peaks were integrated using Varian Star Chromatography Workstation version 6.0 software. Blank water vials were run between each sample duplicates to check for any potential carryover.

7.3.8 Neurocognitive assessment

The Cambridge Neuropsychological Test Automated Battery (CANTAB) was used to assess cognitive performance (https://www.cambridgecognition.com/cantab/). The test battery was presented on a high-resolution touch screen monitor and a test administrator provided verbal instructions from a standardised script, along with specific verbal prompts and encouragement when needed. To avoid effects of fatigue for tests completed later in the session, the tests were presented in different orders for different participants, using a Latin square design. The full battery lasted approximately 40 minutes and participants were assessed on the following tests from the battery: the motor screening test (MOT), rapid visual information processing (RVP), paired associates learning (PAL), spatial span (SS), the intra-extra dimensional set shift (IED) and the emotion recognition task (ERT). A detailed description of each task can be found in Supplementary Material and Methods.

7.3.9 Self-report questionnaires during study visits

Participants completed self-reported paper-based questionnaires to assess perceived stress (Cohen's Perceived Stress Scale, Cohen, Kamarck and Mermelstein, 1983), and depression and anxiety levels (Beck's Depression Inventory II) (Beck, Steer and Brown, 1996), Hospital Anxiety and Depression Scale, (Bjelland *et al.*, 2002). Dietary intake was quantified using a food frequency questionnaire

(Harrington *et al.*, 2011). Gastrointestinal tolerability was assessed using a GI symptom visual analogue scale (VAS). Changes in stool type were captured by the Bristol Stool chart. The Hospital Anxiety and Depression Scale (HADS) is a 14-item self-report screening tool which is widely in use in the clinic to assess levels of depression and anxiety. Beck Depression Inventory (BDI)-II is a self-report measure consisting of 21 items rated on a 4-point scale from 0 (absence of symptom) to 3 (severe manifestation of symptom) (Beck, Steer and Brown, 1996). Scores range from 0-63. Cut-off scores indicating clinically relevant levels of depression have been determined as 0-13 (minimal); 14-19 (mild); 20-28 (moderate); and 29-63 (severe). The State Trait Anxiety Inventory (STAI) is a self-report measure consisting of two subscales each with 20 items, one measuring trait anxiety and the other measuring state anxiety (Spielberger *et al.*, 1983). Participants rate how they feel either right now (state) or generally (trait), in response to each item on a 4-point scale from 'not at all' to 'very much.' The range of scores for each sub-scale is 20-80 with higher scores indicating greater anxiety. The Perceived Stress Scale (PSS) is a self-reported measure in which participants rate, on a 5 point scale ranging from 0 (never) to 4 (very often), how often they have particular thoughts or feelings described by each of the 10 items (Cohen, Kamarck and Mermelstein, 1983). Scores range from 0-40 with higher scores indicating greater stress over the previous month. The Pittsburgh Sleep Quality Index (PSQI) assesses sleep quality over the prior month (Buysse *et al.*, 1989). The PSQI is a self-reported measure comprised of 19 items which are designed to measure seven key components indicating problematic or non-problematic sleep; sleep latency, sleep duration, sleep efficiency, sleep disturbances, subjective sleep quality, use of sleep medication, and daytime dysfunction due to sleep disturbance. Scores on each component are combined to give a global score with >5 indicating significant disturbance of sleep quality during the prior month.

The Food Frequency Questionnaire (FFQ) used in this study is the semi-quantitative version based on the SLAN study (Harrington *et al.*, 2011). The FFQ provides an assessment of habitual dietary intake comprising the major food categories. Participants rate the frequency of consumption of each food type on a scale from

'never or less than once per month' to 'six + per day'. Primary Appraisal Secondary Appraisal (PASA) is a self-reported measure of stress appraisal based on the transactional model of stress and coping (Gaab *et al.*, 2005; Lazarus and Folkman, 1984). It consists of 16 items which are rated on a scale from 'totally disagree' to 'totally agree' immediately prior to completing the stress procedure. Positive Negative Affect Schedule (PNAS) consists of two mood scales with 10 items in each, one assessing negative affect and one assessing positive affect. Each mood dimension (e.g. alert, proud, strong, upset) is rated on a 5-point Likert scale ranging 1 = very slightly or not at all to 5 = extremely. The PNAS gives a total score for positive affect and negative affect ranging from 10-50 for each dimension (Watson, Clark and Tellegen, 1988). As part of the GI Symptoms Visual Analogue Scales (GI-VAS), participants recorded their GI symptoms using an IBS symptom severity scale consisting of five questions relating to digestive comfort. Finally, participants rated the form of their stool sample using the Bristol Stool Chart which is a useful clinical and research tool also used in the diagnosis of irritable bowel syndrome.

7.3.10 Acute Stress Response

The socially evaluated cold pressor test (SECPT) (Schwabe, Haddad and Schachinger, 2008) which combines a psychological and physiological stressor was utilised to elicit an acute stress response as previously described (Allen *et al.*, 2016). Participants completed pre- and post-test questionnaires. It consists of preparatory and baseline phases, a cold pressor exposure and recovery phase. During the preparatory phase, the procedure was introduced to the participant, and psychophysiological recording equipment was attached. During the baseline phase, galvanic skin response, and saliva were collected as a baseline measure. Galvanic skin response was measured during the stress procedure using the NeXus 4 Software (Biotrace, Mind Media, Netherlands). During the cold pressor phase participants immersed their dominant hand in cold (0-4°C) water for up to five minutes. Participants were informed that they can remove their hands before the requested time is complete if they wish. Participants were filmed during this phase, to create socially evaluative conditions as part of the stressor. A total of eight saliva

samples were taken during the stressor in 10-minute increments, with the first sample taken five minutes before the beginning of the stressor. Finally, during the recovery stage participants provided saliva samples for a further 60 minutes.

7.3.11 Cortisol Awakening Response

Morning saliva samples were collected using the Salivette system (Sarstedt, Germany). Participants were instructed to collect the saliva samples in the morning of the day of their study visit. A total of four samples were collected, with the first collected upon wakening, the second 30 minutes later, and the third and fourth in 15-minute increments. Saliva samples were centrifuged at 1500g for five minutes, aliquoted and stored at -80°C for later analysis.

7.3.12 Cortisol analysis

Salivary cortisol (CAR and SECPT saliva) was analysed using the ENZO Life Sciences enzyme-linked immunosorbent assay (ELISA) kits (Exeter, UK) per manufacturer's instructions. The lower limit of detection was 0.156 nmol/L. Inter- and intra-assay coefficients of variability were 13.4% and 10.5%.

7.3.13 Statistical Analysis

Statistical analysis was performed using the Calypso online software (version 8.68) (Zakrzewski *et al.*, 2016), R studio (Version 1.0.143) and SPSS 25 (IBM, Armonk, NY, USA). All samples sequenced had >40,000 reads. Taxa present at <0.01% were removed and up to 20,000 taxa are included in the analysis, unless otherwise stated. Data were CSS+ log transformed to account for the non-normal distribution of taxonomic count data for alpha and beta diversity testing and repeated measures statistical analysis (Paulson *et al.*, 2013). Multivariate analysis including both RDA and CCA methods were used to investigate the complex associations between microbiota composition and various explanatory variables. Multiple alpha diversity measures were calculated, including phylogenetic diversity and observed

species. Alpha diversity was measured using Shannon diversity (which measures the overall diversity of a community, including the number of taxa/OTUs), Chao1 (which can be described as a quantification measurement with respect to the numbers of species present in a community, omitting the abundance of each species), Simpson (used to determine the species evenness and diversity of the microbial dataset, taking into account the relative abundances present in a community) (Lozupone and Knight, 2008), and Evenness (which measures how evenly abundant the taxa/OTUs are). Beta diversity was measured based on Bray–Curtis distance matrices which were constructed at both the genus level and the OTU level using R Studio (version 1.0.143), and Calypso. Base images were generated using the "ggplot2" package. Each matrix was analysed using permutational multivariate analysis of variance (ADONIS) with 1000 permutations. Hierarchical clustering of the 50 most abundant genera ($p < 0.05$; analysis of variance) were visualised using a heatmap to determine patterns in gut microbiota based on the age of the participant. Repeated measures statistical analysis was performed on participants sampled at ≥2 time points. The 1000 most abundant taxa were included in the analysis. Calculated p values are adjusted for multiple testing using the false discovery rate (FDR) correction. All p values provided are following FDR correction. Significance was accepted as $p < 0.05$ following FDR correction. LEfSe was used to identify predominant taxa between active and placebo groups considering biological consistency and effect relevance.

Differences in outcome measures between pre-and post-intervention were analysed using repeated measured analysis of covariance (ANCOVA). CANTAB outcomes were analysed using baseline scores as co-variates. Where parametric assumptions were violated and data transformation did not achieve normal distribution, the Friedman and Wilcoxon rank sum test were applied. Areas under the curve for cortisol measurements were calculated with respect to ground (AUCg) (Pruessner *et al.*, 2003). Categorical data (i.e. Bristol stool chart) was analysed using the Chi-squared test. Correlation between microbial abundance and other outcome measures were performed using spearman rank correlation. $P<0.05$ was considered significant and $p<0.1$ was considered a trend. Data are expressed as mean ± SEM.

7.4 Results

7.4.1 Participant Characteristics

A total of 18 healthy female volunteers completed the study. The CONSORT diagram is shown in Figure 2 and baseline demographics are outlined in Table 1. Participants were of good health, had a normal BMI (mean = 23.7) and baseline psychological profile, and were free of gastrointestinal symptoms or disorders.

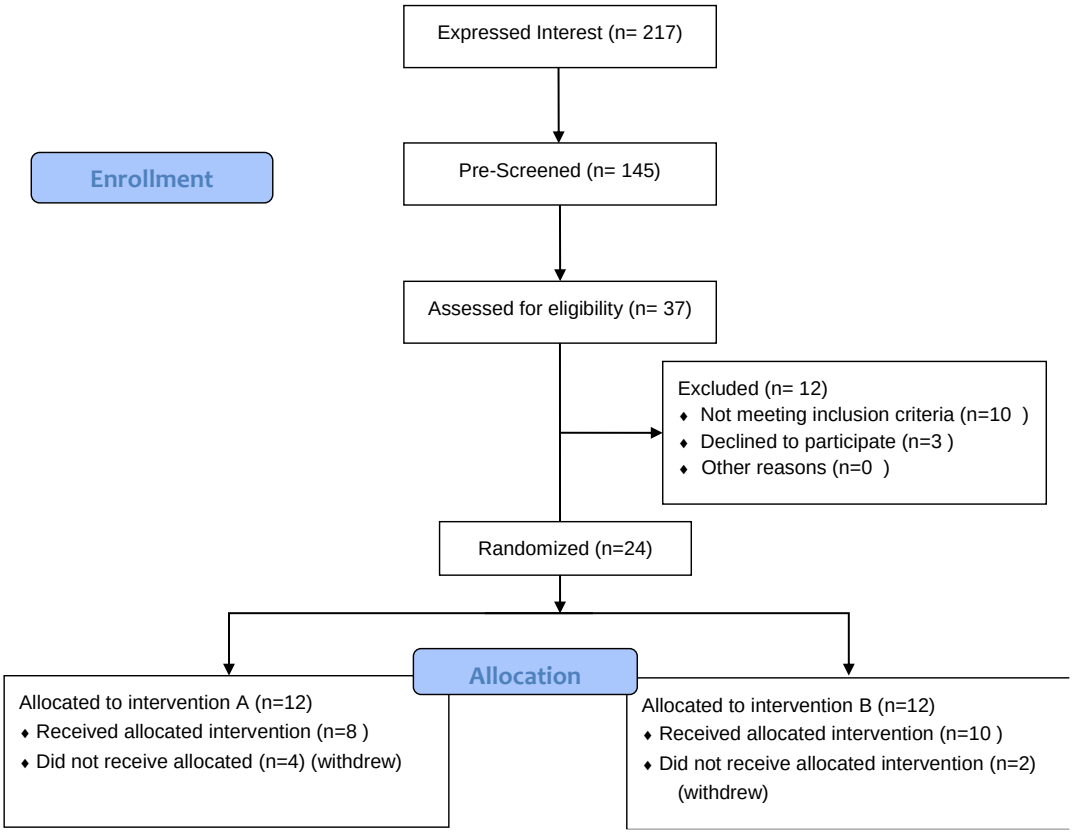


Figure 2: CONSORT diagram outlining screening process, randomisation, enrolment and allocation to groups.

Faecal samples were collected from individuals at visits two (n = 18), three (n = 17), four (n = 17) and five (n = 15). Of the 18 participants, one was identified as an outlier following downstream analysis of 16S data and was excluded due to antibiotic administration. With respect to age, the participants had a mean age of 26 ± 1.3 and 100% of the participants were vaginally delivered. Metadata including

details of delivery mode, age, occupation, years of education, weight, height, and BMI are provided in Table 1.

Table 1: Summary of participant characteristics including age, mode of delivery, race, occupation, education, weight, height, and BMI measurements.

Characteristic	**Mean ± SEM or % (n)**	**Characteristic**	**Mean ± SEM**
Age	26 ±1.3	**IQ**	103 ± 2.1
Mode of Delivery		**AQ**	12.5 ± 1.4
Vaginal	100%		
Race		**STAI (trait)**	34 ± 1.6
Caucasian	94 (17)		
Afro-Caribbean	6 (1)	**IRI**	
		Perspective taking	19.6 ± 1.1
Occupation		Fantasy	22.6 ± 0.7
Student	77 (14)	Empathetic Concern	9.8 ± 1.4
Other	13 (4)		
		Personal Distress	15.9 ± 1.3
Years of Education (years)	18 ± 0.6	**TIPI**	
		Extraversion	4.9 ± 0.3
		Agreeableness	4.9 ± 0.2
Weight (kg)	66.7 ± 3.5	Conscientiousness	6.1 ± 0.2
		Emotional	5.1 ± 0.3

		Stability	
Height (m)	1.67 ± 2.49	Open to experience	5.5 ± 0.2
BMI (kg/m^2)	23.7 ± 1.4		

7.4.2 MiSeq 16S rRNA analysis of faecal microbiota of active and placebo groups pre- and post-supplementation

Following the extraction of DNA from the faecal samples, MiSeq 16S rRNA sequencing was carried out to determine the effects of prebiotic supplementation on faecal microbiota compared to placebo administration. Samples were sequenced using the Illumina MiSeq platform, resulting in >40,000 sequencing reads per sample and revealing approximately 230 bacterial genera of 12 different phyla in active and placebo groups.

At phylum level, active and placebo groups shared common phyla pre- and post-treatment. The most prevalent phyla in both groups were Bacteroidetes (~23%) and Firmicutes (~68%), with Actinobacteria, Verrucomicrobia, Proteobacteria, Cyanobacteria, Synergistetes and Saccharibacteria contributing smaller proportions of the sequencing reads (Figure 3). There were no significant differences in phyla based on treatment or time.

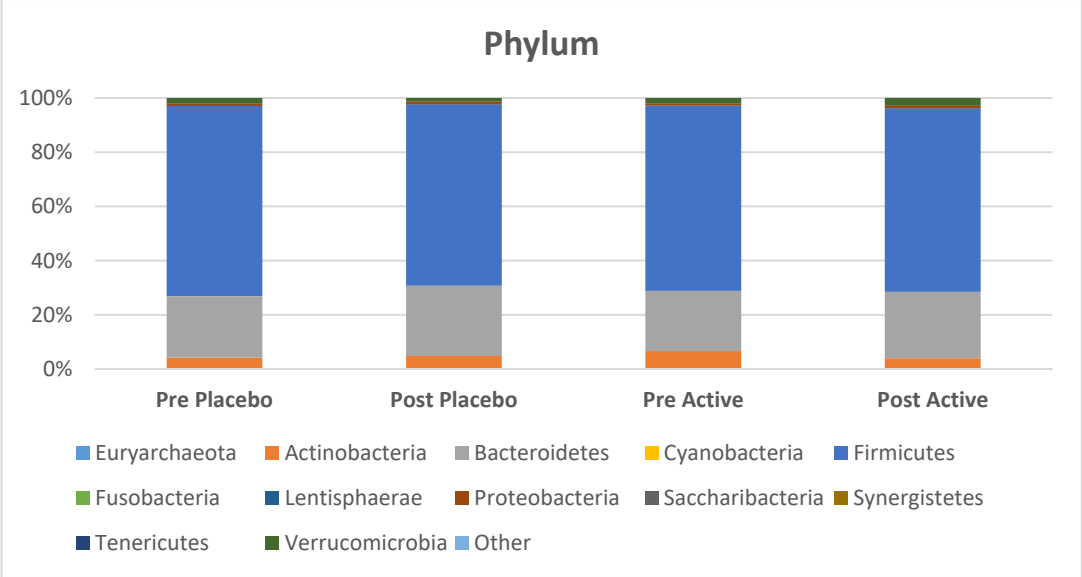


Figure 3: Relative abundances (%) distribution of bacterial phyla in active and placebo groups pre versus post treatment. There were no statistically significant differences at phylum level between treatment groups ($p<0.05$).

At genus level, PDX and placebo groups shared common genera pre- and post-intervention (Figure 4). The most prevalent genera were *Bacteroides* (~18%), *Faecalibacterium* (11%), *Blautia* (~5%), *Pseudobutyrivibrio* (~5%), *Subdoligranulum* (~5%) and *Bifidobacterium* (~5%) with *Alistipes* (~3%), *Lachnoclistridium* (~2%), *Ruminococcus* (~3%) and *Akkermansia* (~2%) contributing smaller proportions of the sequencing reads. Repeated Measures analysis with Bonferroni post-hoc correction revealed significant changes in only one genus after PDX intervention (Table 2). A significant increase in *Ruminiclostridium 5* ($p = 0.00006$, FDR = 0.0068) only after PDX intervention, but not placebo supplementation, was observed (Figure 5 A-C). To determine whether there were bacteria present in the gut microbiota that could discriminate based on PDX versus placebo supplementation, a feature selection statistical analysis (LDA Effect Size (LEfSe)) was used, which determined the genera most likely to explain differences between groups. LEfSe identified R*uminiclostridium* 5 as the discriminating bacteria for the post PDX treatment group (Figure 5 D).

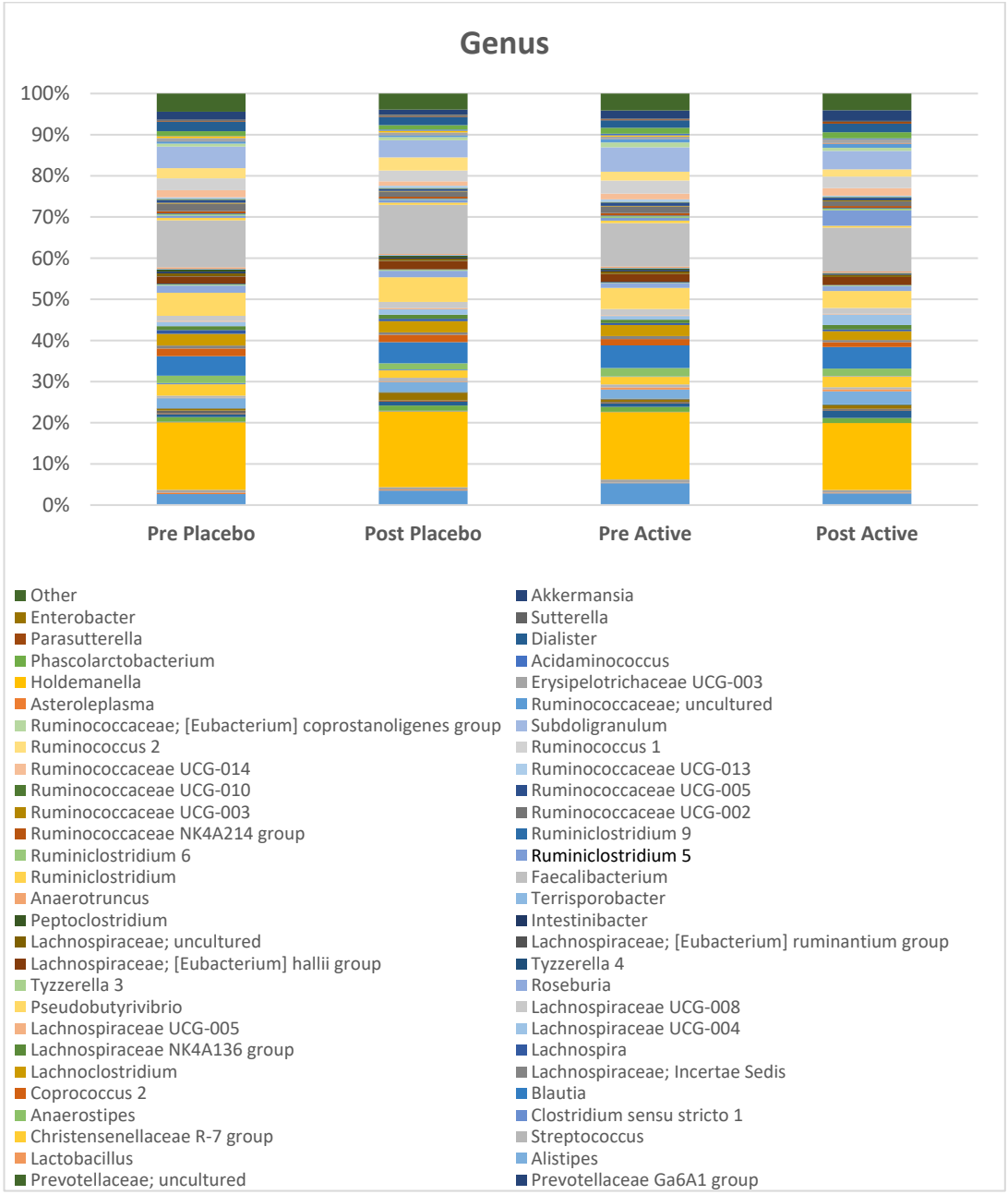


Figure 4: Relative abundances (%) distribution of bacterial genera in PDX and placebo groups pre versus post treatment. Represented only taxa >1% in at least one group. The Other category contains all other genera present at <1% of assignable reads at genus level.

Table 2: Repeated Measures analysis was performed in Calypso on relative abundance data at genus level to determine if statistically significant differences occurred between prebiotic and placebo administration. Statistical significance was accepted as $p<0.05$ and data is adjusted using Bonferroni and False Discovery Rate (FDR).

Taxa	P Value	Bonferroni	FDR
Ruminiclostridium 5	0.00006	0.0068	0.0068
Ruminococcaceae UCG013	0.012	1	0.68
Bifidobacterium	0.02	1	0.69
Lachnospiraceae FCS020 group	0.036	1	0.69
Actinomyces	0.045	1	0.69
Slackia	0.047	1	0.69
Acidaminococcus	0.065	1	0.69
Parabacteroides	0.066	1	0.69
Pseudobutyrivibrio	0.067	1	0.69
Lachnospiraceae UCG004	0.073	1	0.69
Lactobacillus	0.089	1	0.69
Lachnoclostridium	0.091	1	0.69
Lachnospiraceae UCG001	0.1	1	0.69
Shuttleworthia	0.11	1	0.69
Lachnospiraceae UCG008	0.12	1	0.69
Subdoligranulum	0.13	1	0.69
Christensenellaceae R7 group	0.13	1	0.69
Streptococcus	0.14	1	0.69
Oscillospira	0.15	1	0.69
Coriobacteriaceae UCG002	0.15	1	0.69
Candidatus Soleaferrea	0.15	1	0.69
Veillonella	0.16	1	0.69
Erysipelatoclostridium	0.16	1	0.69

Ruminococcus 2	0.17	1	0.69
Lactonifactor	0.17	1	0.69
Family XIII UCG001	0.17	1	0.69
Blautia	0.17	1	0.69
Ruminiclostridium	0.18	1	0.69
Ruminococcaceae UCG002	0.19	1	0.69
Defluviitaleaceae UCG011	0.19	1	0.69
Bacteroides	0.19	1	0.69
Dorea	0.22	1	0.76
Intestinimonas	0.23	1	0.76
Butyricimonas	0.23	1	0.76
Holdemanella	0.25	1	0.77
Turicibacter	0.26	1	0.77
Eubacterium hallii group	0.26	1	0.77
Dialister	0.26	1	0.77
Prevotella 7	0.28	1	0.78
Lachnospiraceae UCG010	0.29	1	0.78
Fusicatenibacter	0.29	1	0.78
Ruminococcaceae UCG014	0.3	1	0.78
Roseburia	0.3	1	0.78
Ruminococcaceae UCG010	0.31	1	0.78
Lachnospira	0.31	1	0.78
Akkermansia	0.33	1	0.8
Barnesiella	0.35	1	0.8
Anaerostipes	0.35	1	0.8
Lachnospiraceae NC2004 group	0.36	1	0.8
Intestinibacter	0.36	1	0.8
Clostridium sensu stricto 1	0.36	1	0.8
Unclassified uncultured bacterium	0.38	1	0.81
Rhodococcus	0.38	1	0.81
Erysipelotrichaceae UCG003	0.4	1	0.84

Ruminiclostridium 6	0.42	1	0.85
Parasutterella	0.42	1	0.85
Incertae Sedis	0.44	1	0.85
Ruminiclostridium 9	0.45	1	0.85
Ruminiclostridium 1	0.45	1	0.85
Methanobrevibacter	0.45	1	0.85
Ruminococcaceae UCG003	0.48	1	0.89
Lactococcus	0.49	1	0.89
Terrisporobacter	0.51	1	0.89
Prevotella 9	0.51	1	0.89
Marvinbryantia	0.51	1	0.89
Enterorhabdus	0.52	1	0.89
Coprobacter	0.54	1	0.89
Coprococcus 2	0.55	1	0.89
Ruminococcaceae UCG004	0.56	1	0.89
Eubacterium ventriosum group	0.56	1	0.89
Tyzzerella 3	0.57	1	0.89
Paraprevotella	0.57	1	0.89
Prevotella 2	0.6	1	0.92
Faecalibacterium	0.61	1	0.92
Coprococcus 1	0.62	1	0.92
Alistipes	0.62	1	0.92
Odoribacter	0.63	1	0.92
Hungatella	0.64	1	0.92
Senegalimassilia	0.65	1	0.92
Sutterella	0.66	1	0.92

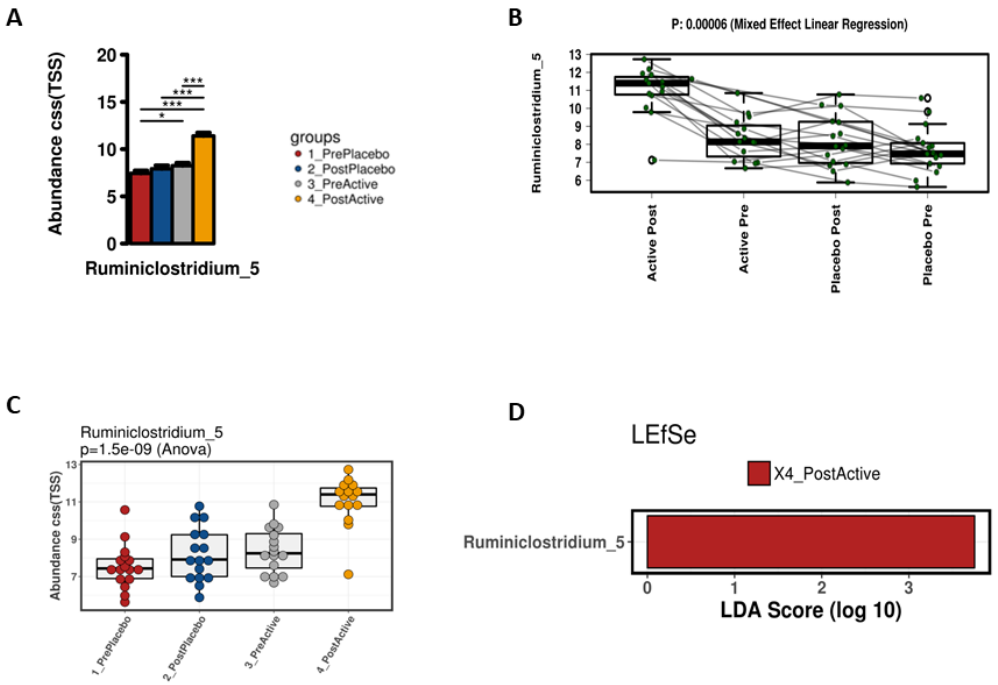


Figure 5: Barchart (A), mixed effects linear regression (B), boxplot (C) and LEfSE plot (D) for *Ruminiclostridium 5* which was found to be significantly more abundant in the post PDX group. LDA Score = 3.75, p = 0.0068 after Bonferroni and FDR adjustment.

Finally, hierarchical clustering of the 50 most abundant genera ($p < 0.05$; analysis of variance) was visualised using a heatmap to determine patterns in gut microbiota based on placebo or prebiotic supplementation (Figure 6). Rows represent explanatory variables, columns represent taxa, red represents a positive correlation and blue represents a negative correlation. Genera highlighted in the second row were present at greater abundances in the PDX group, while genera highlighted in the fifth row were present at greater abundances in the placebo group.

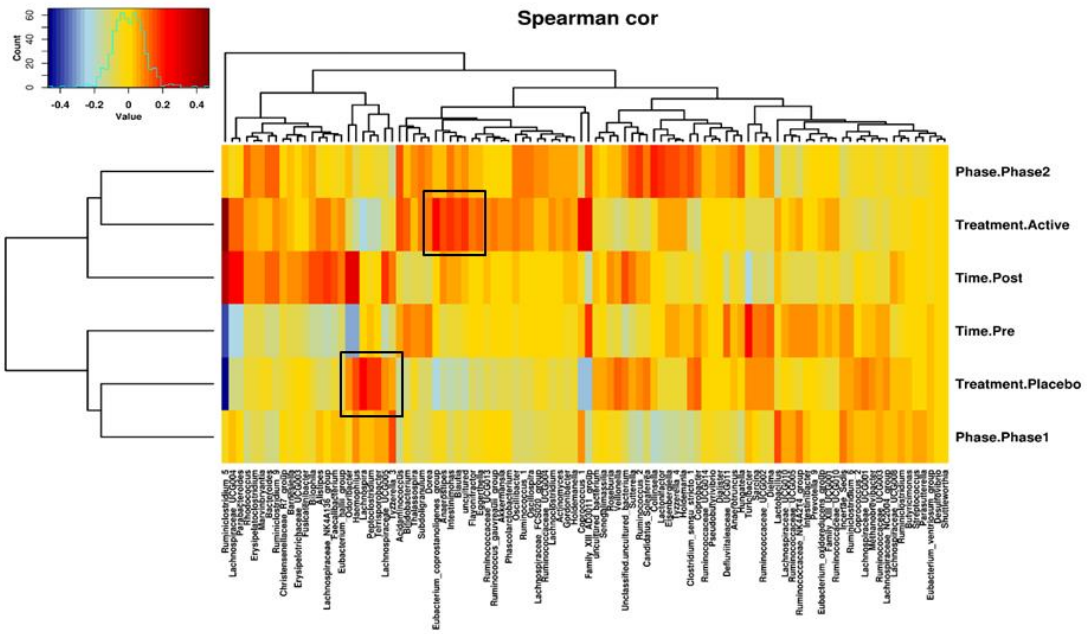


Figure 6: Microbiota associated with prebiotic or placebo administration. Heatmap based on genus relative abundance data. Rows represent explanatory variables, columns represent taxa, red represents a positive correlation and blue represents a negative correlation.

7.4.3 Prebiotic supplementation had no effect on alpha diversity

To determine if alterations in microbial diversity occurred as a result of prebiotic administration compared to placebo, alpha and beta diversity were investigated using Calypso. The Shannon index was employed to measure the overall diversity of the community, including both the number of present taxa and evenness, while Chao1 is a quantification measurement with respect to the number of species present in a community, omitting the abundance of each species (richness). Richness measures the number of present taxa, while Evenness measures how evenly abundant the present taxa are. Microbial α-diversity within each subject quantified before and after each treatment course did not reveal any significant differences between treatments (Chao1 p=0.999; Richness p=0.962; Evenness p=0.527; Simpson p=0.415; Shannon p=0.708; Figure 7).

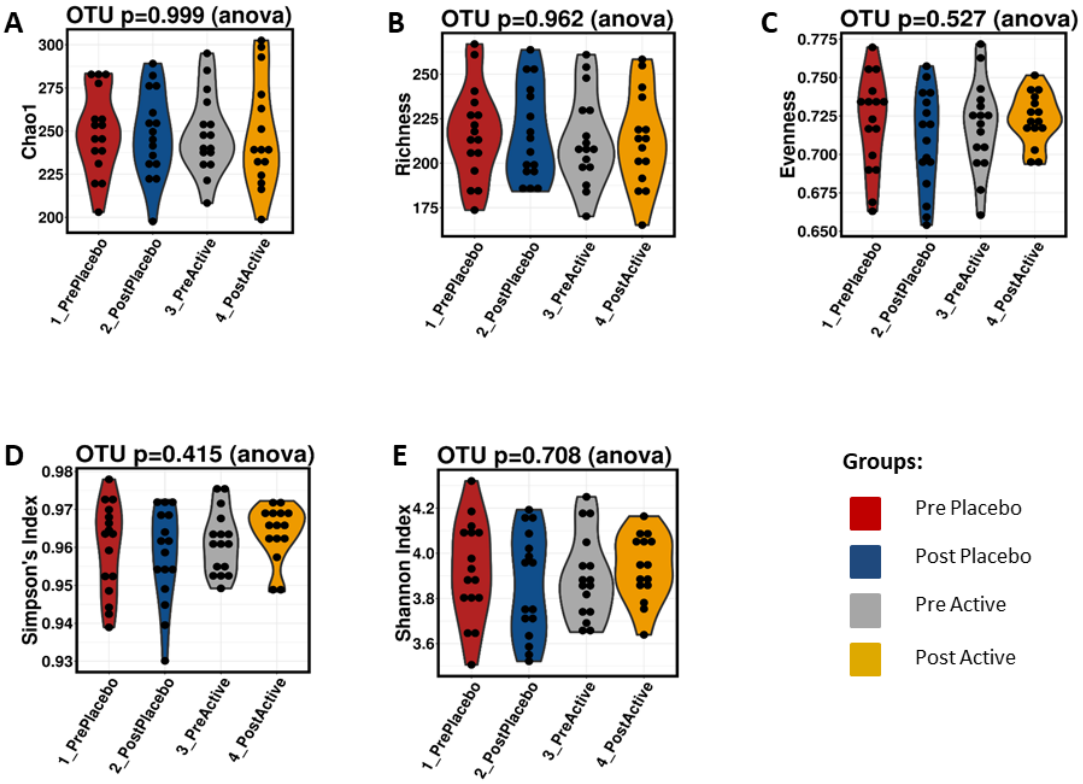


Figure 7: Prebiotic administration had no effect on microbial alpha diversity. Alpha diversity estimates for placebo and prebiotic treatment, before and after intervention, based on Chao1 (A), Richness (B), Evenness (C), Simpson (D), and Shannon (E) indices.

7.4.4 Prebiotic supplementation had no effect on beta diversity

To determine if any significant differences in beta diversity occurred based on prebiotic intervention, PCoA plots were constructed based on Bray Curtis distance matrices. We sought to investigate if samples from the same treatment groups would cluster together, and if there would be separation based on prebiotic and placebo treatment. No statistically significant differences in beta diversity were detected, as measured using Bray Curtis distance matrices, between PDX and placebo groups pre vs. post treatment ($p = 0.781$) and between PDX vs. placebo treatments ($p = 0.403$). All groups displayed a heterogeneous microbiota profile,

and no clustering was observed based on either time or treatment. Furthermore, no significant separation was observed between PDX and placebo groups post intervention (Figure 8). Adonis variance analysis based on Bray-Curtis distance matrices was carried out at genus level to compare PDX versus placebo groups over time. Adonis is a multivariate technique analogous to MANOVA and describes if variation in community composition can be attributed to different experimental treatments or control variables, identifying if the community composition is different between groups. R^2 is the proportion of inter-group variance explained by the microbiota composition. These are measures of the global shift in microbiota composition, a non-significant p value means there is no overall difference but there may still be changes in the Relative Abundance of individual taxa. This was performed using the adonis () function in the 'vegan' package (version 2.3-2) for R (version 3.2.3). However, no significant differences were observed.

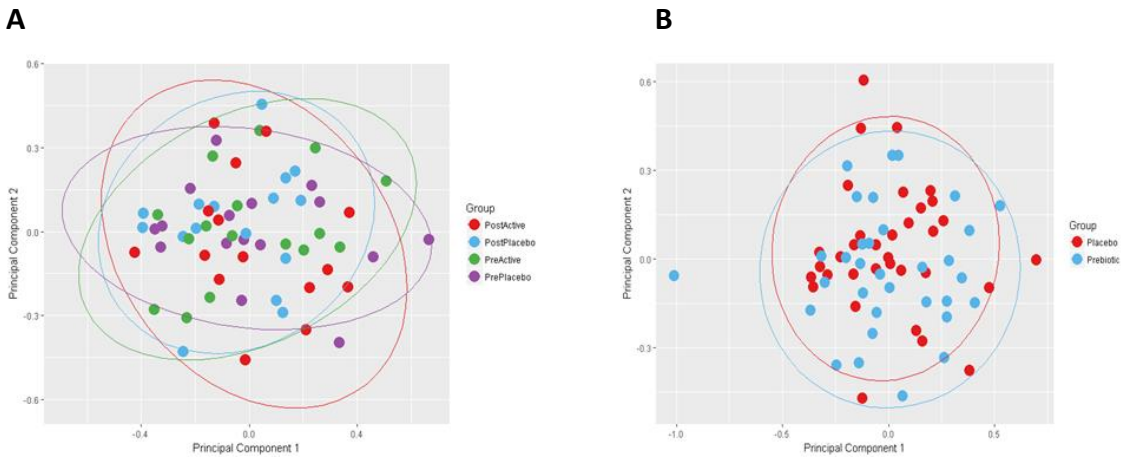


Figure 8: Visualisation of the PCoA analysis based on Bray-Curtis distance matrices at genus level to determine if there is separation of samples based on treatment and time. Purple represents pre placebo, blue represents post placebo, green represents pre active and red represents post active.

To further interrogate the factors associated with the clustering of samples based on diversity, a number of additional multivariate analyses were carried out,

including Canonical Correspondence Analysis (CCA), CCA+, Principal Component Analysis (PCA), PCA+, Redundancy Analysis (RDA) and RDA+ to further investigate the complex associations between community composition and various explanatory variables (Figure 9). PCA analysis allows data visualisation as a 2D plot, identifying sample clusters and determining outliers, which may need to be excluded from downstream analysis, while CCA tests if variations in the data matrix can be explained by selected variables, and RDA indicates how well samples can be separated according to the defined explanatory variables. However, no significant differences were observed in these analytical tests.

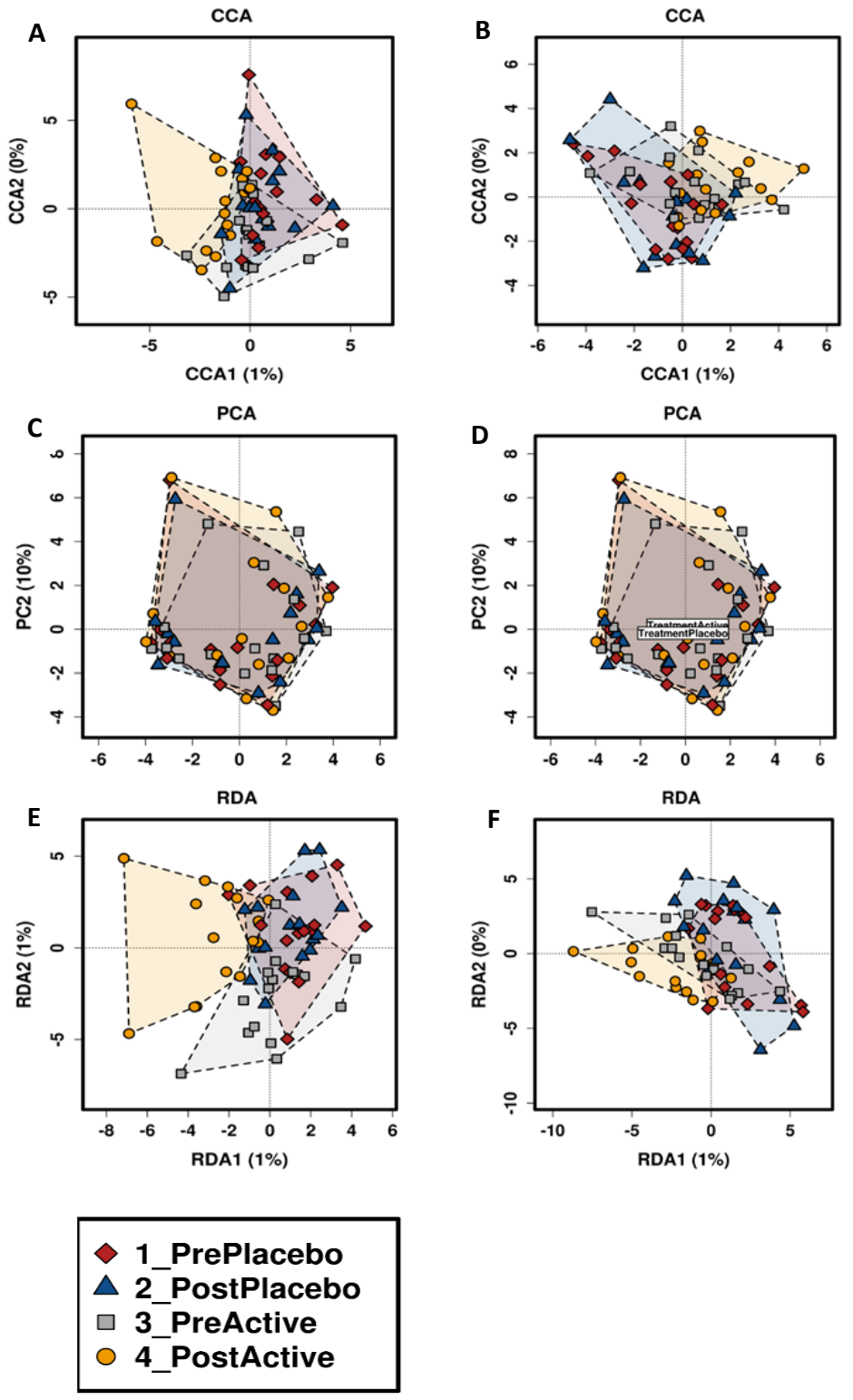


Figure 9: Multivariate Analysis performed in Calypso: (A) Canonical Correspondence Analysis (CCA), (B) CCA+, (C) Principal Component Analysis (PCA), (D) PCA+, (E) Redundancy Analysis (RDA) and (F) RDA+ for all groups and for treatment in order

to further investigate the complex associations between community composition and various explanatory variables. The pre placebo group is shown in red, the post placebo group in blue, the pre PDX group in grey and the post PDX group in yellow.

7.4.5 Plate Count Analysis

Faecal samples were plated on specific media to determine if prebiotic administration altered total anaerobes (WCA), total aerobes (BHI), total lactobacilli (LBS) and total bifidobacteria (MMRS) (Figure 10). There were no statistically significant differences in post-intervention WCA ($F(1, 28) = 1.612$, $p=0.215$), MMRS ($F(1, 27) = 0.04$, $p=0.843$), LBS ($F(1, 28) = 0.959$, $p=0.336$), and BHI ($F(1, 28) = 1.189$, $p=0.285$) plate counts.

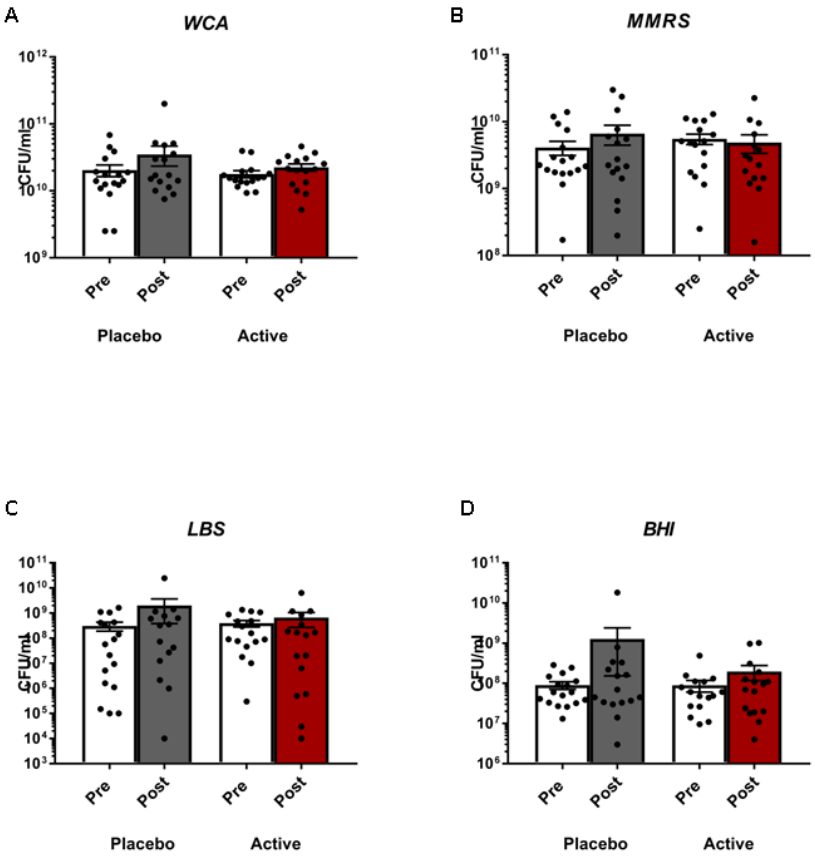


Figure 10: Plate count data for active and placebo cohorts pre versus post treatment. Plate counts for WCA (total aerobes), MMRS (total *Bifidobacterium*), LBS (total *Lactobacillus*), and BHI (total aerobes) are displayed as CFU/ml.

7.4.6 SCFA Analysis

Prebiotic administration had little effect on SCFA concentration (Figure 11) after adjustment for pre-intervention SCFA concentration. Data are adjusted mean ± standard error of the mean (SEM), unless otherwise stated. After adjustment for pre-intervention SCFA concentration there was not a statistically significant difference in post-intervention acetate ($F(1, 26) = 2.314$, $p=0.14$), isobutyrate ($F(1, 26) = 2.593$, $p=0.119$), butyrate ($F(1, 26) = 2.814$, $p=0.105$), isovalerate ($F(1, 26) = 0.573$, $p=0.456$), and total BCFA ($F(1, 26) = 1.397$, $p=0.248$) concentrations between the interventions. There was a trend towards significance for total SCFA ($F(1, 26) = 3.4$, $p=0.073$) and for propionate ($F(1, 26) = 3.839$, $p=0.061$). Valerate concentration was statistically significantly greater in the control group (4.374±0.363 µmol/g wet mass) compared to post-PDX supplementation (2.954±0.35 µmol/g wet mass), a mean difference of 1.42 (95% CI, 0.36 to 2.4790) µmol/g wet mass, $p = 0.011$, $p<0.05$ *.

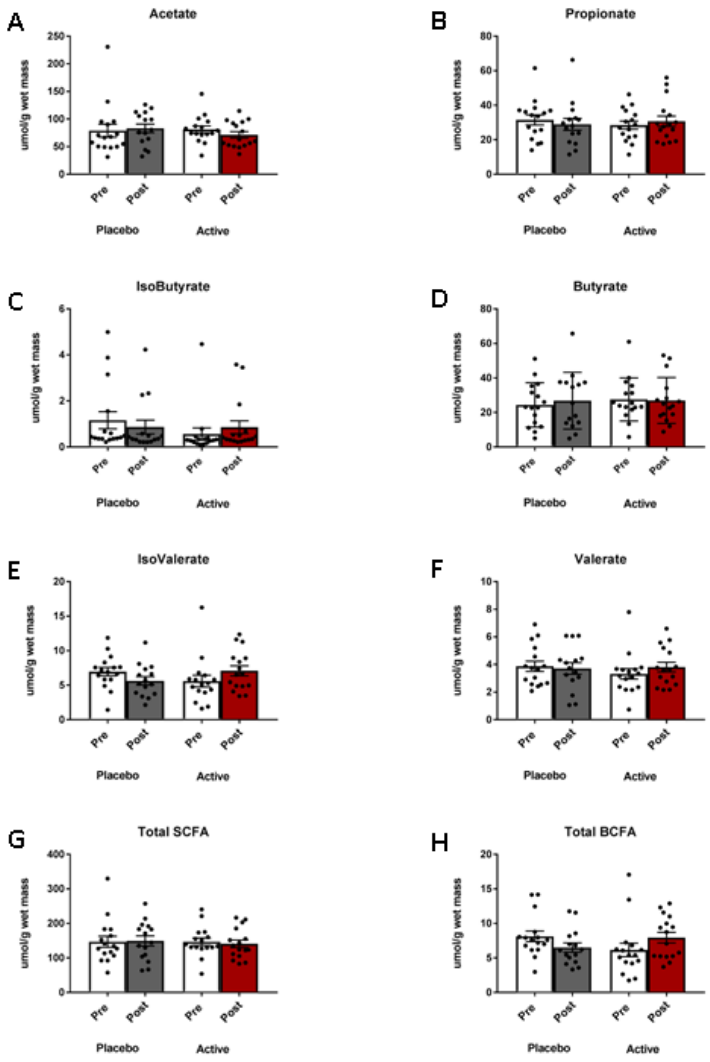


Figure 11: SCFA data for PDX and placebo cohorts pre versus post treatment. SCFA data for acetate (A), propionate (B), isobutyrate (C), butyrate (D), isovalerate (E), valerate (F), total SCFA (G) and total BCFA (H) are displayed as µmol/g wet mass.

7.4.7 Neurocognitive performance

In the CANTAB battery, an improvement in IED (cognitive flexibility) and RVP (sustained attention) after PDX supplementation was observed. There was a significant interaction effect between time (pre- vs. post-intervention) and product (PDX vs. placebo) for completed stage errors ($F(1,16) = 5.8$, $p=0.03$) and completed total trials ($F(1,15) = 9.6$, $p=0.007$) of the IED task (Figure 12 A, B). Post-hoc analysis

revealed that compared with the placebo phase, participants made less errors on the stages successfully completed (completed stage errors, t (1,17) = -3.9, p=0.001) and successfully completed the stages using a lower number of trials (completed stage trials, t (1,17) = -4.9, p<0.001) after the PDX phase. Similarly, improvements in the number of correct responses, total correct rejections, and failure to respond in the RVP task after prebiotic supplementation were recorded (Figure 12 C-E). The number of correct responses (probability of hit, t (1,17) = -3.5, p=0.003) and total correct rejections (t (1,17) = -3.9, p= 0.001) increased after the PDX but not placebo condition. There was also a decrease in failure to respond (total misses (t (1,17) = 3.4, p=0.003) after PDX supplementation. However, neither of these outcome measures were significantly different between treatments.

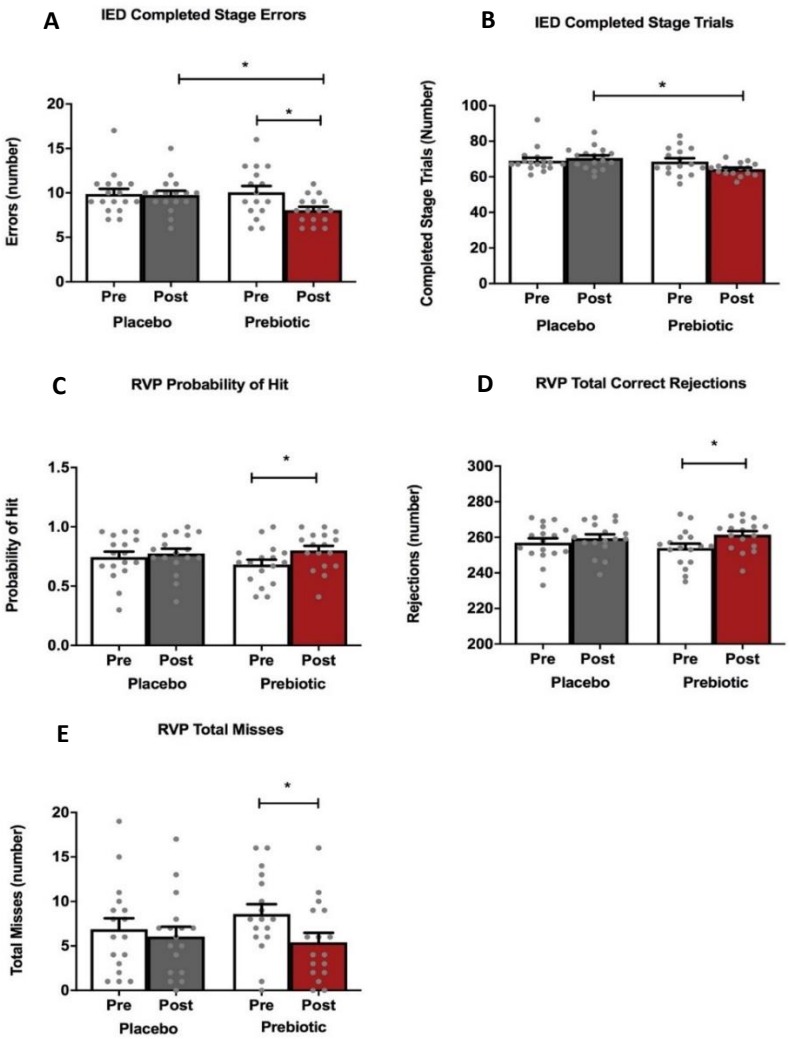


Figure 12: PDX supplementation improved neurocognitive performance in the Intra-Extra Dimensional shift task and Rapid Visual processing task of the CANTAB battery. After PDX supplementation, in the IED task participants made less errors on stages successfully completed (A) and had a lower number of trials completed on all attempted stages (B). In the RVP task, PDX increased the number of correct responses (C), and total correct rejections (D) and lowered the number of misses (E). Individual dots represent a participant; data shown as mean ± SEM; *p<0.05; IED – Intra Extra Dimensional Set Shift; RVP – Rapid Visual Processing.

7.4.8 Stress responses

Neither cortisol awakening response nor salivary cortisol concentrations after the acute stressor were affected by PDX supplementation (Figure 13). Likewise, the galvanic skin response was not affected by the stressor or PDX administration.

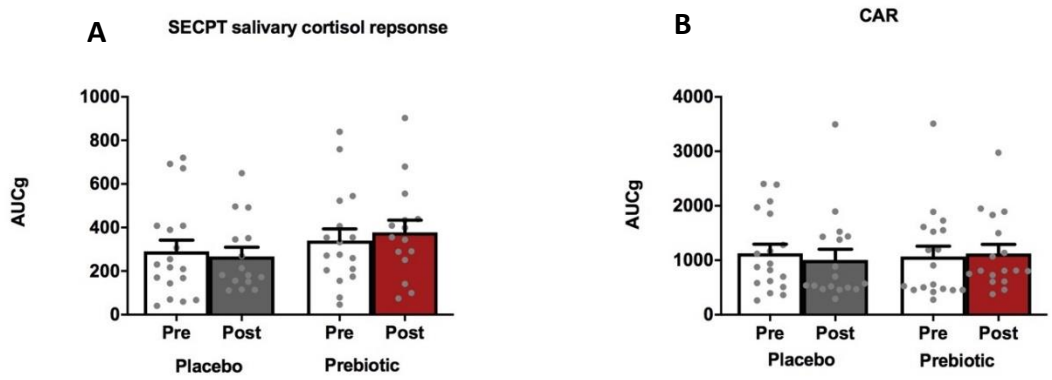


Figure 13: Salivary cortisol responses to acute stressor and morning awakening response did not change after PDX supplementation. Salivary cortisol for each visit as measured by the area under the curve with respect to ground (AUCg). Neither total salivary output in response to the socially evaluated cold pressor test (SECPT, 13A), nor cortisol awakening responses (CAR, 13B) were affected by PDX supplementation.

Perceived psychological (questionnaires) and biological measures (salivary cortisol, galvanic skin response) of the acute stressor response were not affected by the stressor or PDX administration (Figure 14). Questionnaires included stress visual analogue scales (Stress VAS; Figure 14E), state anxiety (Figure 14F), positive affect schedule (PAS score; Figure 14G), negative affect schedule (NAS score; Figure 14H), or primary appraisal secondary appraisal (PASA Global Stress Index; Figure 14I).

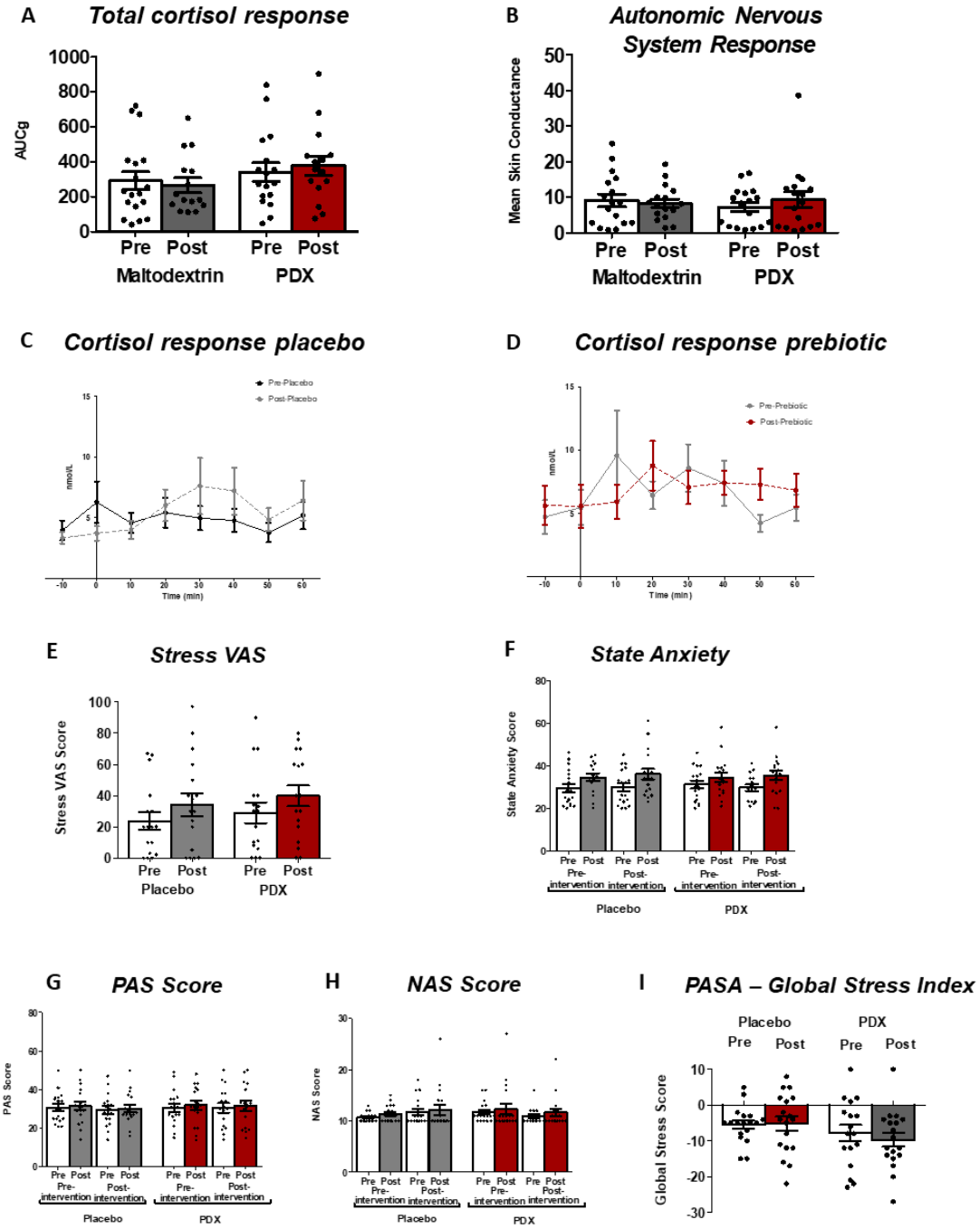


Figure 14: There were no significant differences in response to the cold pressor acute stressor between PDX and placebo groups for total cortisol response (A), autonomic nervous system response (B), cortisol response placebo (C), or prebiotic (D), stress visual analogue scales (E), state anxiety (F), positive affect schedule (G), negative affect schedule (H), or primary appraisal secondary appraisal (I).

7.4.9 Compliance and tolerability

Overall, participants showed good compliance to study product (>70%), however one participant showed lower compliance at 60% during the prebiotic phase. No treatment-related adverse events occurred during the PDX treatment arm. Overall, PDX supplementation was well tolerated as measured by the GI VAS at each visit. Only two participants reported GI symptoms after PDX supplementation as assessed by the GI VAS, of which one reported severe abdominal pain. However, these symptoms were only present one out of every 10 days. Three participants reported development of bloating after the PDX intervention. However, these participants also reported bloating at other time points of the study, suggesting that bloating might be due to other causes. Generally, satisfaction with bowel habits was very high and GI symptoms did not interfere with life satisfaction in this cohort. PDX supplementation did not change GI symptom satisfaction and GI symptom life interference. Stool consistency as measured by the Bristol Stool chart did not change throughout the study period (mean Type 3 ("Like a sausage but with cracks on its surface"); Table 3).

Table 3: Gastrointestinal symptoms

	PLACEBO		PREBIOTIC	
	Pre-Intervention	**Post-Intervention**	**Pre-Intervention**	**Post-Intervention**
Abdominal pain at	5	11	11	17

present time (%)				
Bloating (%)	28	33	22	22
Satisfaction with bowel habits	33.6 (quite happy)	35.1 (quite happy)	29.7 (very to quite happy)	38.6 (quite happy)
Interference of bowel habits with life in general	20.9 (little)	16.1 (very little)	8.5 (very little)	10.1 (very little)
Currently urge to have bowel movement	10.8 (very little)	17.2 (very little)	10.3 (very little)	12.1 (very little)
Bristol Stool Chart	3.3 ± 0.4	3.6 ± 0.4	3.9 ± 0.3	3.9 ± 0.4

GI symptoms measured by GI Visual Analogue Scale (range 0-100); data expressed as mean ± SEM

7.4.10 Mood and Psychological Symptoms

PDX supplementation did not affect levels of anxiety (HADS-A score), depression (HADS-D score, BDI-II score), perceived stress (PSS score) or psychological symptoms and distress (SCL-90 global score) (Table 4, Figure 15).

Table 4: Measures of perceived anxiety, depression, and stress assessed by self-administered questionnaires was not affected by PDX. Data expressed as mean ± SEM. HADS-A – Hospital Anxiety and Depression Scale: A – Anxiety, D –Depression (range 0-21, score below 7 are considered normal); BDI-II – Beck's Depression Inventory (range: 0-63; score below 10 are considered normal mood changes); PSS – Cohen's Perceived Stress Scale (range: 0-40 with scores less than 13 considered low stress levels); SCL-90 – Symptom Checklist 90; GSI – General Severity Index.

	PLACEBO		PREBIOTIC		
Psychological measure	**Pre-intervention**	**Post-intervention**	**Pre-intervention**	**Post-intervention**	**p-value**
HADS-A	3.9±0.8	3.3±1.3	3.9±0.7	3.5±0.8	0.33
HADS-D	1.20.3	1.2±0.4	1.3±0.4	1.3±0.3	0.81
BDI-II	4.1±1.04	2.9±0.8	4.2±1.2	2.8±0.8	0.22
PSS	11.8±1.5	9.8±1.3	11.4±1.6	10.6±1.6	0.21
SCL-90 GSI	0.25±0.06	0.25±0.06	0.26±0.07	0.22±0.05	0.10

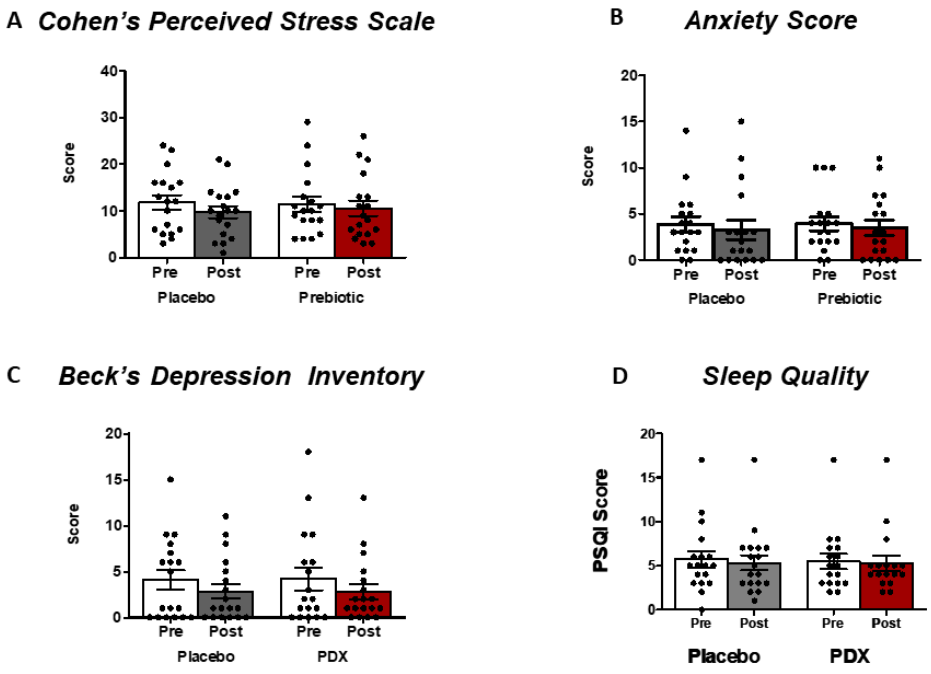


Figure 15: There were no significant differences in self-report stress (A), anxiety (B), depression (C), and sleep quality (D) between prebiotic and placebo administration.

7.4.11 Dietary intake

Intake of total calories, macronutrient, and dietary fibre intake as assessed by the food frequency questionnaires was mostly consistent throughout the study period. There was a slight decrease in vitamin E (Z = -2.4, p = 0.02), polyunsaturated fatty acids (Z = -2.4, p = 0.02), and monounsaturated fatty acids (Z = -1.7, p = 0.09) during the PDX period (Table 5).

Table 5: Nutrient intake assessed by a food frequency questionnaire did not change substantially during the study period. Recommended dietary intake based on Healthy Eating Guidelines Ireland; Data expressed as mean ± SEM.

		PLACEBO		PREBIOTIC		
Nutrient	**Recommended Daily Intake**	**Pre-intervention**	**Post-Intervention**	**Pre-intervention**	**Post-Intervention**	**p-value**
Kilocalories	1,800 – 2,000 (depending on activity level)	2031 ± 153	2109 ± 298	1949 ± 166	1773 ± 202	0.12
Protein (g)	10-35% of total energy	86 ± 8.6	100 ± 19	82 ± 7.5	81 ± 12	0.28
Fat (g)	20-35% of total calories	77 ± 5.1	78 ± 7.5	71 ± 6.9	63 ± 5.9	0.18

Carbohydrate (g)	45-65% of total calories	257 ± 24	260 ± 44	255 ± 25	227± 30	0.23
Monounsaturated fatty acids (g)	>12% of total energy	30 ± 2.4	31 ± 3.1	26 ± 3.0	24 ± 2.4	0.39
Polyunsaturated fatty acids (g)	>6% of total energy	14 ± 1.2	15 ± 2.1	14 ± 1.4	12 ± 1.6	0.05
Saturated fatty acids (g)	<10% of total energy	27 ± 1.8	27 ± 2.1	25 ± 2.5	22 ± 1.8	0.27
Cholesterol (mg)	300 mg	297 ± 42	320 ± 53	271 ± 35	246 ± 30	0.17
Total sugar (g)	<10% of total energy	114 ± 13	121 ± 25	113 ± 12	95 ± 13	0.16
Starch (g)		140 ± 13	137 ± 19	140 ± 160	130 ± 17	0.56
Fibre (g)	>25g	19.8 ± 2.7	22.2 ± 5.5	22.1 ± 3.1	21.3 ± 4.5	0.27
Vitamin A (µg)	800 µg	848 ± 79	1161 ± 214	1033 ± 96	1011 ± 131	0.16
Thiamine (mg)	1.1 mg	1.7 ± 0.2	1.8 ± 0.3	1.8± 0.2	1.8 ± 0.3	0.19
Riboflavin (mg)	1.4 mg	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.1 ± 0.3	0.78

Niacin (mg)	16 mg	27 ± 3.0	29 ± 6.1	26 ± 2.9	26 ± 4.5	0.54
Vitamin B6 (mg)	1.4 mg	2.8 ± 0.3	2.8 ± 0.5	2.8 ± 0.3	2.6 ± 0.4	0.40
Vitamin B12 (µg)	2.5 µg	7.7 ± 1.8	10.1 ± 3.2	5.6 ± 0.6	7.3 ± 1.6	0.43
Folate (µg)	200 µg	323 ± 30	319 ± 52	307 ± 34	312 ± 50	0.79
Vitamin C (mg)	80 mg	81 ± 14	121 ± 42	107 ± 19	106 ± 30	0.13
Vitamin D (µg)	5 µg	4.1 ± 1.4	5.5 ± 2.4	2.3 ± 0.3	3.9 ± 1.3	0.71
Vitamin E (mg)	12 mg	13 ± 1.1	13 ± 1.2	13 ± 1.4	11 ± 1.6	0.07
Phosphorous (mg)	700 mg	1515 ± 116	1666 ± 254	1446 ± 118	1416 ± 171	0.10
Calcium (mg)	1000 mg	930 ± 65	954 ± 91	873 ± 68	823 ± 78	0.42
Iron (mg)	7 mg	13.9 ± 1.3	13.8 ± 2.1	13.4 ± 1.5	13.1 ± 2.0	0.85
Selenium (µg)	55 µg	64 ± 12	79 ± 26	53 ± 5.4	59 ± 13	0.24
Zinc (mg)	10 mg	9.4 ± 0.9	9.9 ± 1.6	9.8 ± 0.9	9.4 ± 1.2	0.16
Sodium (mg)	1600 mg	2790 ± 190	2853 ± 482	2699 ± 236	2455 ± 224	0.45

Potassium (mg)	2000 mg	2593 ± 389	4137 ± 894	3633 ± 326	3496 ± 514	0.09
Magnesium (mg)	375 mg	338 ± 33	371 ± 69	348 ± 31	325 ± 46	0.08
Copper (mg)	1 mg	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.2 ± 0.2	0.09
Chloride (mg)	800 mg	412.4 ± 298	416.6 ± 701	393.4 ± 359	359.6 ± 339	0.52
Manganese (mg)	2 mg	3.1± 0.3	3.5 ± 0.6	3.1 ± 0.3	3.1 ± 0.3	0.19
Iodine (µg)	15 µg	148 ± 17	177 ± 42	141 ± 13	121 ± 14	0.04

7.5 Discussion

Compelling evidence of the impact of the gut microbiome on vital aspects of health such as metabolism, the immune system, behaviour, cognition, and brain function has resulted in considerable interest in investigating methods of modulating gut microbiota composition and metabolic function using a variety of therapeutic tools such as probiotics, prebiotics, and dietary intervention. Therefore, microbiome manipulation using probiotics or prebiotics to strengthen host-microbe symbiosis has a potential role in the future prevention and treatment of immune and psychiatric disorders. However, although prebiotic supplementation benefits for host physiology have previously been illustrated in animals (Savignac *et al.*, 2013; Mika *et al.*, 2014) and humans (Holscher, 2017; Hull *et al.*, 2012; Hibberd *et al.*, 2019; Savignac *et al.*, 2013), evidence for the impact on mental health is limited. Therefore, in this study we aimed to investigate the impact of a prebiotic fibre, PDX, on cognitive function, acute stress response and microbiota composition and

diversity. Overall, PDX had limited effects on the outcome measures assessed in this cohort. Diversity of the microbiota was unchanged after PDX supplementation and minimal alterations in composition were detected, namely an increase in *Ruminiclostridium* 5. However, while acute stress responses were not impacted in this cohort, PDX supplementation modestly improved cognitive flexibility and sustained attention of participants.

Several beneficial physiological effects of PDX have previously been demonstrated in human studies on measures such as constipation (Timm *et al.*, 2013) and satiety. For example, Hull *et al.* (2012) found that consuming PDX increased satiety, and decreased appetite and energy intake in humans compared to controls, suggesting a role for PDX in satiety. Ranawana *et al.* (2013) also demonstrated beneficial effects of PDX for satiety in males as consuming a polydextrose-containing smoothie resulted in a significantly lower energy intake at lunch compared to the control. Furthermore, beneficial behavioural and cognitive effects of PDX have been observed in animal models. PDX when combined with other pre- or probiotics, affected cognitive functions such as exploratory behaviour, recognition memory and neurochemistry (Fleming *et al.*, 2019) and also attenuated the effects of early life stress on anxiety-like behaviour and learning (McVey Neufeld *et al.*, 2019). A recent study by Fleming *et al.* (2019) showed that PDX/GOS supplementation in early life had a demonstrable effect on behaviour and physiology whereby pigs fed a diet containing PDX/GOS exhibited improved performance on the Novel Object Recognition test. Furthermore, a combination of PDX/GOS and *Lactobacillus rhamnosus* attenuated the effects of early-life maternal separation on anxiety-like behaviour and hippocampal-dependent learning in rats accompanied by changes to hippocampal mRNA expression of genes related to stress circuitry, anxiety and learning (McVey Neufeld *et al.*, 2019).

In the present study, a subtle improvement in cognitive flexibility as measured by the IED task and in sustained attention as shown by the increased number of total correct rejections in the RVP task after PDX supplementation was detected. Cognitive flexibility is an adaptive behaviour characterised by the ability to shift attention from a previously learned representation to select behaviourally relevant

stimuli in a particular context (Cools, Clark and Robbins, 2004). The IED task specifically tests behavioural set shifting ability, which measures component executive function processes including working memory, problem solving, reasoning and inhibition to shift attention between different stimulus dimensions. The RVP task, on the other hand, is a test of executive function and assesses rule acquisition and reversal, attentional set formation, maintenance and shifting. It requires both selective and sustained attention as well as working memory to be successfully completed. Sustained attention is the ability to maintain attention through a set of stimuli over a set period of time and is predominantly regulated by the right prefrontal and superior parietal cortex (Pardo, Fox and Raichle, 1991). Observations from previous studies demonstrating improved cognitive flexibility after prebiotic use aligns with data from the present study which found an enhanced ability of participants to transition their thinking from one concept to another and sustaining attention following PDX supplementation. For example, in the same attentional set-shifting task adapted for rodents as the one utilised in this study, rats consuming B-GOS displayed greater cognitive flexibility by shifting from an intra-dimensional set to an extra-dimensional set in fewer trials than controls (Gronier *et al.*, 2018). Similarly, in healthy volunteers B-GOS supplementation lowered the salivary cortisol awakening response and decreased attentional vigilance to negative versus positive information in a dot-probe task compared to placebo intake (Schmidt *et al.*, 2015). Taken together, results from the cognitive assessment in this study provide evidence for a psychobiotic and cognitive-enhancing effect of PDX supplementation.

In contrast to previous studies which demonstrated that PDX increases microbial diversity and supports the growth of beneficial bacteria such as *Faecalibacterium, Akkermansia* and *Dialister* (Roytio and Ouwehand, 2014), no statistically significant changes in microbiota diversity and only minimal changes in composition were observed in this cohort. Previously, high-throughput pyrosequencing analysis revealed that PDX consumption resulted in significant shifts in the gut microbiota composition of healthy adult males (Hooda *et al.*, 2012). Several microbes in the Firmicutes phylum were altered; the abundance of *Faecalibacterium,*

Clostridiaceae, Akkermansia and *Dialister* genera was increased and the abundance of *Ruminococcus, Eubacterium* and *Coprococcus* genera were reduced after PDX consumption. Furthermore, the abundance of *Faecalibacterium prausnitzii*, known for its ant-inflammatory properties, and *Clostridium leptum* was greater after PDX supplementation, but the levels of *Bifidobacterium, Eubacterium rectale* and *Ruminococcus* species decreased. However, it should be noted that the effect of PDX intervention on microbial composition varies greatly from study to study (Roytio and Ouwehand, 2014), mainly due to different dosages of PDX (8 g (Costabile *et al.*, 2012) vs. 21 g (Holscher *et al.*, 2015)), differences in microbiota analysis (next generation sequencing (Holscher *et al.*, 2015) vs. denaturing gradient gel electrophoresis (Costabile *et al.*, 2012), classical plating method, quantitative PCR, fluorescence in situ hybridisation (Roytio and Ouwehand, 2014)) and differences in characteristics of subject populations (healthy vs. obese, age, gender etc.). Furthermore, PDX has been shown to only impact outcome measures of weight management, gut barrier function and inflammation in combination with other prebiotic supplementations (Stenman *et al.*, 2016), or elicited microbial changes in obese populations, in which an aberration in microbiota composition is commonly observed (Hibberd *et al.*, 2019). It is also increasingly understood that the magnitude of response to prebiotic supplementation depends on factors such as baseline microbiota composition and dietary habits. Therefore, baseline microbial diversity, richness and prior stability could be predictors of the responsiveness of a participant to a prebiotic intervention (Tap *et al.*, 2015; Salonen *et al.*, 2014). Indeed, the amount of PDX excreted in faeces has been shown to vary substantially between subjects (Costabile *et al.*, 2012), implying that different gut microbiota exhibit different abilities to degrade PDX. The lack of vast microbial changes observed in the present study could therefore be explained by the relatively low dose of the prebiotic, an unresponsive baseline microbial community, or the healthy status of the population.

Interestingly, we found a significant increase in *Ruminiclostridium* 5 abundance after PDX supplementation. Although little is known about the function of *Ruminiclostridium* 5 in humans, decreased levels were found in patients with kidney

stones (Tang *et al.*, 2018) and in a rat model of acute necrotising pancreatitis (Chen *et al.*, 2017), potentially indicating an important role for this bacterium in inflammatory diseases. Additional studies are needed to decipher the potential function of *Ruminiclostridium* 5 in human physiology. It is worth mentioning that dietary intake over the study period did not significantly change, so that the increase in *Ruminiclostridium* 5 could mainly be attributed to PDX. Similarly, minimal changes in SCFA concentration were observed in this study, which could be explained by the fact that, in line with gas production as mentioned previously, the rate of SCFA production from PDX is also more moderate than from short chain oligosaccharides, such as fructo- and galacto-oligosaccharides (Hernot *et al.*, 2009; Mäkeläinen *et al.*, 2010). Furthermore, fermentation products are rapidly absorbed and/or utilised by colonocytes or other cells in the body, thus the concentrations measured in faecal samples do not describe the total production rate in human and animal trials. Fava *et al.* (2007) demonstrated that fermentation of PDX reduced the concentrations of all SCFA in the colon of pigs which was accompanied by increased levels of acetate and lactate in blood samples, suggesting an improved absorption rate from the gastrointestinal tract rather than reduced production by microbes. Similar results have been reported in human dietary interventions whereby PDX had a neutral or decreasing effect on the faecal SCFA concentrations (Boler *et al.*, 2011; Costabile *et al.*, 2012; Hengst *et al.*, 2009). Therefore, serum levels of SCFA and other fermentation metabolites could be similarly increased in humans so it would be useful to measure blood SCFA levels in future studies.

There are several limitations to this study such as the relatively small sample size. However, this is partly mitigated by the cross-over study design employed here which reduces confounding variables and provides statistical control for between-participant variables, thus allowing for a lower sample size to achieve similar power as non-crossover design studies. Secondly, while a four-week washout period was utilised in this study, the potential of carryover effects cannot be ruled out. The lack of an impact of PDX on measures of psychopathology, anxiety, or stress could be partially explained by the healthy cohort of this study, who experienced low baseline depression, perceived stress, and anxiety levels which may make it difficult

to observe significant changes. Additionally, the limitations of 16S rRNA sequencing could have contributed to the limited amount of changes observed in the microbiota composition, as although these compositional analyses provide a large volume of data, they fail to generate information regarding the viability and functionality of the microbiota present (Patterson *et al.*, 2014). More detailed taxonomic classification using shotgun metagenomic sequencing, which targets small fragments of metagenomic DNA to characterise the full genetic content and functional potential of the microbial community (Qin *et al.*, 2010), might be required to fully understand the impact of PDX on the microbiota composition. Nevertheless, the subtle changes in the microbiome composition observed here are in line with previous observations (Roytio and Ouwehand, 2014). Our knowledge is also limited by the difficulty of obtaining samples from different regions of the gastrointestinal tract in human intervention studies, which could explain the limited changes in SCFA concentrations detected in the faecal samples from this study. Additionally, most clinical studies have been performed in non-psychiatric patients; therefore, it will be important in the future to translate findings to large scale longitudinal studies in well-phenotyped psychiatric populations. Interventional studies in psychiatric patients with prebiotics, and monitoring changes in brain response after these interventions could provide additional information on mechanisms of action. Proving a causal link between gut microbiota modulation and the beneficial physiological effect remains a considerable challenge in microbiome research, therefore research needs to progress beyond characterising microbiota composition and investigate potential mechanisms by which altered microbiota composition affects host health, and by which potential prebiotic interventions exert their beneficial effects, potentially by metabolite production (Kelly *et al.*, 2015). Nevertheless, patents and patent applications relating to gut microbiome research have increased substantially in recent years (Walker and Lawley, 2013), and progress in our understanding of host-microbial interactions is facilitating the translation of basic knowledge into novel therapies and functional foods (Bindels *et al.*, 2015).

7.6 Conclusion

Despite these limitations, to the best of our knowledge the data generated from this study is the first human study investigating the impact of PDX on brain health and suggests that PDX could represent a potential psychobiotic candidate to support cognitive function, thereby providing preliminary evidence to inform future clinical trials. Previously, potential mechanisms by which PDX can exert beneficial effects on the host have been suggested to include microbial metabolites affecting hormonal concentrations in the blood (Tolhurst *et al.*, 2012). Although the data provided from this study are insufficient to draw conclusions on underlying mechanisms, it could be hypothesised that the prebiotic PDX improved cognitive function through reducing inflammatory status, potentially mediated by *Ruminiclostridium* 5 abundance. Further studies are warranted to fully understand the potential benefits of PDX on cognitive function and unravel underlying mechanisms. Bindels *et al.* (2005) proposed that prebiotic research will require insight from both human trials to establish correlations, and from mechanistic studies in animal trials to prove causation. The use of nondigestible compounds that are metabolised by microorganisms in the gut is therefore an exciting prospect for modulating the gut microbiota.

Author contributions

N. Wiley performed DNA extractions, library preparation, microbiota sequencing, SCFA analysis, plate count analysis, interpreted the data and contributed to the writing of the chapter. K. Berding contributed to the data analysis, interpreted the stress and cognition data, and contributed to the writing of the stress and cognition analysis. C.M. Long-Smith designed the intervention study, performed the participants visits and interpreted the stress and cognition data. C.R. Strain contributed to biological analyses, C. Cabria contributed to the analysis of the psychological questionnaires and CANTAB, F. Fouhy performed the bioinformatics for the microbiota data. C. Stanton contributed to study design and data

interpretation, J.F. Cryan and T.G. Dinan contributed to the design of the study, interpreted the data, and contributed to writing of the manuscript.

Conflict of Interest

APC Microbiome Ireland has conducted studies in collaboration with several companies, including GSK, Pfizer, Cremo, Wyeth, Mead Johnson, Nutricia, 4D Pharma, and DuPont. T. G. D. has been an invited speaker at meetings organised by Servier, Lundbeck, Janssen, and AstraZeneca and has received research funding from Mead Johnson, Cremo, Wellness, Nutricia, and 4D Pharma. J. F. C. has been an invited speaker at meetings organised by Mead Johnson, Yakult, and Alkermes, and has received research funding from Mead Johnson, Cremo, Nutricia, and DuPont. C.S. has been an invited speaker at meetings organised by Nutricia and received research funding from Mead Johnson, Cremo, Nutricia, and DuPont. All other authors report no conflict of interest.

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7.8 Supplementary Materials and Methods

7.8.1 Inclusion and exclusion criteria

To be considered eligible for enrolment into the study, subjects must be able to give written informed consent; be female; be between 18 and 40 years of age; and be in generally good health as determined by the investigator. Subjects were excluded from the study if they fell outside the required age range; had a significant acute or chronic coexisting illness; had a condition or taking a medication that the investigator believes would interfere with the objectives of the study, pose a safety risk or confound the interpretation of the study results; current prebiotic or probiotic use; females who were peri-menopausal, menopausal or post-menopausal; females who were pregnant or planning a pregnancy, or lactating; participants who were not fluent in English; were colour blind; had dyslexia or dyscalculia; were vegan; were a current or past habitual daily smoker; individuals who, in the opinion of the investigator, were considered to be poor attendees or unlikely for any reason to be able to comply with the trial; subjects receiving treatment involving experimental drugs; or had a malignant disease or any concomitant end-stage organ disease.

7.8.2 Summary of Visits

Visit 1 Screening Visit (Day -14)

The aims of the study and the procedures to be undertaken were explained to all potential subjects, who then signed the informed consent form. Once the participant was deemed eligible, after reviewing the inclusion and exclusion criteria, the subject was offered participation in the study. Demographic data was collected including age, sex, race, occupation, and social status. The participant was asked general questions concerning their health, including medical history, family medical history and current medical status and medication use. Details of current and/or past medications were also collected. Participants were asked to confirm method of

delivery at birth (natural or C-section), as delivery by Caesarean section can affect the microbiota. Gastrointestinal symptoms were also assessed. Participants were then asked to complete a structured clinical interview (MINI) regarding their psychiatric history (including substance use), and they completed questionnaires regarding early life traumatic experiences, personality, and sociability. Participants completed a brief verbal IQ test and a short practice of cognitive tests using touch screen computer apparatus (CANTAB, Cambridge Cognition). At the end of screening visit participants were given packs and instructions to collect saliva and stool samples to bring to the next visit.

Visit 2 Pre-Intervention Phase 1 (Day 0)

Participants were asked to refrain from strenuous exercise and alcohol for 24 hours prior to the experimental session and from caffeine for three hours prior to testing. Upon arrival participants underwent a pregnancy test, and if this was positive, they were withdrawn from the study into the care of their GP. Stool and saliva samples produced that morning were also collected. The Bristol stool chart was used to check for healthy bowel habit. Venous blood samples (total 20 ml) were drawn for later analysis. Participants were asked to complete questionnaires regarding current levels of mood, anxiety and depression, recent stressful life events, sleeping habits, physical activity, and diet. They completed a battery of CANTAB cognitive tests using touch screen computer apparatus and a short pen and paper assessment of social-cognitive function. They were then asked to take part in a stress procedure (SECPT) and were only informed of what was required at the beginning of the procedure and the reason for the stress procedure at the end of the study. This was to ensure that they were not able to prepare for the procedure which may have affected the results. During the SECPT period subjective stress and galvanic skin response were analysed. Saliva samples were taken immediately post-stressor as well as 10, 20 30, 45, and 60 minutes post-stress. The participants were given packs and instructions to collect saliva and stool samples which they would bring to the next visit. Sufficient prebiotic or placebo sachets were supplied for Intervention Phase 1, participants were instructed to take the treatment once a day (in the morning) (12.5 g polydextrose (Litesse®Ultra)) every day for four weeks. All

participants were also instructed not to make any significant changes to their diet during the supplementation phase (e.g. starting a new diet regime, taking/ceasing to take a supplement, giving up a frequently eaten food etc.), or take any probiotic or prebiotic products beside the current dietary supplementation. Participants were given mood, anxiety, stress, and symptom severity questionnaires to fill in each week of the intervention phase.

Visit 3 Post-Intervention Phase 1 (Day 28)

This visit consisted of a similar format to Visit 2. Additionally, participants fasted overnight. Participants were given packs and instructions to collect saliva and stool samples which they would bring to the next visit. They then commenced a four-week wash-out period.

Visit 4 Pre-Intervention Phase 2 (Day 56)

This visit took the same format as Visit 2. Additionally, diet over the supplementation phase was assessed to ensure no major changes to diet had been made aside from the supplementation. They received packs and instructions to collect saliva and stool samples which they would be required to bring to the next visit. They were then given the supplement along with full instructions on how to take the supplement and commenced supplementation the day after this visit.

Visit 5 Post-Intervention Phase 2 (Day 98)

This visit took the same format as Visit 3. Participants were fully de-briefed as regards the SECPT procedure. Participants were given questionnaires to complete four weeks later for follow-up. This marked the end of the intervention phases.

7.8.3 List of Specific Procedures

- Psychiatric History
  MINI International Psychiatric Interview

- Self-Report Scales

  Childhood Trauma Questionnaire (CTQ-SF)

  Recent Life Events (RLE)

  Ten Item Personality Index (TIPI)

  Creative Achievement Questionnaire (CAQ)

  ROME IV

  Cambridge Behaviour Scale (CBS)

  Interpersonal Reactivity Index (IRI)

  Autism Quotient (AQ)

  Handedness questionnaire

  Ways of Coping (WAYS)

  Hopkins Symptom Checklist (HSCL-90)

  Hospital Anxiety and Depression Scale (HADS) Beck Depression Inventory (BDI)-II

  State-Trait Anxiety Inventory (STAI)

  Perceived Stress Scale (PSS)

  Pittsburgh Sleep Quality Index (PSQI)

  International Physical Activity Questionnaire (IPAQ) Food Frequency Questionnaire (FFQ)

  Primary Appraisal Secondary Appraisal (PASA) Positive and Negative Affect Schedule (PANAS) Bond-Lader Visual Analogue Scales (BL-VAS)

  GI symptoms Visual Analogue Scale (GI-VAS) Bristol Stool Chart

  Palatability Visual Analogue Scale

- Cognitive Performance

  Cambridge Neuropsychological Test Automated Battery (CANTAB) Cognitive assessment

  MOT, IED, SS, ERT, PAL, RVP Reading the Eyes in the Mind

  Confidential Verbal IQ Assessment (National Adult Reading Test-2)

- Experimental Procedures:

  Acute psychological stress (Socially evaluated cold pressor test (SECPT))

  DNA extractions

  16s sequencing

  Culturing and plate counts

  SCFA analysis

7.8.4 Neurocognitive assessment

The MOT allows participants to familiarise with using the touchscreen as well as screening for difficulty with movement, comprehension, and vision. Participants were presented with a series of crosses in different locations on the screen and instructed to touch them in turn. The outcome measures assessed included mean speed of response and mean error (distance between centre of cross and point where participant touches screen).

The RVP test assesses sustained attention. Participants were presented with digits appearing on screen one at a time and were required to press the button on the press pad as soon as they detected target sequences of digits (e.g. 3-5-7). Outcome measures assessed included targets correctly detected, false alarms, reaction time (these were assessed for the test overall but also per block, as performance typically declines as the test continues), A' (a measure of sensitivity to the target), B'' (a measure of the strength of stimuli needed to detect the target).

The PAL assesses conditional learning of pattern-location associations and gives an indication of list memory and list learning. White boxes are presented on-screen and each opens in a randomised order. In one (or more) a pattern is shown, and participants must remember patterns associated with different locations. In the test phase, one pattern is presented three times and after each presentation the participant must select the box it appeared in. The number of patterns participants must remember is then increased to two for two trials, three for two trials, six for one trial and eight for one trial. If the participant makes an error the patterns are

presented again to remind the participant of their locations. When all pattern locations are correctly identified the test may proceed to the next stage. If participants do not correctly identify the pattern locations within 10 trials the test terminates. Outcome measures assessed are PAL total errors for each stage (1, 2, 3, 6 or 8 shapes), mean trials required to locate all patterns correctly, first trial memory score and stages completed.

The Spatial Span task assesses working memory for visual stimuli. A pattern of white boxes is shown on the screen, which change colour one by one in a variable sequence. The participant is required to press the boxes in either the same or the reverse order to which they changed colour. Outcome measures assessed include span length (longest sequence successfully recalled), total errors, total usage errors (selecting a box which was not in the sequence), number of attempts, mean time to first response, and mean time to last response.

The IED is a test of executive function and assesses rule acquisition and reversal, attentional set formation, maintenance and shifting. Participants must use feedback to work out a rule that determines which stimulus is correct. The stimulus and/or rule changes after six correct responses. The first stages involve a single stimulus dimension (e.g. shape) followed by two stages in which the previous correct stimulus dimension must be selected. The next two stages introduce critical shifts in which the participant has to switch attention to new stimuli from the same stimulus dimension (e.g. shape, intra-dimensional shift) as well as new stimuli from a different stimulus dimension (e.g. lines, extra-dimensional shift). The outcome measures assessed were reversal learning (errors made on stage 2, 5, 7 and 9) attentional flexibility (errors made on stage 6 and 8) and total errors (adjusted).

Finally, the emotion recognition task (ERT) assesses visual appraisal of emotion in the facial expressions of others. The participant was briefly shown faces displaying distinct emotions, for example happiness or anger. The participant was then required to identify the emotion as quickly as possible from a list of possible options. The outcomes measured included total correct, total errors, and mean speed of response.

8. General Discussion

8.1 Overview and summary

The brain-gut-microbiota axis comprises an extensive communication network between the brain, the gut, and the microbiota residing there. Microbial metabolic and neuroactive metabolite production represents one of the main mechanisms through which the gut microbiota can influence this axis and consequently impact the health status of the host. Therefore, Chapter 3 aimed to identify novel probiotics by screening gut-derived lactobacilli for bioactive metabolite production, namely GABA and serotonin, identifying one *Lactobacillus plantarum* GABA producer and two *Lactobacillus paracasei* serotonin producers which were subsequently characterised and assessed for probiotic characteristics. This chapter also compared and contrasted two individual screening methods for serotonin production, namely ELISA and HPLC analysis, and whole genome sequencing was utilised to further assess serotonin production capability, identify activity of other tryptophan metabolism pathways such as the kynurenine pathway, as well as probiotic characteristics including antimicrobial production, acid tolerance and adhesion ability.

The identified GABA producer was not as efficient as the positive control *Lactobacillus brevis* DPC6108, however future studies could aim to optimise GABA synthesis by altering various fermentation parameters including initial glutamic acid level, culture temperature, initial pH, and incubation time (Wu *et al.*, 2018). Potential applications for such a probiotic strain include the treatment of metabolic syndrome as well as psychological disorders such as anxiety and depression as previous studies have demonstrated beneficial effects of GABA-producing strains of relevance to these disorders (Bravo *et al.*, 2011; Patterson *et al.*, 2019; Ko, Lin and Tsai, 2013; Yunes *et al.*, 2020). GABA has consequently been classified as a health-promoting bioactive component in foods and pharmaceuticals (Li and Cao, 2010). Several commercial formulations of GABA are available which claim to have a calming and tranquilising effect, for example, PharmaGABA™ has been approved by

the Food and Drug Administration (FDA) as a food ingredient (Linares *et al.*, 2016). Furthermore, *Lactobacillus plantarum* is one of the most studied species extensively used in food industry as a probiotic microorganism and/or microbial starter and there are currently several probiotics available on the market (Behera, Ray and Zdolec, 2018).

Similarly, probiotics and food supplements consisting of *Lactobacillus paracasei* are also available on the market, such as *Lactobacillus paracasei* CASEI 431®, which has been used in food and dietary supplements since 1995. It is one of the most extensively documented *Lactobacillus* probiotic strains, having been described in over 80 scientific publications as well as being tested on thousands of people in over 20 clinical trials. Research has shown that this strain supports the immune system, helping to improve immune responses and reduce duration of cold and flu symptoms (Jespersen *et al.*, 2015). While serotonin supplements such as 5-HTP are available, to our knowledge, there is not currently a probiotic specifically involved in serotonin synthesis on the market, although some strains may indirectly regulate host serotonin production through the production of secondary metabolites (Yano *et al.*, 2015; Hsiao and Yano, 2016). Serotonin regulates several diverse functions, including enteric motor and secretory reflexes (Gershon and Tack, 2007), platelet aggregation (Mercado *et al.*, 2013), blood pressure regulation (Watts *et al.*, 2012), immune responses (Baganz and Blakely, 2012), bone development (Chabbi-Achengli *et al.*, 2012; Yadav *et al.*, 2008), and cardiac function (Côté *et al.*, 2003). Furthermore, disruption of peripheral serotonin is evident in several disease states, including irritable bowel syndrome (Stasi *et al.*, 2014), cardiovascular disease (Ramage and Villalón, 2008), and osteoporosis (Ducy and Karsenty, 2010). Therefore, serotonin-producing probiotic strains are likely to provide several health benefits, particularly for anxiety. For example, administration of *Lactobacillus plantarum* PS128 significantly increased serotonin and dopamine levels in the striatum of germ free mice, resulting in reduced anxiety-like behaviour in the Elevated Plus Maze (Liu *et al.*, 2016).

It is expected in the coming years that probiotic research will focus on the development of targeted and specific probiotic dietary supplements which will have

the potential to be used as a concomitant therapy alongside medical treatments (Grumet, Tromp and Stiegelbauer, 2020). Before a probiotic can be brought to market several robust processes must first occur. As well as health beneficial probiotic factors, candidate strains must possess adaptation factors that will allow them to survive low pH, bile salts and digestive enzymes which can be assessed using *in vitro* survivability assays. Subsequent *in vitro* assays should be used to study host-microbe interactions, for example the use of human cell line assays to study bacterial adhesion, the effect of microbes on epithelial barrier function, and the use of *in vitro* co-culture assays to study interactions with the immune system (Ouwehand and Salminen, 2003). Furthermore, in addition to screening the functional properties of probiotic strains using *in vitro*, *ex vivo* or animal models, probiotic functionalities can also be assessed at the genomic level with the application of *in silico* functional screening. It is vital to establish the safety profile of candidate novel probiotics, therefore the identity and taxonomic position of the strains must be established using state-of-the-art methods in molecular biology, biochemistry and physiology such as 16S rRNA sequencing or whole genome sequencing (Donelli, Vuotto and Mastromarino, 2013). Following detailed strain identification, a thorough safety assessment should be performed to rule out adverse properties such as production of metabolites which are deleterious to the host, pathogenicity, stimulation of excessive immune responses, and transferable antimicrobial resistance genes (Grumet, Tromp and Stiegelbauer, 2020). *In vivo* efficacy and proof of concept studies can be carried out in germ free or specific pathogen free animal models before graduating to animal studies using models specific to disorders of interest such as BALB/c mice for anxiety, or C57BL/6J mice for metabolic syndrome. Subsequent clinical human trials should be double-blind, randomised, placebo-controlled, have appropriate sample sizes and collect different sample types to provide information on biomarkers such as microbes, cytokines and metabolites which may establish putative mechanisms of action (Lebeer, Vanderleyden and De Keersmaecker, 2008). As well as being safe for human consumption and possessing desirable functional properties, candidate strains must also have traits which allow them to make it through the manufacturing process to produce viable, concentrated cells that are stable, will

have consistent performance in the intended application and a long shelf-life (Grumet, Tromp and Stiegelbauer, 2020).

The microbial community in the gut is influenced by environmental factors and diet, which can moderate host behaviour and cognition through the microbiome-gut-brain axis. Chapter 4 explored the effect of environment on the gut microbiota and aimed to determine whether enriched environmental conditions can alter gut microbiota composition in high-anxiety related behaviour mice and if these alterations are associated with changes in anxiety and depression. Exposure to an enriched environment resulted in improved anxiety-like behaviour in both the Light Dark Test and Open Field Test. However, no effect was observed on alpha diversity while differences in beta diversity were based on mouse phenotype and not environment. Chapter 5 investigated the other main influence of the gut microbiome, namely diet, by administering one of two diets that differed in their relative levels of fat, protein and fibre content to wild caught great tit birds: an insect only diet (low content), or a seed, nut, and beef suet diet (high content). Exploration behaviour and corticosterone response were also assessed to gain an insight into the interactions between the gut microbiome, behaviour, and the stress response in this ecologically relevant model. Microbial communities changed substantially and were less diverse among individuals given the insect but not the seed diet and several differences at both phylum and genus levels were also evident between post-seed and post-insect cohorts. Diet had no effect on corticosterone response, but differences were observed in baseline corticosterone levels for pre- versus post- insect diet groups.

Preclinical data strongly support the view that microbe manipulation using probiotics and prebiotics to enhance the host-microbe symbiotic relationship has great potential in the future prevention and treatment of metabolic, immune, and stress-related psychiatric disorders. Therefore, Chapters 6 and 7 investigated the potential of manipulating the gut microbiota by probiotic or prebiotic administration, respectively and the subsequent impact on metabolic and neuropsychological parameters. The data presented in Chapter 6 outline a potential role of metabolic- and neuroactive-microbial metabolite production in the

modulation of diet-induced metabolic dysfunction, including abnormal behaviour. Intervention with GABA-producing *Lactobacillus brevis* strains attenuated several abnormalities associated with metabolic dysfunction, causing a reduction in the accumulation of mesenteric adipose tissue, increased insulin secretion following glucose challenge, improved plasma cholesterol clearance and reduced despair-like behaviour and basal corticosterone production during the forced swim test. Chapter 7 investigated an alternative method of gut microbiota manipulation, namely prebiotic administration with polydextrose which resulted in a modest improvement in cognitive flexibility and an increased ability for sustained attention in this double-blind, randomised, placebo-controlled clinical trial. Although there was no change in microbial diversity, abundance of *Ruminiclostridium* 5 significantly increased after polydextrose supplementation compared to placebo. Therefore, the results indicate that polydextrose could benefit gut-to-brain communication and modulate behavioural responses.

8.2 Screening and characterising gut-derived Lactobacillus strains for bioactive metabolite production

There is considerable interest in recent years in developing novel probiotic bacteria which are capable of bioactive metabolite production because it is hypothesised that they will be of great benefit to neuropsychiatric disorders. This was the subject addressed in Chapter 3 which aimed to screen gut-derived microbiota isolated from healthy adult males for psychoactive metabolite production targeting stress-related disorders, specifically GABA and serotonin. A commercially available ELISA kit was employed to screen gut-derived putative lactobacilli compared with positive controls selected from the literature in order to assess the suitability of this method for serotonin detection and to identify optimal growth requirements. The positive controls which were selected included *Lactococcus lactis* subsp*. cremoris* MG1363, *Lactococcus lactis* subsp. *cremoris* 1614, *Lactococcus lactis* subsp. *cremoris* 712, *Streptococcus thermophilus* 5655, *Streptococcus thermophilus* 5656, *Lactobacillus plantarum* 891, and *Lactobacillus plantarum* 6805 (Özoğul *et al.*, 2012). Strains

were grown in standard broth as well as tryptophan supplemented broth to stimulate serotonin production. Caution is required when interpreting the results from this assay as high background levels of serotonin were detected in all samples, including the blanks. *Lactococcus lactis* subsp. *cremoris* 1363 and *Lactococcus lactis* subsp. *cremoris* 1614 were identified as potential serotonin producers as higher serotonin levels were detected in these cultures compared to the negative controls. A serotonin increase of 45.84% was calculated in standard broth and 45.88% in tryptophan supplemented broth for *Lactococcus lactis* subsp. *cremoris* MG1363, while *Lactococcus lactis* subsp. *cremoris* 1614 had increases of 3.75% and 56.53% for standard versus tryptophan supplemented broth, respectively. Of the MyNewGut isolates tested, two strains had increased levels of serotonin compared to the blank with an increase of 8.96% in standard broth and 2.57% in tryptophan supplemented broth for *Lactobacillus paracasei* 110, and an increase of 24.7% in tryptophan supplemented broth for *Lactobacillus paracasei* 231 indicating that these strains may be potential serotonin producers. An important observation from this screen was that tryptophan supplementation increased serotonin detection but variability masked improved measurement so should be interpreted with caution. Although the results from this assay were promising in that they seemed to confirm the serotonin production capability of two positive controls from the literature as well as identifying two potential producers from the MyNewGut biobank, the results from this assay should be viewed as preliminary and further analysis was carried out using HPLC.

The strains of interest from the ELISA screen were further analysed using HPLC as an adjunct method to assess the reliability of the ELISA assay. An additional advantage of using the HPLC method was the ability to detect 5-hydroxyindoleacetic acid, which is the main metabolite of serotonin and could provide further insight into the serotonin metabolism capabilities of the strains tested here. Similar findings were observed compared to the ELISA method where there was a strong effect of tryptophan supplementation and high background levels of serotonin or 5-hydroxyindoleacetic acid were detected in all samples, including the blanks, highlighting that caution is required when interpreting results

from this HPLC screen. An important observation from both of these screening methods is the necessity of using blank broths to control for the high background interference. Nevertheless, it was possible to integrate a serotonin peak for the putative positive controls, and a serotonin concentration of 3.4 µg/ml and 12.7 µg/ml were detected for *Lactococcus lactis* subsp*. cremoris* 1614 and *Lactococcus lactis* subsp*. cremoris* MG1363 respectively, when grown in GM17 broth. Therefore, this HPLC assay provided similar overall findings to the serotonin ELISA, although the concentrations detected differed. Similarly, 5-hydroxyindoleacetic acid was also detected in all samples, including the blanks, albeit at lower levels than in the strains tested. Interestingly, tryptophan supplementation consistently resulted in lower serotonin, when detectable, and higher 5-hydroxyindoleacetic acid in the inoculated samples compared to the blanks. It was hypothesised that this effect is possibly a result of tryptophan supplementation shifting bacterial metabolism away from serotonin and towards other pathways, leading to increased bacterial indole production which the HPLC method was unable to separate from 5-hydroxyindoleacetic acid. Taken together, the results from this HPLC screen indicate that *Lactococcus lactis* subsp. *cremoris* 1614 and *Lactococcus lactis* subsp. *cremoris* MG1363 are likely to be serotonin producers which is in agreement with the literature (Özoğul *et al.*, 2012). Further investigation will be required in order to determine whether the HPLC method was detecting 5-hydroxyindoleacetic acid or indole.

In order to provide further information about the potential serotonin production capabilities of these strains, *L. paracasei* 110 and *L. paracasei* 231 underwent whole genome sequencing to search for genes involved in tryptophan metabolism using *Lactococcus lactis* subsp. *cremoris* MG1363 (Genbank accession number AM406671) as reference. Artemis confirmed the presence of the following genes of the tryptophan pathway in *L. paracasei* 110: *trpA, trpB, trpC, trpD, trpF,* and *trpS2*. In contrast, Artemis revealed that *L. paracasei* 231 only contained *trpA* and trpS2. Taken together, these results indicate that *L. paracasei* 110 is capable of both tryptophan and serotonin synthesis because it contains the *trp* operon and the gene which encodes for tryptophan synthase (O'Mahony *et al.*, 2015; Gao *et al.*,

2020). While *L. paracasei* 231 does contain the gene *trpA* it does not contain the genes to encode the remaining enzymes involved in tryptophan synthesis. Therefore, it is likely that this strain is capable of synthesising indole as this gene contributes to tryptophan-independent indole biosynthesis and that tryptophan supplementation is leading to increased bacterial indole production which the HPLC method is unable to separate from 5-hydroxyindoleacetic acid.

Data from the whole genome sequencing were also used to investigate whether the strains of interest contained genes which encoded enzymes involved in other pathways of tryptophan metabolism apart from serotonin synthesis, namely the kynurenine pathway, indole production and tryptamine production. It was decided to investigate whether the strains contained genes responsible for the synthesis of these metabolites because approximately 90% of tryptophan is metabolised along the kynurenine pathway, indole is the main metabolite produced by gut bacteria from tryptophan, and tryptamine is a monoamine which is structurally similar to serotonin (Gao *et al.*, 2020), therefore these metabolites could be interfering with serotonin detection in the ELISA and HPLC methods. *KynA* and *KynB*, which encode the enzymes tryptophan 2,3-dioxygenase and kynurenine formamidase respectively (Kaur, Bose and Mande, 2019), were not found to be present in either *L. paracasei* 110 or *L. paracasei* 231, indicating that these strains may not be involved in kynurenine production. The genes *KMO* and *Kyn*, which encode the enzymes kynurenine 3-monooxygenase and kynurinase for quinolinate production were also not found in either of the genomes of interest, indicating that these strains may not play a role in quinolinate production and are not involved in the kynurenine pathway (Kaur, Bose and Mande, 2019). The gene *tnaA*, which encodes the enzyme tryptophanase which is responsible for indole synthesis from tryptophan, was not found to be present in either of the genomes of interest (Kaur, Bose and Mande, 2019; Gao *et al.*, 2020). However, the gene *trpA* which contributes to tryptophan-independent indole biosynthesis is present in both of the strains so indole production through this gene cannot be ruled out, particularly for *L. paracasei* 231 which does not contain *trpB*, the enzyme which catalyses the final step in tryptophan biosynthesis. Similarly, the genes involved in indole-3-acetic acid

synthesis, *iaaM* and *ipdC*, which encode tryptophan-2-monooxygenase and indolepyruvate decarboxylase respectively, were not found in the genomes of these strains (Kaur, Bose and Mande, 2019). The gene *fldABC*, which encodes the enzyme phenyllactate dehydratase for indole-3-propionic acid production, was not present in the genomes (Kaur, Bose and Mande, 2019). Finally, the ability of the strains to synthesise tryptamine was assessed by searching for the genes *TDC, CLOSPO-0283,* and *RUMGNA-01526* which encode tryptophan decarboxylase (Williams *et al.*, 2014). Neither *L. paracasei* 110 nor *L. paracasei* 231 were found to possess the genes known to be involved in tryptamine synthesis; therefore, it is unlikely that either of the strains of interest are capable of tryptamine production. Taken together, these results indicate that the strains described here do not play a role in the kynurenine pathway or are involved in the degradation of tryptophan to form the metabolites indole-3-acetic acid, indole-3-propionic acid, or tryptamine. Therefore, it is unlikely that any of these metabolites were mistaken for serotonin or 5-hydroxyindoleacetic acid in the ELISA and HPLC analysis as mentioned previously. Additionally, an important conclusion that can be drawn from these results is the value of using multiple screening approaches in combination, including laboratory techniques such as ELISA and HPLC, as well as metagenomic approaches such as whole genome sequencing to search for genes of interest. This provides a greater insight into the metabolic capabilities of strains of interest by determining whether they contain the genes to participate in the relevant pathways and allows a deeper interrogation of the metabolites detected from screening methods which could be structurally similar to the metabolites of interest.

A probiotic which is capable of GABA synthesis has potential for treating disorders such as anxiety and depression, therefore amino acid analysis was used to assess the ability of 34 human-derived *Lactobacillus* strains obtained from the MyNewGut biobank as well as strains isolated from fermented food products to generate GABA from monosodium glutamate compared to the positive control *Lactobacillus brevis* DPC6108, which is a highly efficient GABA producer (Patterson *et al.*, 2019; Barrett *et al.*, 2012). This assay identified one putative *Lactobacillus* strain as a GABA producer with an average monosodium glutamate to GABA conversion rate of

38.4%. Sanger sequencing subsequently identified the strain as *Lactobacillus plantarum*. However, this strain is not as efficient a GABA producer as the positive control *L. brevis* DPC6108, with a conversion rate of monosodium glutamate to GABA of up to 90% (Barrett *et al.*, 2012), so would be unlikely to be a more effective probiotic than *L. brevis* DPC6108 which was subsequently administered to a mouse model of metabolic syndrome in this thesis (Chapter 6).

Bacteriocin production is considered a desirable probiotic trait and has been shown to promote probiotic survival within the gastrointestinal tract, contribute to the control of pathogens and influence host gene expression in the gut (Hegarty *et al.*, 2017). Therefore, in order to further characterise the isolates as potential probiotic candidates, a series of assays to detect antimicrobial activity were carried out on both the MyNewGut isolates (n=26) and the fermented food isolates (n=46) using deferred antagonism assay and well diffusion assay approaches. All MyNewGut isolates tested produced zones in the deferred antagonism assay, however the zones were cloudy which signified acid production. All but two fermented food isolates also produced zones in this assay, however no zones were visible in the subsequent well diffusion assay. *Lactobacillus paracasei* 110 was identified as a potential bacteriocin producer using the well diffusion technique and further assays were carried out on this strain including heat treating the supernatant at 99°C and 80°C as well as using Proteinase K. No zones were observed when the supernatant was heat treated although small zones were visible when the supernatant was maintained at room temperature, indicating weak antimicrobial activity. However, in order to gain a greater insight into their antimicrobial potential, the genomes of both *Lactobacillus paracasei* strains were also analysed using BAGEL to identify genes encoding antimicrobial activity. Both *L. paracasei* 110 and 231 contained a putative carnobacteriocin B2 immunity protein within their genomes at positions 2446898-2447233 and 2615586-2615921, respectively. Two areas of interest were found using BAGEL within *L. paracasei* 110 and were identified as Enterolysin A, with a match of 56.82%, and LSEI 2386, with a match of 100%. Similarly, three areas of interest were uncovered in *L. paracasei* 231 and were subsequently identified as Carnocin CP52, LSEI 2163, and Enterolysin A. Interestingly, Nilsen *et al.* (2003)

initially only detected Enterolysin A antimicrobial activity on agar plates in overlay assays, but activity was later optimised by growing the bacteria in a fermenter under optimised production conditions. This could explain why large zones of clearing were only detected in the deferred antagonism assay while minimal zones were observed in the cell-free supernatant well diffusion assay in the present study. In order to confirm this hypothesis, the antimicrobial assays should be repeated using neutralised supernatant (pH 6.9–7.2 using 1 M NaOH) to eliminate inhibition due to acids. Furthermore, optimisation techniques could be carried out on the strain in a manner similar to Nilsen *et al.* (2003) (MRS broth with 4% [wt/vol] glucose, pH 6.5, and a temperature between 25 and 35°C) before repeating the well diffusion assay to determine whether the area of the zones increased following optimisation. Future studies could also test antimicrobial activity of the strain against a wider variety of indicator strains to provide a deeper insight into its spectrum of activity.

The strains were also analysed for genes promoting probiotic characteristics such as acid and bile tolerance and adhesion capability. These included *dltA* and *dltD* which confer tolerance to acid, which is an important feature for a candidate probiotic to possess for survival during gastrointestinal transit and subsequent colonisation of the intestinal mucosa (Goel, Halami and Tamang, 2020). Interestingly, both *L. paracasei* 110 and 231 contained the genes *dltA* and *dltD* which indicates that they are tolerant of low pH. However, the genes *clpL*, promoting acid and bile tolerance, and *bsh*, supporting tolerance to bile were not present (Bustos *et al.*, 2018). Adhesion ability of the strains was also investigated and both strains were found to contain the gene *fbp* which promotes adhesion ability, an additional characteristic which is important for candidate probiotics to possess (Turpin *et al.*, 2012). Therefore, the presence of bacteriocin genes as well as genes promoting tolerance to acid and adherence to intestinal mucosa may provide a competitive advantage to the strains identified in this study, promoting survival in the complex, competitive environment of the large intestine, which may indicate the promise of these strains as potential probiotics (Dobson *et al.*, 2012; Jia *et al.*, 2020). However, in order to confirm these findings, a number of *in vitro* assays would first need to be carried

out, including survival assays in simulated gastric juice as well as adhesion assays using tissue cell culture models of intestinal epithelial cells such as Caco-2 and HT-29 cells (Ouwehand and Salminen, 2003).

8.3 Investigating the influence of environmental conditions and diet on the gut microbiome, stress, and anxiety-related behaviour

Environmental enrichment has been shown to provide several beneficial physiological, neurobiological and behavioural effects such as altering brain structure, promoting superior learning and cognitive function (Renner and Rosenzweig, 1987), reducing anxiety (Benaroya-Milshtein *et al.*, 2004; Chamove, 1989; Nilsson *et al.*, 1999), facilitating recovery from brain lesions (Will *et al.*, 1977), combatting addiction (Thiel *et al.*, 2009), and reversing the detrimental effects of past stressors (Escorihuela, Tobeña and Fernández-Teruel, 1994) such as prenatal stress by increasing social interaction, decreasing corticosterone secretion and strengthening the immune system (Laviola *et al.*, 2004; Morley-Fletcher *et al.*, 2003). Furthermore, the influence of environmental conditions on gut microbiome diversity and composition is well established (Rothschild *et al.*, 2018; Xie *et al.*, 2016). While previous studies have explored the impact of environmental enrichment on psychological and physiological factors, there has been limited investigation into the effects of environmental enrichment on gut microbiome composition and diversity, and whether these changes may play an underlying role in mediating the beneficial effects of environmental enrichment. Therefore, Chapter 4 assessed whether environmental enrichment can attenuate enhanced anxiety and depression-like behaviour in high anxiety-related behaviour phenotype mice compared to normal anxiety-related behaviour mice and, if so, whether alterations in gut microbial composition and diversity could be involved in the underlying mechanisms. Additionally, we aimed to assess whether the gut microbiota profile differed between high anxiety-related behaviour and normal anxiety-related behaviour mice.

As expected, there was a pronounced impact of mouse phenotype on anxiety-like behaviour in the highly anxious phenotype compared to the non-anxious mice within the Elevated Plus Maze. Anxiety behaviour was then assessed post enrichment using the Light Dark Test and Open Field Test. Interestingly, environmental enrichment appeared to have a positive effect on the high anxiety phenotype mice as they did not display any significant differences to normal anxiety-related behaviour mice post enrichment in the Light Dark Test, while multiple differences were observed between normal anxiety-related behaviour and high anxiety-related behaviour mice post standard conditions. Similarly, high anxiety mice showed reduced anxiety-like behaviour in the Open Field Test following enriched but not standard housing. When comparing normal anxiety and high anxiety mice post environmental enrichment, significant differences were observed in number of visits into the centre whereby high anxiety mice visited the centre more than normal anxiety-related behaviour mice following environmental enrichment. In contrast, when comparing high anxiety-related behaviour and normal anxiety-related behaviour mice post standard housing, a significant difference in total distance travelled in the Open Field Test was observed whereby normal anxiety mice travelled a greater distance than high anxiety mice. Taken together, the results from these behavioural tests suggest that environmental enrichment reduced anxiety-like behaviour of highly anxious mice to levels comparable to or even lower than their non-anxious counterparts. However, when examining associations with the microbiome results, we noticed patterns of responders and non-responders and it was determined that this may be due to a colonisation effect from where the animals were reared. Upon further investigation it was found that a subsection of the high anxiety-related behaviour cohort was born in a different facility (CCB) to the remainder of the high anxiety-related behaviour mice and the entire normal anxiety-related behaviour cohort (PM1), which was consequently skewing the results. High anxiety mice born in the CCB facility maintained a very different microbiota profile despite being raised in the PM1 facility and clustered separately from the remainder of the high anxiety-related behaviour mice on PCoA plots. Therefore, it was decided to re-analyse the results with the high anxiety mice from the CCB facility omitted which resulted in a

low n number, reducing the power of this study so consequently these results should be regarded as preliminary.

The behavioural analysis was repeated with the high anxiety mice from the CCB facility cohort omitted and similar findings were observed in the Elevated Plus Maze test, reaffirming the altered anxiety profile of high anxiety-related behaviour mice compared to normal anxiety-related behaviour mice. To determine whether enrichment influenced anxiety behaviour in highly anxious mice, the Light Dark Test and Open Field Test data were again analysed without the high anxiety mice from the CCB facility. However, many of the positive effects of enrichment observed in these behavioural tests with the full dataset were lost. Normal anxiety-related behaviour mice showed reduced anxiety compared to the high anxiety phenotype following both enriched and standard conditions in the Light Dark Test. However, an interesting finding was observed when high anxiety-related behaviour mice who had been exposed to environmental enrichment spent a greater amount of time in the light compared to high anxiety mice who were housed in standard conditions. Interestingly, high anxiety mice post enrichment demonstrated improved anxiety-like behaviour in the Open Field Test compared to normal anxiety mice post enrichment, possibly indicating that environmental enrichment had a greater effect in reducing anxiety-like behaviour in the high anxiety-related behaviour phenotype compared to the normal anxiety-related behaviour phenotype. These diverging behavioural results after the removal of the high anxiety mice from the CCB facility compared to the full dataset could suggest that the facility factor skewed the results or that the power is too low to obtain reliable results. In order to determine this, the present study would need to be repeated with a larger cohort of mice from the same facility.

Analysis of the mouse samples at baseline prior to environmental enrichment provided an insight into the association of mouse phenotype on microbiome diversity and composition. Many of the significant differences were lost after removal of the CCB mice, however a significantly higher abundance of *Chlamydiae* was present in high anxiety compared to normal anxiety mice which contrasts to three significant differences with CCB mice included. Similarly, when

comparing high anxiety-related behaviour and normal anxiety-related behaviour phenotypes at genus level pre-enrichment, 21 significant differences were observed including *Staphylococcus*, *Lactococcus*, and *Tyzzerella* while 41 differences were observed with the entire dataset included. No significant differences in alpha diversity were observed when comparing normal anxiety-related behaviour and high anxiety-related behaviour mice pre-environmental enrichment or pre standard housing, indicating that phenotype does not have a substantial influence on microbial alpha diversity. However, differences in beta diversity were observed based on mouse phenotype between high anxiety-related behaviour and normal anxiety-related behaviour mice in standard conditions at baseline, indicating that mouse phenotype may have an influence on microbial beta diversity.

As expected, environmental enrichment had little impact on the microbiome of normal anxiety-related behaviour mice as minimal differences were observed between normal anxiety mice pre versus post enrichment. No significant differences in alpha diversity were evident between normal anxiety-related behaviour mice pre versus post enriched or standard housing. Together, these results indicate that environmental enrichment had no effect on microbial diversity in normal anxiety, which is in agreement with other studies which suggest that the protective effects of environmental enrichment are particularly apparent in animals that are highly anxious or when the task is especially challenging for the subject (Chapillon *et al.*, 1999; Klein *et al.*, 1994). These results suggest that environmental enrichment may be therapeutically anxiolytic only in situations of pronounced anxiety (Fox, Merali and Harrison, 2006).

When examining the effect of environmental enrichment on high anxiety-related behaviour mice, many of the significant differences were lost after removal of the CCB mice. However, there was a significant difference in *Chlamydiae* and *Other* between enriched cohorts, with a higher abundance present in high anxiety-related behaviour mice. Interestingly, 29 significant differences were observed at genus level post enrichment between high anxiety and normal anxiety phenotypes while there were no significant differences between high anxiety mice pre versus post enrichment, indicating that environmental enrichment had little impact on the

microbiome composition of high anxiety-related behaviour mice while phenotype had a substantial influence. No significant differences in alpha diversity were observed in high anxiety-related behaviour mice based on phenotype or environment. Similarly, adonis tests showed no significant differences in beta diversity between high anxiety-related behaviour and normal anxiety-related behaviour mice pre versus post enrichment, although there was a trend towards significance between the high anxiety and normal anxiety mice post environmental enrichment where high anxiety mice clustered separately to normal anxiety mice. However, there were significant differences observed between the standard housing groups, namely between high anxiety-related behaviour and normal anxiety-related behaviour mice in standard housing conditions at baseline and between high anxiety and normal anxiety mice post standard conditions, while no separation was visible between high anxiety-related behaviour mice pre versus post standard. These results indicate that mouse phenotype may have a greater influence on microbial beta diversity than housing in an enriched environment versus a standard environment because samples clustered based on phenotype but not based on environment. However, it is possible that environmental enrichment may reduce differences in beta diversity between the high anxiety and normal anxiety phenotypes compared to those not exposed to enrichment because no significant differences were observed between the high anxiety and normal anxiety mice post enrichment whereas differences were observed post standard housing. This will be an interesting finding to investigate further. However, these results should be interpreted cautiously due to the low n number of this study. Furthermore, differences in beta diversity which were previously observed between high anxiety-related behaviour and normal anxiety-related behaviour mice post enrichment were lost once mice from the CCB facility were removed, suggesting that this separation was due to the different facilities and not due to the study variables.

Following on from investigating the impact of differential environmental conditions on the gut microbiome and the subsequent impact on behaviour, the other main variable which is widely regarded to have a strong impact on the microbiome was

explored in Chapter 5, namely diet. This study demonstrated that an experimentally induced dietary change caused significant alterations to the gut microbiome diversity, and to phylum- and genus-level abundance in a wild bird species. Importantly, it provides experimental evidence in a non-model lab organism that microbial communities in the gut are influenced by diet. Furthermore, this study describes an interaction between microbiota composition and a behavioural trait, in this case exploration behaviour as alterations in gut microbiota composition at the genus level were observed based on fast versus slow exploration behaviour. The present study demonstrated that microbiota composition changes at phylum and genus levels as well as diversity changes were attributed to dietary intervention, possibly due to differences in relative protein, fat, and fibre content. Higher abundance of Bacteroidetes were present in subjects with lower fat, protein and fibre intake following administration of an insect diet in line with (Clarke *et al.*, 2014). In contrast to that study, however, no decrease in Firmicutes was observed, while an increase in Proteobacteria, possibly due to differences in the core microbiome between birds and mammals (Waite and Taylor, 2014), and the type of fat and/or fibre (Singh *et al.*, 2017) did occur. Birds in the insect diet group showed a decrease in alpha diversity compared to birds administered a seed diet, potentially because the insect diet itself was less diverse and lower in dietary fibre than the seed and nut diet. Furthermore, a trend towards significance was observed in beta diversity between birds post insect diet compared to birds post seed diet. Several significant differences were also observed at both phylum and genus levels, although most of these differences were between the post seed and post insect groups. LeFSe analysis revealed 19 discriminating taxa for the pre seed group, including *Nocardioides*, *Devosia*, and *Micromonospora*. Seven discriminating taxa were identified for the post seed group including *Paracoccus* and *Myroides*. Finally, nine discriminating taxa were revealed for the post insect group including *Lactococcus*. In summary, this manipulation showed clearly that diet modifies the microbiome in this model species of field ecology and suggests that environmental drivers of variation in diet, such as seasonality and food availability, may hold a significant role in driving these effects.

The metabolic function of the microbial community is largely dependent on host diet and likely plays a role in the gut microbiome's influence on behaviour via the microbiota-gut-brain axis (Roager and Dragsted, 2019). Therefore, an additional aim of this study was to test diet-microbiome-behaviour relationships within an ecologically relevant context that would translate to wild animals in their natural environment. Exploration behaviour was investigated as part of this study; however, few differences were observed in microbial composition or diversity between fast and slow exploration groups, apart from seven differences at genus level, suggesting that the microbiome may not have a strong influence on exploration behaviour in this model. Similarly, dietary intervention had no significant impact on corticosterone response. There was a significant difference in baseline corticosterone concentrations (CORT 0) between pre- and post-insect groups, indicating an effect of time rather than diet as there were no differences in CORT 0 between insect and seed groups. This is likely due to the effect of captivity which increased baseline corticosterone in the insect group but not the seed group, suggesting a possible role of the seed diet in protecting from the effects of captivity on stress.

8.4 Investigating the effect of probiotic and prebiotic intervention on the gut microbiome, metabolic and psychological health

The potential role of neuroactive microbial metabolite production was explored in Chapter 6 using a preclinical mouse trial, namely in the modulation of diet-induced metabolic dysfunction, including abnormal behaviour. This exploratory study demonstrates that daily administration of GABA-producing lactobacilli has a moderate efficacy in improving insulin sensitivity and glucose tolerance in metabolically dysfunctional mice. Two GABA-producing *Lactobacillus brevis* strains of different origins were selected to compare their therapeutic potential in a mouse model of diet-induced obesity. The GABA-production capability of *L. brevis* DSM32386 was comparable to that of *L. brevis* DPC6108 *in vitro*. However, DSM32386 appeared to have a greater impact, albeit modest, in improving some of

the metabolic and behavioural outcomes associated with metabolic dysfunction in this mouse model.

In this study, 12 weeks of high fat feeding was sufficient to induce glucose intolerance and insulin insensitivity and subsequent intervention with GABA-producing lactobacilli had positive metabolic outcomes. The results indicated that microbial intervention with these GABA-producing *Lactobacillus* strains improved glucose homeostasis by attenuating diet-induced obesity associated hyperglycaemia and insulin insensitivity. During the glucose tolerance test, intervention with both *L. brevis* strains significantly enhanced insulin secretion in response to glucose load. This could represent an increase in the sensitivity of the endocrine response to promote glucose clearance following GABA-producing *L. brevis* intervention. Beneficial effects were also observed for obesity as although there were no differences in the final bodyweight gain between the high fat diet fed groups, *L. brevis* had a significant effect on the distribution of adiposity. Importantly, intervention with both DSM32386 and DPC6108 resulted in less mesenteric adipose tissue accumulation, compared with the high fat control which is known to correlate well with the development of insulin resistance and type-2 diabetes (Wajchenberg, 2000). Therefore, it appears that GABA-secreting microbial interventions altered fat distribution in a manner which contributes beneficially to metabolic fitness. Furthermore, *L. brevis* had a beneficial impact on host lipid metabolism, as both strains were associated with reductions in fasting circulating cholesterol levels and an overall improvement in post-prandial cholesterolaemia and this exploratory study is the first to demonstrate a potential role of microbial GABA-production in addressing diet-induced hypercholesterolaemia.

We hypothesised that high fat feeding would significantly affect the gut microbiota composition of the mice in this study. Taking a metataxanomic approach, we aimed to determine whether improvements in the metabolic profile following microbial intervention could be attributed to a beneficial impact on the gut microbiota. Our data demonstrated that the high fat diet induced alterations in the intestinal microbial composition, significantly increasing Firmicutes, with a stable abundance of Bacteroidetes in the high fat control and in the groups exposed to *L. brevis* and

high fat diet. Actinobacteria decreased in all groups fed the high fat diet irrespective of microbial intervention compared with the low fat control. Deferribacteres decreased in all groups fed the high fat diet compared with the low fat control and again, microbial intervention did not significantly reverse that change. A high inter-group variation was observed in terms of *Odoribacter* abundance, although DSM32386 intervention trended towards a reversal. Members of the *Odoribacter* spp. can cause health problems in humans and animals, but it has been reported that they may be beneficial in the prevention of hypertension (Göker *et al.*, 2011; Hardham *et al.*, 2008) and modulating host blood glucose levels (Manfredi *et al.*, 2015; Salomäki-Myftari *et al.*, 2016). However, *L. brevis* did not modulate the genus *Akkermansia* and thus does not explain the improved metabolic profile.

A further objective was to identify whether changes in specific microbial genera were related to any metabolic parameters measured during the study. Considering both microbial and metabolic factors, the low fat control and *L. brevis* groups clearly separated from the high fat control on PCA. *Parabacteroides* appeared to be related to the increase of glycaemia after IP-ITT, the reduction of resting insulin levels and the increase of cholesterolaemia. *Bifidobacterium* and *Muribaculum* were related to reduced glycaemia (upon IP-GTT and/or IP-ITT) and the reduction of cholesterolaemia. Finally, *Erysipelotrichaceae* was related to reduced cholesterolaemia, and *Odoribacter* appeared to be related to reduced glycaemia (upon IP-GTT and/or IP-ITT). Since those genera tended to be increased in the low fat control and *L. brevis* groups, respectively, they could be involved in the metabolic response of metabolically dysfunctional mice, although until proven, this point remains hypothetical. Nevertheless, if the gut microbiota changes would be the only correlation with the changes identified in the metabolic parameters, then intervention with DSM32386, which had improved metabolic parameters, would have had a different microbial profile than DPC6108. Thus, we hypothesise that the changes in the metabolic parameters observed in DSM32386 may be related to the higher luminal content of GABA in the small intestine and thus, endogenous microbial GABA production. Therefore, alterations to the gut microbiota

composition following *L. brevis* intervention do not solely explain the improvements to metabolic function.

Clinical data from patients with metabolic syndrome and preclinical data from animal models of obesity have demonstrated an increased risk for neuropsychiatric disorders (Castanon, Luheshi and Layé, 2015; Farooqui *et al.*, 2012), therefore considering the associated risk of behavioural abnormalities with obesity, we aimed to identify whether microbial GABA production in the intestine could relieve behavioural abnormalities associated with obesity and metabolic dysfunction. Regarding anxiety-related behaviour, neither high fat diet nor *L. brevis* intervention had any impact in the Open Field and Elevated Plus Maze tests. While mice in the DSM32386 group produced less faecal pellets during the Forced Swim Test, compared to the high fat control, the method of counting faecal pellets produced during the Forced Swim Test is considered a crude measure of anxiety-related behaviour and is not typically used as a robust output from this test which is predominantly used preclinically to assess antidepressant-like activity (Cryan, Markou and Lucki, 2002). Furthermore, there was no effect of *L. brevis* on faecal pellet production in the aversive Open Field test and we therefore cannot conclude that either strain had an impact on anxiety-like behaviour. However, intervention with DSM32386 had a modest effect towards improving recognition memory in the Novel Object Recognition test. This is particularly interesting since hyperglycaemia is a risk factor for cognitive impairment (Davari, Talaei and Alaei, 2013; Biessels *et al.*, 1999; Selvarajah *et al.*, 2011). Finally, *L. brevis* improved high fat diet-induced behavioural despair as observed in the Forced Swim Test, and basal corticosterone levels taken before the induction of Forced Swim Test-induced stress were lower following *L. brevis* intervention compared with the high fat control. The observation that both DPC6108 and DSM32386 reduced despair behaviour and basal corticosterone to levels which were comparable to the low fat control is intriguing and opens the possibility that these strains could modulate brain behaviour and signalling, mediated through microbial GABA production.

Several beneficial physiological effects on measures such as constipation and satiety have previously been demonstrated in human studies following administration of the prebiotic polydextrose (Timm *et al.*, 2013). Furthermore, beneficial behavioural and cognitive effects of polydextrose have been observed in animal models whereby polydextrose administration combined with other pre- or probiotics, affected cognitive functions such as exploratory behaviour, recognition memory and neurochemistry (Fleming *et al.*, 2019) and also attenuated the effects of early life stress on anxiety-like behaviour and learning (McVey Neufeld *et al.*, 2019). However, while prebiotic supplementation benefits for host physiology have previously been illustrated in animals (Savignac *et al.*, 2013; Mika *et al.*, 2014) and humans (Holscher, 2017; Hull *et al.*, 2012; Hibberd *et al.*, 2019; Savignac *et al.*, 2013), evidence for the impact on mental health is limited. Therefore, the impact of the prebiotic polydextrose on cognitive function, acute stress response as well as microbiota composition and diversity were investigated in Chapter 7. While acute stress responses were not impacted in this cohort, subtle improvements in cognitive flexibility and in sustained attention were detected in tests of the CANTAB battery following polydextrose supplementation. Therefore, data from this study align with observations from previous studies demonstrating improved cognitive flexibility after prebiotic use because the present study detected an enhanced ability of participants to transition their thinking from one concept to another and sustaining attention following polydextrose supplementation (Schmidt *et al.*, 2015; Gronier *et al.*, 2018). Taken together, results from the cognitive assessment in this study provide evidence for a psychobiotic and cognitive-enhancing effect of polydextrose supplementation.

No statistically significant changes in microbiota diversity and only minimal changes in composition were observed in this cohort in contrast to previous studies which demonstrated that polydextrose increases microbial diversity and supports the growth of beneficial bacteria such as *Faecalibacterium, Akkermansia* and *Dialister* (Roytio and Ouwehand, 2014). However, the effect of polydextrose intervention on microbial composition has been shown to vary greatly from study to study (Roytio and Ouwehand, 2014), due to variables such as different dosages of polydextrose

(Costabile *et al.*, 2012; Holscher *et al.*, 2015), differences in microbiota analysis (Costabile *et al.*, 2012; Holscher *et al.*, 2015; Roytio and Ouwehand, 2014), and differences in the health status of subject populations. Indeed, polydextrose has predominantly been shown to impact outcome measures of weight management, gut barrier function and inflammation in combination with other prebiotic supplementations (Stenman *et al.*, 2016), and has only elicited microbial changes in populations where an aberration in microbiota composition is commonly observed such as in obese subjects (Hibberd *et al.*, 2019). Dietary habits, baseline microbial diversity, richness and prior stability could be predictors of the responsiveness of a participant to a prebiotic intervention (Tap *et al.*, 2015; Salonen *et al.*, 2014). The lack of vast microbial changes observed in the present study is therefore likely explained by the relatively low dose of the prebiotic, an unresponsive baseline microbial community, or the health status of the population.

Interestingly, polydextrose supplementation significantly increased *Ruminiclostridium* 5 abundance and although little is known about the function of this bacterium in humans, decreased levels of *Ruminiclostridium* 5 have previously been found in patients with kidney stones (Tang *et al.*, 2018) and in a rat model of acute necrotising pancreatitis (Chen *et al.*, 2017), potentially indicating an important role for this bacterium in inflammatory diseases. Similarly, minimal alterations in SCFA concentration were detected in this study, which could be explained by the fact that, in line with the observation of limited gas production experienced by subjects, the rate of SCFA production from polydextrose is also more moderate than from short chain oligosaccharides, such as fructo- and galacto-oligosaccharides (Hernot *et al.*, 2009; Mäkeläinen *et al.*, 2010). Furthermore, the concentrations of fermentation products measured in faecal samples do not describe the total production rate in human and animal trials as they are rapidly absorbed by host cells such as colonocytes. Previous studies have demonstrated that fermentation of polydextrose is associated with reduced colonic SCFA concentration coupled with increased levels in blood circulation (Fava *et al.*, 2007), suggesting an improved absorption rate from the gastrointestinal tract rather than reduced production by microbes. Therefore, serum levels of SCFA and other

fermentation metabolites could be similarly increased so it would be useful to measure blood SCFA levels in future studies.

8.5 Future Directions

Microbiome manipulation using probiotics, particularly strains which produce neuroactive metabolites, are likely to play a promising role in the future prevention and treatment of immune and psychiatric disorders. However, further research is necessary to ascertain whether manipulation of human neurotransmission by gut microbiota is associated with additional physiological implications and how it may be employed therapeutically, and there are still several challenges that need to be addressed. Research needs to progress beyond characterising the microbiota composition in various disease states and investigate potential mechanisms by which altered microbiota composition contributes to pathophysiology of disease, and by which potential probiotic bacteria exert beneficial effects on host health, potentially by metabolite production (Kelly *et al.*, 2015) through the use of metabolomics, culturomics, and metatranscriptomics (O'Callaghan *et al.*, 2016). Importantly, Chapter 3 highlighted the value of combining laboratory screening techniques with metagenomic analysis to gain further mechanistic insight into the psychobiotic potential of strains of interest. Whole genome sequencing allows identification of enzymes involved in the relevant metabolic pathways within the genomes of potential probiotics, enabling the distinction between the metabolite of interest and other secondary metabolites which are structurally similar, therefore it is likely to play a crucial role in future probiotic characterisation. Future studies will be required to optimise the methods described in this study, particularly to reduce background readings from media which were observed in the serotonin ELISA and HPLC screens, potentially by using minimal growth media to reduce the background readings in the broth. This study also highlights the importance of including uninoculated growth media as blanks to monitor the background levels detected and provide a more accurate description of serotonin detection within the samples. Optimisation of the synthesis capabilities of potential probiotics will be necessary by adjusting growth conditions, growth medium and precursor concentration. For

example, future studies could further characterise the GABA producing *Lactobacillus plantarum* and work to optimise GABA production by testing different concentrations of monosodium glutamate supplied as a precursor.

It will also be important to assess whether positive producers are capable of synthesising neuroactive metabolites in the presence of other microbiota by using techniques such as faecal fermentation. Further important steps in probiotic characterisation include assessing the safety profile of candidate probiotics such as testing for antibiotic resistance and determining whether they possess allergenic or toxigenic genes. Further tests will be necessary to assess their survivability and resistance to stressors such as low pH, bile salts and digestive enzymes. Finally, their adhesion capability will need to be assessed using tissue cell culture techniques as well as investigating the interaction with the host immune system and other commensal microbiota. Proof of concept and *in vivo* efficacy can be assessed using germ free and specific pathogen free animals before progressing to animal model studies for specific disorders, for example using BALB/c mice as a model for anxiety or employing C57/BL6 mice for metabolic syndrome. Additionally, animal studies which combine microbiome intervention with neurotransmitter antagonists will provide further insight into mechanisms of communication between gut microbiota and the host (Strandwitz, 2018). Subsequent probiotic clinical trials should be double-blind, randomised, placebo-controlled, of the appropriate sample size and collect a variety of sample types to provide information on biomarkers such as microbes, cytokines, and metabolites as well as putative mechanisms of action.

In terms of future studies, the work carried out in Chapter 4 will need to be repeated with a higher n number using mice from the same facility in order to determine whether the observed differences in behaviour and microbiome were due to the facility factor or due to the environmental enrichment itself. Once this is determined, it will be important to identify species of bacteria that are strongly associated with these changes and future studies could employ strains which were present in a higher abundance in normal anxiety-related behaviour mice compared to high anxiety-related behaviour mice, or which increased in the high anxiety

phenotype following enrichment, to test for probiotic potential as these could be providing protective effects. A significant challenge in microbiome research is determining whether microbiota alterations are causal or consequential of behavioural changes, therefore future studies could administer these strains to examine whether they are causal or protective in the observed effects. LEfSe analysis was carried out to identify key phylotypes that contributed to the differences between the high anxiety-related behaviour and normal anxiety-related behaviour gut microbiome profiles and which were most likely to explain the differences between individual groups in order to identify potential candidates for future probiotic studies. *Rikenellaceae* RC9 gut group was associated with normal anxiety-related behaviour mice prior to environmental enrichment. The specific function of this taxon remains poorly understood; however, it has previously shown to be decreased in rats with hypertriglyceridemia-related acute necrotising pancreatitis, and high *Rikenellaceae* abundance is reportedly associated with healthy metabolic states (Huang *et al.*, 2017; Lippert *et al.*, 2017). Furthermore, members of the *Rikenellaceae* family can produce propionate, acetate and/or succinate as fermentation end products (Graf, 2014). Other genera which were discriminating in this group include *Lactococcus*, which has been demonstrated to have anti-inflammatory effects and consequently identified as a potential treatment for Inflammatory Bowel Disease (Luerce *et al.*, 2014). It has probiotic characteristics such as gastric acid resistance and survival in NaCl and bile salts. It has also been identified as a bacteriocin producer against *Listeria monocytogenes* and a producer of neuroactive bioamines such as dopamine, noradrenaline, serotonin (Stoyanova *et al.*, 2017), and GABA (Song *et al.*, 2017). Furthermore, several studies have demonstrated various strains of this genus improve depressive- and anxiety-like behaviour (Ramalho *et al.*, 2019; Colica *et al.*, 2017; Liu, Walsh and Sheehan, 2019). *Parabacteroides* was one of the discriminating genera in normal anxiety-related behaviour mice post enrichment and certain strains have been shown to possess beneficial metabolic effects such as reducing obesity, inflammation and insulin resistance (Wu *et al.*, 2019). *Parabacteroides* has also been demonstrated to be inversely associated with stress, depression and anxiety in adults (Taylor *et al.*, 2019).

Ruminiclostridium was identified as a discriminating genus in high anxiety-related behaviour mice post enrichment. Decreased levels of *Ruminiclostridium* 5 were found in patients with kidney stones (Tang *et al.*, 2018) and in a rat model of acute necrotising pancreatitis (Chen *et al.*, 2017), potentially indicating an important role for this bacterium in inflammatory diseases. Similarly, *Roseburia* was also identified as a discriminating factor of the highly anxious mice post enrichment. This genus has been identified as a butyrate producer (Tamanai-Shacoori *et al.*, 2017), is negatively associated with Irritable Bowel Syndrome severity (Liu *et al.*, 2020), and is reduced in ulcerative colitis patients (Machiels *et al.*, 2014). Regarding anxiety, *Roseburia* has been found to be more abundant in healthy controls compared to people with generalised anxiety disorder (Jiang *et al.*, 2015). Any of the aforementioned bacteria could be contributing to the reduced anxiety profile in normal anxiety-related behaviour mice and in high anxiety-related behaviour mice post environmental enrichment and it would be interesting to investigate these further in future animal studies examining environmental enrichment, particularly the strains which have previously been shown to be beneficial for anxiety and depression-like behaviour.

Chapter 5 aimed to mimic variation in individual food consumption and in seasonal food availability by examining wild birds in captivity over a short time period. However, in order to confirm whether similar microbial diversity and taxa changes would occur in the wild under natural conditions, longitudinal sampling of individuals in the wild across seasons would be necessary, particularly given that the invertebrate species accessible in the wild would be different from those provided in this experiment. It would also be useful to employ metabolomics profiling in future studies to provide a functional assessment of microbial products such as short chain fatty acids involved in gut-brain axis communication (Stilling *et al.*, 2016; Heintz-Buschart and Wilmes, 2018; Cryan *et al.*, 2019) as well as a metagenomic approach to provide insight into the functional genes present in the microbiome, all of which will aid in studying potential mechanisms of the link between diet, the microbiome, behaviour and the stress response. Furthermore, in order to effectively examine the evolutionary and ecological patterns in vertebrate

microbiomes, broader sampling is needed. Exponential advances have been made in next generation sequencing technologies in recent years so that broader and deeper sampling have now become both more cost and time effective. Most next generation sequencing studies of the animal microbiome have focused solely on community composition using 16S rRNA sequencing. However, a metagenomic approach is being increasingly incorporated in human studies and would be a useful addition to animal studies by providing an insight into the functional genes present in the microbiome, which could further elucidate the influence of diet, geography and phylogeny on the host microbiome. This will aid in studying multiple potential mechanisms, which is necessary to uncover the interactions between cognition, behaviour, diet and the microbiome, and will help to elucidate the environmental and genetic effects determining individual differences in cognition and behaviour (Davidson *et al.*, 2018).

The preclinical mouse trial outlined in Chapter 6 demonstrated that increased microbial GABA production can impact host metabolism and behaviour. However, further substantiation of these exploratory health benefits in mice need to be confirmed before moving towards the clinic. This is the first study to our knowledge identifying a potential role of microbial GABA-production in alleviating some symptoms of metabolic dysfunction. Future experiments should utilise this dataset to focus on the potential mechanism of action of *L. brevis* DPC6108 and DSM32386 on the outcomes observed in this study. Furthermore, the potential for other metabolites to have a causative effect on the outcomes observed in this study should be explored. Therefore, future studies should employ a metagenomic approach coupled with metabolomic analysis to further elucidate the mechanisms of action of these metabolic- and psychological- health promoting *L. brevis* strains.

Finally, Chapter 7 presents polydextrose as a potential psychobiotic candidate to support cognitive function and is, to the best of our knowledge, the first human study investigating the impact of polydextrose on brain health. The use of nondigestible compounds that are metabolised by microorganisms in the gut is therefore an exciting prospect for modulating the gut microbiota and it will be important to carry out future studies to fully identify the underlying mechanisms

involved. The effect of microbial metabolites on hormonal concentrations in the blood have previously been suggested as potential mechanisms by which polydextrose can exert beneficial effects on the host (Tolhurst *et al.*, 2012). Although the data provided from this study are insufficient to draw conclusions on underlying mechanisms, it could be hypothesised that the prebiotic polydextrose improved cognitive function through reducing inflammatory status, potentially mediated by *Ruminiclostridium* 5 abundance. However, additional studies are required to decipher the potential function of *Ruminiclostridium* 5 in human physiology. Further studies are also warranted to fully understand the potential benefits of polydextrose on cognitive function and unravel underlying mechanisms.

8.6 Limitations of the reported studies

The predominant limitation encountered in Chapter 3 related to high background levels of serotonin detection in all samples, including the blanks, highlighting that caution is required when interpreting results from this assay. Tryptophan supplementation increased serotonin detection but variability masked improved measurement so should be used with caution. An additional ELISA was carried out to attempt to optimise this method by reducing the high background readings in the blank broths. A number of alterations were implemented for this ELISA, including using a lower concentration of tryptophan broth (0.1% instead of 0.75%) to try to reduce background levels. Furthermore, the standards and controls were prepared with broth instead of with the dilution buffer supplied in the kit, and water was also tested as a negative control to test for kit contamination. However, this ELISA was unsuccessful because the samples had readings above the four parameter logistic standard curve, so it was impossible to determine the percentage increase, possibly due to preparing the standards and controls with broth instead of reagents from the kit. Although the ELISA results were promising in that they seemed to confirm the serotonin production capability of the positive controls from the literature as well as identifying two potential producers from the MyNewGut biobank, the results from this assay should be viewed as preliminary.

Further work will be necessary to optimise this method, potentially by using minimal growth media to reduce the background readings in the broth. Similar to the ELISA method, HPLC analysis revealed a strong effect of tryptophan supplementation in the media as it was not possible to get a reliable reading of serotonin concentration for samples grown in tryptophan-supplemented broth, possibly due to interfering peaks which made it impossible to integrate a serotonin peak. Furthermore, high background levels of serotonin or 5-hydroxyindoleacetic acid were detected in all samples, including the blanks, highlighting that caution is required when interpreting results from this HPLC screen. An important observation from both of these screening methods is the necessity of using blank broths to control for the high background interference.

The main limitation of the environmental enrichment study described in Chapter 4 was the low n number of the experimental design which makes it challenging to draw definite conclusions from statistical analysis as the power is low. As mentioned previously, a pattern of responders and non-responders emerged when examining associations with the microbiome results and it was determined that this may be due to a colonisation effect from where the animals were reared. Upon further investigation it was found that a subsection of the high anxiety-related behaviour mice cohort was born in a different facility (CCB) to the remainder of the high anxiety-related behaviour mice and the entire normal anxiety-related behaviour cohort (PM1), which was consequently skewing the results. High anxiety-related behaviour mice born in the CCB facility maintained a very different microbiota profile despite being raised in the PM1 facility and clustered separately from the remainder of the high anxiety mice on PCoA plots. As a result of this, it was impossible to determine the true effect of environmental manipulation on microbiome composition and the subsequent impact on anxiety-related behaviour. Therefore, it was decided to re-analyse the results with the high anxiety mice from the CCB facility omitted which resulted in a low n number, reducing the power. This study highlights the important effect that the origin facility has on preclinical mouse trials such as this as it was determined separation observed on PCoA plots was as a result of different facilities of origin of the mice and not due to environmental

enrichment. Furthermore, microbiome composition was also more affected by facility of origin and differences were observed once the CCB mice were removed, strengthening the hypothesis that environment has a strong influence on the gut microbiome. Nevertheless, interesting results were observed from this study so future studies will be required to further investigate the impact of environmental enrichment on anxiety-like behaviour and the potential underlying role of the gut microbiota. The limitation of this study can be overcome by ensuring that facility of origin remains constant and subsequent studies are sufficiently powered. Once this is further understood, it will be interesting to carry out subsequent preclinical studies administering potential probiotic strains identified from this work to determine whether they can improve anxiety and depression-like behaviour. A significant challenge in microbiome research remains to determine whether microbiota alterations are causal or consequential of behavioural changes, but future studies administering candidate probiotics which were altered as a result of environmental intervention may provide clarity on this.

The main limitation of the bird study described in Chapter 5 is related to the sequencing method where 16S rRNA sequencing was employed which only provides data on community composition. A metagenomic approach would be a useful addition to animal studies such as this by providing an insight into the functional genes present in the microbiome, which could further elucidate the influence of diet, geography and phylogeny on the host microbiome as well as providing an insight into potential underlying mechanisms of action. It would also be useful to employ metabolomics profiling which would provide additional functional assessment of microbial products such as short chain fatty acids involved in gut-brain axis communication. Due to the large sample size of this study (~400 bird faecal samples), it was not possible to employ methods such as shotgun sequencing, but this would be useful for future studies, even if only performed on a subset of samples.

There are a number of limitations to the prebiotic study presented in Chapter 7 such as the relatively small sample size, however, this is partly mitigated by the cross-over study design employed here which allows for smaller variance in

outcome measures. Secondly, although 16S rRNA sequencing provides a large volume of data, it fails to generate information regarding the viability and functionality of the microbiota present and this limitation could have contributed to the limited amount of changes observed in the microbiota composition (Patterson *et al.*, 2014). Therefore, a more detailed taxonomic classification approach using shotgun metagenomic sequencing, which targets small fragments of metagenomic DNA to characterise the full genetic content and functional potential of the microbial community (Qin *et al.*, 2010), would be required to fully understand the impact of polydextrose on the microbiota composition. However, the lack of a dedicated reference database of gut microbial neuroactive metabolic potential represents a significant challenge for functional interpretation of metagenomes in microbiota-gut-brain axis research (Valles-Colomer *et al.*, 2019). Nevertheless, the subtle changes in the microbiome composition observed here are in line with previous observations (Roytio and Ouwehand, 2014). Our knowledge is also limited by the difficulty of obtaining samples from different regions of the GI tract in human intervention studies, which could explain the limited changes in SCFA concentrations detected in the faecal samples from this study. Additionally, most clinical studies have been performed in non-psychiatric patients; therefore, it will be important in the future to translate findings to large scale longitudinal studies in well-phenotyped psychiatric populations. Interventional studies in psychiatric patients with prebiotics coupled with monitoring changes in brain response after these interventions could provide additional information on mechanisms of action.

8.7 Conclusion

The data presented in this thesis reveal the impact of environmental and dietary factors on intestinal microbiota composition and the subsequent effect on anxiety and stress-related behaviour while also highlighting how modulation of the intestinal microbiota through prebiotic and bioactive metabolite-producing probiotic supplementation can positively shape health status. Furthermore, this thesis outlines the screening process of novel potential psychobiotic bacteria with a

focus on microbial GABA and serotonin production which are hypothesised to be particularly beneficial for psychological health. Positive microbial producers were then further characterised using whole genome sequencing, and probiotic characteristics such as antimicrobial production, acid tolerance and adhesion capability were assessed at the genomic level. These screening processes are fundamental for discovering the bioactive potential of future probiotics and microbiome manipulation using probiotics, particularly strains which produce neuroactive metabolites, are likely to play a promising role in the future prevention and treatment of immune, metabolic and psychiatric disorders. Before this can be achieved, a greater understanding of how probiotic bacteria mechanistically alter metabolic processes as well as how they interact with the host and the intestinal microbial ecosystem to impact health will be required. Recent advances in high throughput sequencing technology and metagenomic analysis will likely play central roles in identifying and characterising novel probiotics and investigating their subsequent impact on physiological and psychological health using optimally designed *in vivo* studies which utilise metagenomic and metabolomic approaches.

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Appendix 1: Publication Abstracts

The microbiota-gut-brain axis as a key regulator of neural function and the stress response: implications for human and animal health

Niamh C. Wiley, Timothy G. Dinan, R. Paul Ross, Catherine Stanton, Gerard Clarke, John F. Cryan

The brain-gut-microbiota axis comprises an extensive communication network between the brain the gut, and the microbiota residing there. Development of a diverse gut microbiota is vital for multiple features of behaviour and physiology, as well as many fundamental aspects of brain structure and function. Appropriate early-life assembly of the gut microbiota is also believed to play a role in subsequent emotional and cognitive development. If the composition, diversity, or assembly of the gut microbiota is impaired, this can have a negative impact on host health and lead to disorders such as obesity, diabetes, inflammatory diseases, and even potentially neuropsychiatric illnesses including anxiety and depression. Therefore, much research effort in recent years has focused on understanding the potential of targeting the intestinal microbiota to prevent and treat such disorders. This review aims to explore the influence of the gut microbiota on host neural function and behaviour, particularly those of relevance to stress-related disorders. The involvement of microbiota in diverse neural functions such as myelination, microglia function, neuronal morphology, and blood brain barrier integrity across the lifespan, from early life to adolescence to old age will also be discussed. Nurturing an optimal gut microbiome may also prove beneficial in animal science as a means to manage stressful situations and to increase productivity of farm animals. The implications of these observations are manifold, and researchers are hopeful that this promising body of preclinical work can be successfully translated to the clinic and beyond.

Production of Psychoactive Metabolites by Gut Bacteria

Niamh C. Wiley, John F. Cryan, Timothy G. Dinan, R. Paul Ross, Catherine Stanton

The gut microbiome plays a vital role in numerous aspects of physiology, including functions related to metabolism, the immune system, behaviour, brain structure and function. Furthermore, it is now becoming increasingly clear that alterations in microbial composition or diversity are implicated in several disease states including anxiety, depression, Autism Spectrum Disorder (ASD), Alzheimer's disease (AD), Parkinson's disease (PD), obesity and diabetes. Therefore, therapeutic targeting of the gut microbiota has the potential to be useful in the treatment of both stress-related disorders and metabolic diseases. An important method by which the gut microbiome can influence the gut-brain axis is through microbial production of psychoactive metabolites. Several bacteria have been shown to produce metabolites which can impact host health such as short chain fatty acids (SCFA), conjugated linoleic acid (CLA), antimicrobials, exopolysaccharides, and vitamins. Furthermore, several molecules with neuroactive functions, including serotonin, gamma-aminobutyric acid (GABA), catecholamines and acetylcholine have been isolated from bacteria within the human gut. This review aims to explore the psychoactive metabolites reported to be produced by gut bacteria, particularly those of relevance to stress-related disorders. Screening methods for psychoactive metabolite production, as well as the challenges and limitations of this research will also be addressed. Finally, the implications of metabolite production for neuropsychiatric disorders such as depression, anxiety, and stress; behavioural disorders such as ASD; and neurodegenerative disorders such as AD and PD will be discussed.

Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain–gut axis alterations

Marcel Van De Wouw, Marcus Boehme, Joshua M. Lyte, **Niamh Wiley**, Conall Strain, Orla O'Sullivan, Gerard Clarke, Catherine Stanton, Timothy G. Dinan, John F. Cryan

There is a growing recognition of the involvement of the gastrointestinal microbiota in the regulation of physiology and behaviour. Microbiota-derived metabolites play a central role in the communication between microbes and their host with short-chain fatty acids (SCFAs) being perhaps the most studied. SCFAs are primarily derived from fermentation of dietary fibres and play a pivotal role in host gut, metabolic and immune function. All these factors have previously been demonstrated to be adversely affected by stress. Therefore, we sought to assess whether SCFA supplementation could counteract the enduring effects of chronic psychosocial stress. C57BL/6J male mice received oral supplementation of a mixture of the three principle SCFAs (acetate, propionate and butyrate). One week later, mice underwent three weeks of repeated psychosocial stress, followed by a comprehensive behavioural analysis. Finally, plasma corticosterone, faecal SCFAs and caecal microbiota composition were assessed. SCFA treatment alleviated psychosocial stress-induced alterations in reward-seeking behaviour, increased responsiveness to an acute stressor and *in vivo* intestinal permeability. In addition, SCFAs exhibited behavioural test-specific antidepressant and anxiolytic effects, which were not present when mice had also undergone psychosocial stress. Stress-induced increases body weight gain, faecal SCFAs and the colonic gene expression of the SCFA receptors free fatty acid receptor 2 and 3 remained unaffected by SCFAs supplementation. Moreover, there were no collateral effects on caecal microbiota composition. Taken together, these data show that SCFAs supplementation alleviates selective and enduring alterations induced by repeated psychosocial stress and these data may inform future research into microbiota-targeted therapies for stress-related disorders.

Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome

Elaine Patterson*, Paul M. Ryan*, **Niamh Wiley**, Ilaria Carafa, Eoin Sherwin, Gerard Moloney, Elena Franciosi, Rupasri Mandal, David S. Wishart, Kieran Tuohy, R. Paul Ross, John F. Cryan, Timothy G. Dinan, Catherine Stanton. *Joint first authors

Metabolic and neuroactive metabolite production represents one of the mechanisms through which the gut microbiota can impact health. One such metabolite, gamma-aminobutyric acid (GABA), can modulate glucose homeostasis and alter behavioural patterns in the host. We previously demonstrated that oral administration of GABA-producing *Lactobacillus brevis* DPC6108 has the potential to increase levels of circulating insulin in healthy rats. Therefore, the objective of this study was to assess the efficacy of endogenous microbial GABA production in improving metabolic and behavioural outcomes in a mouse model of metabolic dysfunction. Diet-induced obese and metabolically dysfunctional mice received one of two GABA-producing strains, *L. brevis* DPC6108 or *L. brevis* DSM32386, daily for 12 weeks. After 8 and 10 weeks of intervention, the behavioural and metabolic profiles of the mice were respectively assessed. Intervention with both *L. brevis* strains attenuated several abnormalities associated with metabolic dysfunction, causing a reduction in the accumulation of mesenteric adipose tissue, increased insulin secretion following glucose challenge, improved plasma cholesterol clearance and reduced despair-like behaviour and basal corticosterone production during the forced swim test. Taken together, this exploratory dataset indicates that intervention with GABA-producing lactobacilli has the potential to improve metabolic and depressive- like behavioural abnormalities associated with metabolic syndrome in mice.

Diet induces parallel changes to the gut microbiota and problem-solving performance in a wild bird

Gabrielle L. Davidson, **Niamh Wiley**, Amy C. Cooke, Crystal N. Johnson, Fiona Fouhy, Michael S. Reichert, Ivan de la Hera, Jodie M. S. Crane, Ipek G. Kulahci, R. Paul Ross, C. Stanton, John L. Quinn

The microbial community in the gut is influenced by environmental factors, especially diet, which can moderate host behaviour through the microbiome-gut-brain axis. However, the ecological relevance of microbiome-mediated behavioural plasticity in wild animals is unknown. We presented wild-caught great tits (*Parus major*) with a problem-solving task and showed that performance was weakly associated with variation in the gut microbiome. We then manipulated the gut microbiome by feeding birds one of two diets that differed in their relative levels of fat, protein and fibre content: an insect diet (low content), or a seed diet (high content). Microbial communities were less diverse among individuals given the insect compared to those on the seed diet. Individuals were less likely to problem-solve after being given the insect diet, and the same microbiota metrics that were altered as a consequence of diet were also those that correlated with variation in problem solving performance. Although the effect on problem-solving behaviour could have been caused by motivational or nutritional differences between our treatments, our results nevertheless raise the possibility that dietary induced changes in the gut microbiota could be an important mechanism underlying individual behavioural plasticity in wild populations.

A Specific Dietary Fibre Supplementation Improves Cognitive Performance – an Exploratory Randomised, Placebo-Controlled, Crossover Study

Kirsten Berding, Caitriona M. Long-Smith, Carina Cabria*, Thomaz F. S. Bastiaanssen*, Marcel van de Wouw*, **Niamh Wiley***, Conall R. Strain, Fiona Fouhy, Catherine Stanton, John F. Cryan, Timothy G. Dinan.

* Authors contributed equally to this work

Rationale: The impact of the microbiota on the gut-brain-axis is increasingly appreciated. A growing body of literature demonstrates that use of prebiotics can manipulate the microbiota and affect host health. However, the influence on cognition and acute stress response is less well understood.

Objectives: The objective of this study was to investigate the efficacy of a dietary fibre, polydextrose (PDX), in improving cognitive performance and acute stress responses through manipulation of the gut microbiota in a healthy population.

Methods: In this double-blind, randomised, placebo-controlled, crossover design study, 18 healthy female participants received 12.5 g Litesse®Ultra (>90% PDX polymer) or maltodextrin for four weeks. Cognitive performance, mood, acute stress responses, microbiota composition, and inflammatory markers were assessed pre- and post-intervention.

Results: PDX improved cognitive flexibility as evidenced by the decrease in the number of errors made in the Intra-Extra Dimensional Set Shift (IED) task. A better performance in sustained attention was observed through higher number of correct responses and rejections in the Rapid Visual Information Processing (RVP) task. Although there was no change in microbial diversity, abundance of *Ruminiclostridium* 5 significantly increased after PDX supplementation compared with placebo. PDX supplementation attenuated the increase of adhesion receptor CD62L on classical monocytes observed in the placebo group.

Conclusions: Supplementation with the PDX resulted in a modest improvement in cognitive performance. The results indicate that PDX could benefit gut-to-brain communication and modulate behavioural responses.

Volatility as a Concept to Understand the Impact of Stress on the Microbiome

Thomaz F. S. Bastiaanssen*, Anand Gururajan*, Marcel van de Wouw, Gerard M. Moloney, Nathaniel L. Ritz, Caitriona M. Long-Smith, **Niamh C. Wiley**, Amy B. Murphy, Joshua M. Lyte, Fiona Fouhy, Catherine Stanton, Marcus J. Claesson, Timothy G. Dinan, John F. Cryan.
*Joint first authors

The microbiome-gut-brain-axis is a complex phenomenon spanning several dynamic systems in the body which can be parsed at a molecular, cellular, physiological, and ecological level. A growing body of evidence indicates that this axis is particularly sensitive to the effects of stress and that it may be relevant to stress resilience and susceptibility. Although stress-induced changes in the composition of the microbiome have been reported, the degree of compositional change over time, which we define as volatility, has not been the subject of in-depth scrutiny. Using a chronic psychosocial stress paradigm in male mice, we report that the volatility of the microbiome significantly correlated with several readouts of the stress response, including behaviour and corticosterone response. We then validated these findings in a second independent group of stressed mice. Additionally, we assessed the relationship between volatility and stress parameters in a cohort of healthy volunteers who were undergoing academic exams and report similar observations. Finally, we found inter-species similarities in the microbiome stress response on a functional level. Our research highlights the effects of stress on the dynamic microbiome and underscores the informative value of volatility as a parameter that should be considered in all future analyses of the microbiome.

A time-lagged association between the gut microbiome, nestling growth and nestling survival in wild great tits

Gabrielle L. Davidson*, Shane E. Somers*, **Niamh Wiley**, Crystal N. Johnson, Michael S. Reichert, R. Paul Ross, Catherine Stanton, John L. Quinn.

*Joint first authors

1. Natal body mass is a key predictor of viability and fitness in many animals. While variation in body mass and therefore viability of juveniles may be explained by genetic and environmental factors, emerging evidence points to the gut microbiota as an important factor influencing host health. The gut microbiota is known to change during development, but it remains unclear whether the microbiome predicts fitness, and if it does, at which developmental stage it affects fitness traits.

2. We collected data on two traits associated with fitness in wild nestling great tits (*Parus major*): weight and survival to fledging. We characterised the gut microbiome using 16S rRNA sequencing from nestling faeces and investigated temporal associations between the gut microbiome and fitness traits across development at day 8 (D8) and day 15 (D15) post-hatching. We also explored whether particular microbial taxa were 'indicator species' that reflected whether nestlings survived or not.

3. There was no link between mass and microbial diversity on D8 or D15. However, we detected a time-lagged relationship whereby the microbial diversity at D8 was negatively associated with weight at D15, while controlling for weight at D8, therefore reflecting weight gain over the intervening period. Indicator species analysis revealed that while several taxa were unique to birds that either survived or did not survive, there were no universal taxa that were consistently found across all birds within either survival group. This suggests that the presence of particular bacterial taxa may be sufficient, but not necessary for determining future survival, perhaps owing to functional overlap in microbiota.

4. We highlight that measuring microbiome-fitness relationships at just one time point may be misleading, especially early in life. Instead, microbial-host fitness effects should be investigated longitudinally as there may be critical development windows in which key microbiota are established and prime host traits associated with nestling growth. Pinpointing which features of the gut microbial community impact on host fitness, and when during development this occurs, will shed light on population level processes and has the potential to support conservation.

In vitro screening of human-derived lactobacilli for novel psychobiotic potential

Niamh C. Wiley, Timothy G. Dinan, John F. Cryan, Elaine Patterson, R. Paul Ross, Catherine Stanton

Psychobiotics are live microorganisms that when consumed, positively influence the gut microbiome to confer health benefits for neuropsychiatric illnesses. Accumulating preclinical and clinical evidence point towards the effectiveness of psychobiotics as bio-therapeutics to treat depression and anxiety. Commensal gut bacteria may influence the microbiota-gut-brain-axis through mechanisms such as: SCFA production, neurotransmitter release, activation of afferent sensory fibers of the vagus nerve, regulation of the immune system and regulation of tryptophan metabolism. MyNewGut is a project aimed at identifying the effects of environmental factors on the gut microbiome and its influence on brain, immune, and metabolic programming, development, and function. As part of this project, a biobank of future probiotics containing ~300 putative lactobacilli isolated from healthy adult males, born *via* Caesarean section but not prone to stress was created. These strains will be screened for psychobiotic potential; ability to produce neurotransmitters and neuroactive compounds which could influence the microbiota-gut-brain-axis. Serotonin is of particular interest because it plays an important role in the regulation of a number of bodily functions, including gastrointestinal tract motility, secretion, sensation and mood. There is significant overlap between behaviours regulated by the serotonergic system and those that

are influenced by the microbiota. *Lactobacillus* species from the biobank will be compared to the serotonin producer *Lactococcus lactis* subsp*. cremoris* using a commercially available ELISA kit and HPLC. The genomes of positive producers will be sequenced to identify serotonin production machinery. In the future, positive strains will be further analysed for probiotic characteristics (acid and bile tolerance) and studies will be carried out in preclinical models of anxiety and depression.