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The Gut Microbiota in Depression

Thesis presented by

Dr John Kelly

under the supervision of

Prof. Timothy G. Dinan

Dr. Gerard Clarke

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Department of Psychiatry

Head of Department:

Prof. Timothy G. Dinan

Contents

Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

All of the work conducted in this thesis was performed independently by the author with the following exceptions.

Dr Gerard Clarke performed the high performance liquid chromatography (HPLC) analysis on plasma samples to generate the tryptophan and kynurenine data.

Mr Ciaran O' Brien performed the 16sRNA microbiota sequencing.

Dr Elaine Patterson performed the gas chromatography for the SCFA analysis.

In *Chapter 3* Dr Yuliya Borre & Patrick Fitzgerald played a significant role in the animal behavioural work.

Mr Alan E. Hoban performed the Quantitative real-time PCR analysis on the hippocampal samples.

Chapter 4

Dr Andriy Temko assisted with the electroencephalography analysis.

Ms Aoife Collery, Ms Clara Seira, Ms Pauline Luczinski, Ms Livia Morais, and Dr Karen Scott acted as the confederate in the socially evaluated cold pressor test.

Signed

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John Kelly

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I dedicate this thesis to Wesley Van Oeffelen whose journey ended prematurely.

“Existence is quite weird - Man is a little germ that lives on an unimportant rock ball that revolves about an insignificant burning star on the outer edges of one of the smaller galaxies, and it is this funny tiny microbe, growing on this planet, which is way out somewhere, who has the ingenuity by nature of this magnificent organic structure to realize its own presence and to evoke the whole universe”. **Alan Watts**.

Publications and presentations

1. Kelly, J. R., Borre, Y., O'Brien, C., Patterson, E., El Aidy, S., Deane, J., Kennedy, P. J., Beers, S., Scott, K., Moloney, G., Hoban, A. E., Scott, L., Fitzgerald, P., Ross, P., Stanton, C., Clarke, G., Cryan, J. F. & Dinan, T. G. (2016). Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat. J Psychiatr Res 82, 109-118 (Kelly *et al.*, 2016b).
2. Kelly, J. R., Clarke, G., Cryan, J. F. & Dinan, T. G. (2016). Brain-gut-microbiota axis: challenges for translation in psychiatry. Ann Epidemiol 26, 366-72. (Kelly *et al.*, 2016c).
3. Kelly, J. R., Kennedy, P. J., Cryan, J. F., Dinan, T. G., Clarke, G. & Hyland, N. P. (2015). Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. Front Cell Neurosci 9, 392. (Kelly *et al.*, 2015).
4. Kelly, J. R., Allen A. P., Temko, A., Hutch, W., Kennedy P. J., Farid, N., Murphy, E., Bienenstock. J., Cryan, J. F., Clarke, G., Dinan, T. G. (2016). Lost in Translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects (Kelly et al., 2016a).

Conference Posters

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2. Kelly, J. R., Borre, Y., O' Brien, C., Patterson, E., El Aidy, S., Deane, J., Kennedy, P. J., Beers, S., Scott, K., Moloney, G., Scott, L., Fitzgerald, P., Ross, P., Stanton, C., Clarke, G., Cryan, J. F., Dinan, T. G. Gut Microbiome alterations in Major Depressive Disorder: Relevance to Pathophysiology. *9th Neuroscience Ireland Conference, Dublin, 1st – 2nd Sept 2015.*
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P., Stanton, C., Clarke, G., Cryan, J. F., Dinan, T. G. Transferring the Blues: Depression-Associated Gut Microbiota Induces Neurobehavioural Changes in the Rat. *New Horizons Conference 2015, University College Cork, 10th December 2015.*

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8. Kelly, J. R., Allen A. P., Temko, A., Hutch, W., Kennedy P. J., Farid, N., Murphy, E., Bienenstock. J., Cryan, J. F., Clarke, G., Dinan, T. G. (2016). Lost in Translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects. *Winter Conference of the College of Psychiatrists Ireland, Cavan, 10th-11th November 2016.*

Oral Presentations

1. Kelly, J. R., Borre, Y., O' Brien, C., Patterson, E., El Aidy, S., Deane, J., Kennedy, P. J., Beers, S., Scott, K., Moloney, G., Hoban, A. E., Scott, L., Fitzgerald, P., Ross, P., Stanton, C., Clarke, G., Cryan, J. F., Dinan, T. Gut Microbiome alterations in Depression: relevance to pathophysiology. *Irish society of Gastroenterology, Dublin, 19th & 20th November 2015 - (presented by Dr. Gerard Clarke).*
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Abstract

The accruing data linking the gut microbiota to the development and function of the central nervous system has been proposed as a paradigm shift in neuroscience. Neuroimmune, neuroendocrine and neural communication pathways exist between host and microbe. These pathways are components of the brain-gut-microbiota axis and preclinical evidence suggests that the microbiota can recruit this bidirectional communication system to modulate brain development, function and behaviour. Dysfunctional neuro-immune and neuro-endocrine pathways are implicated in stress-related psychiatric disorders. To this end, we proposed that the gut microbiota, by modulating these pathways, plays an influential role in the pathophysiology of depression.

We demonstrated that depression is associated with altered gut microbiota composition with decreased richness and diversity. Furthermore, we have shown that transferring the gut microbiota from depressed patients to microbiota-depleted rats can induce behavioural and physiological features characteristic of depression in the recipient animals, including anhedonia and anxiety-like behaviours, as well as alterations in tryptophan metabolism. Although we provide evidence that the gut microbiota is altered in depression and that this alteration could have a role in prominent features of depression, an interventional study based on targeting the gut microbiota in healthy males using *Lactobacillus rhamnosus* (JB-1) was not superior to placebo in modifying self-reported stress, HPA axis response to an acute stressor, inflammation, cognition or neurophysiological measures.

Taken together, these findings have furthered our understanding of the pathophysiology of depression. By incorporating the gut microbiota into existing neurobiological models of depression a more comprehensive model has been developed. The successful translation of this work could lead to stratification based on gut microbiome composition and could deliver further diagnostic accuracy to improve patient phenotyping for treatment selection in future studies in psychiatric populations.

Furthermore, our findings advance the possibility of targeting the gut microbiome in the treatment and prevention of stress related disorders and offer an important future strategy in psychiatry.

Chapter 1: General Introduction

1.1 Epidemiology of Depression

Depression is a complex, heterogeneous disorder, which accounts for almost 10% of all medical disability, making it the leading cause of medical disability in the United States and Canada, with an economic burden estimated at $210.5 billion dollars (Greenberg *et al.*, 2015, WHO, 2008). In Europe, the economic cost of depression corresponds to approximately 1% of the total economy (Sobocki *et al.*, 2006). Worldwide, depressive spectrum disorders affect 121 million people and by 2020, the WHO has predicted that depression will be second only to cardiovascular disease as the leading causes of total disease burden worldwide, measured by disability adjusted life years (Whiteford *et al.*, 2015, WHO, 2008).

20% of women and 15% of men suffer at least one episode of depression during their lifetime (Hirschfeld, 2012, Kessler *et al.*, 2007) and studies indicate that the prevalence of depression may be increasing, with an increased lifetime risk for younger cohorts (Andrade *et al.*, 2003, Hidaka, 2012). Of those that develop a depressive episode 50% will have another episode (Eaton *et al.*, 2008) and the risk of recurrence increases with residual symptoms (Nierenberg *et al.*, 2010) and with longer, more frequent, (1985, Kessing and Andersen, 2005) and more severe episodes (Kessing, 2004). A large (N=7076) prospective epidemiological survey, The Netherlands Mental Health Survey and Incidence Study (NEMESIS), found that 50% of depressed participants recovered within 3 months, 63% within 6 months, 76% within 12 months, whereas 20% did not recover at 24 months (Spijker *et al.*, 2002).

The median age of onset of depression is approximately 30 years (Kessler *et al.*, 2005), though up to 50% have had depressive symptoms prior to the identified episode (Horwath *et al.*, 1992). The median duration of a depressive episode is approximately 3 months (Eaton *et al.*, 2008, Spijker *et al.*, 2002). On average those with a history of depression will have on average 5 to 9 episodes (Kessler and Walters, 1998, Kessler *et al.*, 1997). The prevalence of depression declines in the community dwelling elderly (>65years) (Blazer and Hybels, 2005) and this decline does not appear to be due to increased confounding with physical disorders (Kessler *et al.*, 2010). However, not all studies are consistent (Forlani *et al.*, 2013).

Epidemiological studies have consistently demonstrated a higher lifetime prevalence of depression in women. Women have a higher lifetime prevalence of depression in high, middle and low income countries (WHO, 2008) and this higher prevalence is due to high risk of first onset of depression and not to persistence or recurrence (Kessler, 2003). The higher prevalence is not limited to depression, as women have higher rates of other stress-related disorders (Weich *et al.*, 2001). The higher rates of depression and anxiety in women have implications for future generations as maternal depression has negative effects on perinatal outcomes (Grigoriadis *et al.*, 2013). Indeed, preclinical studies indicate that maternal stress can be transferred to the next generation by a number of mechanisms, including epigenetic modification (Franklin *et al.,* 2010, Pena *et al.*, 2013).

However, it is interesting to note that women and men experience depression differently (Addis, 2008). Men are more likely to react with anger or self-destructive behaviour, and engage in substance abuse with lower levels of impulse control compared to women (Winkler *et al.,* 2005). In a secondary analysis of the National Comorbidity Survey Replication (Kessler *et al.*, 2003), men reported higher rates of anger, aggression, substance use and risk taking compared to women and when this is taken into account men and women met criteria for depression in equal proportions 30.6% men and 33.3% of women (Martin *et al.*, 2013).

1.2 Symptoms and Classification of Depression

The DSM-5 criteria for Major Depressive Disorder (MDD) requires, at least 5 of the following 9 symptoms, present nearly every day for more than two weeks; depressed mood or irritable most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful), decreased interest or pleasure (anhedonia) in most activities, most of each day, significant weight change (5%) or change in appetite, change in sleep: insomnia or hypersomnia, change in activity: psychomotor agitation or retardation, fatigue or loss of energy, guilt/worthlessness: feelings of worthlessness or excessive or inappropriate guilt, concentration: diminished ability to think or concentrate, or more indecisiveness, suicidality: thoughts of death or suicide, or suicide plan. Mood must represent a change from the person's baseline and impair social and occupational/educational function (American Psychiatric Association, 2013).

The current classification systems separate depression into several categorical subtypes including; melancholic, atypical, psychotic, postpartum, and seasonal affective disorder. The melancholic subtype is defined as lacking mood reactivity and loss of pleasure with at least three of the following: distinct quality of mood, mood worsening in the morning, early morning awakening, psychomotor retardation, significant anorexia or weight loss, and excessive or inappropriate guilt. The atypical subtype is defined as having mood reactivity and at least two of the following: hyperphagia, hypersomnia, leaden paralysis, and a longstanding pattern of interpersonal rejection sensitivity (American Psychiatric Association, 2013).

1.3 Health implications of Depression

The adverse consequences of depression are well established, and broadly encompass psychiatric and medical co-morbidities. Apart from the negative subjective experience and functional disability, chronic or recurrent depression results in an increased mortality rate (relative risk =1.5) (Osby *et al.*, 2001). The lifetime completed suicide rate for men is 7% and 1% for women (Blair-West *et al.*, 1999, Nordentoft *et al.*, 2011). Depression is highly co-morbid with other psychiatric disorders. Nearly half of those meeting lifetime criteria for depression also have met criteria for a comorbid anxiety disorder (Kessler *et al.*, 2008, Kessler *et al.*, 1996, Regier *et al.*, 1998, Shalev *et al.*, 1998) and 20% of those with depression have a co-morbid substance use disorder (Conway *et al.*, 2006, Grant *et al.*, 2004).

1.3.1 Cardiovascular disease

Depression worsens the prognosis of medical co-morbidities such as cardiovascular disease (CVD), diabetes and obesity. Depression is an independent risk factor for cardiac morbidity and mortality (Larsen *et al.*, 2013, Versteeg *et al.*, 2013) and the prevalence of depression in cardiac disease is 15 – 20% (Celano and Huffman, 2011). The relationship between CVD and depression is bidirectional, as depression can increase the risk of CVD and CVD can increase the risk of depression (Plante, 2005). Although a direct causal link between depression and CVD has not been established, the aetiology is likely to be multifactorial, and involves the interaction of the autonomic nervous system, the neuroimmune, the neuroendocrine and the vascular systems.

Depression is associated with over activity of the Hypothalamic Pituitary Adrenal (HPA) axis and Sympathetic Adrenal Medullary (SAM) axes, both of which can result in vascular endothelial cell damage (Joynt *et al.*, 2003). Inflammation, linked to endothelial dysfunction is the principal pathophysiological process in CVD and can lead to atherosclerosis and atherothrombosis (Halaris, 2013, Nymo *et al.*, 2014). Chronic stress can result in a pro-inflammatory state, and the increase of pro-inflammatory cytokines can result in platelet aggregation and contribute to atherosclerosis (Elsenberg *et al.*, 2013). Neurovascular dysfunction and hyper permeability of the blood-brain barrier due to oxidative stress and neuroinflammation are evident in depression (Najjar *et al.*, 2013a). Thus, stress acting via inflammatory pathways could be a common underlying mechanism that contributes to CVD and depression (see section **1.4.8**).

1.3.2 Diabetes

20% of people with diabetes have a co-morbid diagnosis of depression (Ali *et al.*, 2006). Type 2 diabetes mellitus is a risk factor for the development of depression, with a 15–24% increased risk compared to people without diabetes (Mezuk *et al.*, 2008, Nouwen *et al.*, 2011). A meta-analysis investigating the link between prevalence of depression, impaired glucose metabolism, undiagnosed diabetes, previously diagnosed type 2 diabetes and normal glucose metabolism found individuals with previously diagnosed diabetes have an increased risk of depression relative to those with impaired glucose metabolism or undiagnosed diabetes (Nouwen *et al.*, 2011). A co-morbid diagnosis of depression and diabetes is associated with an increased risk of all-cause mortality (hazard ratio = 1.5) (van Dooren *et al.*, 2013) and a number of adverse consequences including; lower levels of physical activity (Koopmans *et al.*, 2009), less healthy eating behaviours (Egede, 2005) and suboptimal glycemic control (Lustman and Clouse, 2005), lower quality of life (Schram *et al.*, 2009) and decreased medication adherence (Makine *et al.*, 2009).

The role of inflammation in the pathogenesis of type 2 diabetes is now well established (Donath, 2014). In a prospective study of community dwelling older adults both high C-reactive protein (CRP) levels and elevated depressive symptoms were associated with risk of diabetes (Au *et al.*, 2014). In a cohort of 1,790 adult participants with newly diagnosed type 2 diabetes, CRP, interleukin (IL) 1β, IL-1 receptor antagonist, and

monocyte chemotactic protein-1 were significantly associated with depressive symptoms in type 2 diabetes (Laake *et al.*, 2014).

1.3.3 Obesity/Metabolic syndrome

A meta-analysis of 15 studies, indicated that obesity was associated with an increased risk of depression and depression was associated with an increased the risk of developing obesity (Luppino *et al.*, 2010). A prospective cohort study involving 3054 participants found that the atypical subtype of depressive disorder at baseline resulted in an increase in adiposity during the 5.5 years of follow-up (Lasserre *et al.*, 2014). Consideration of metabolic parameters is important. A meta-analysis of 30,337 men and women aged 15–105 years, found that the metabolically healthy obese subjects had a slightly increased risk of depressive symptoms compared with non-obese, but the risk of depressive symptoms was greater in the metabolically unhealthy obese individuals (Jokela *et al.*, 2013). A prospective study of community dwelling older adults, found that metabolically unhealthy obese subjects had an increased risk of depressive symptoms at follow-up whereas the metabolically healthy obese did not (Hamer *et al.*, 2012).

Chronic low-grade inflammation has been suggested as a potential mediator linking depression, obesity and metabolic syndrome (Kraja *et al.*, 2007, Wellen *et al.*). The English longitudinal study of ageing found that obesity at baseline was associated with elevated levels of depressive symptoms at 4 year follow-up and that CRP explained approximately 20% of the obesity-related longitudinal change in depression scores (Daly, 2013). Despite the relationship between depression, obesity and metabolic syndrome, studies investigating weight reduction and dietary intervention on depressive symptoms are inconsistent. Although it has been suggested that a Mediterranean diet can reduce the incidence of depression (Sanchez-Villegas *et al.*, 2009), a large meta-analysis and meta-regression found no relationship between changes in weight and changes in symptoms of depression in lifestyle modification interventions (Fabricatore *et al.*, 2011). Conversely, a study using a subgroup of the Reduction of the Metabolic Syndrome in Navarra-Spain cohort (RESMENA-S) showed that a six month hypocaloric diet reduced depressive symptoms, as measured by the Beck Depression Inventory and reduced body fat and CRP levels (Perez-Cornago *et al.*, 2014).

1.3.4 Immune Disorders

As discussed above chronic medical disorders and depression co-occur at high levels (Farmer *et al.*, 2008). Similar to all medical disorders, the relationship between disorders of the immune system and depression is bidirectional and involves the interaction of biological, psychological and sociological factors. Multiple sclerosis, a demyelinating disease with CNS inflammation is associated with a range of neuropsychiatric manifestations (Feinstein *et al.*, 2013). Lifetime prevalence of major depression in multiple sclerosis can be as high as 50% (Feinstein, 2004, Minden and Schiffer, 1990). Accumulating evidence from preclinical studies suggests that some aspects of depression and fatigue in MS may be linked to inflammatory markers (Gold and Irwin, 2009). Similarly, disorders of the immune system that primarily involve systems outside the CNS, such as rheumatoid arthritis, sarcoidosis and psoriasis are associated with higher levels of depression (Chang *et al.*, 2001, Kurd *et al.*, 2010, Margaretten *et al.*, 2011, Soderlin *et al.*, 2000, Zyrianova *et al.*, 2006). Interestingly, patients with psoriasis treated with the monoclonal antibody, ustekinumab, reported significant improvements in symptoms of depression and anxiety compared to placebo (Langley *et al.*, 2010). For further discussion of the relationship between depression and immune dysregulation (see section **1.4.2**).

1.4 Neurobiology of Depression

For nearly four decades the prevailing biological theory stated that depression was a neurochemical disorder arising from dysfunction of brain monoamine systems including the serotonergic, noradrenergic, and/or dopaminergic pathways (Hirschfeld, 2000, Schildkraut, 1965). This hypothesis arose from observations that the administration of classical antidepressants increased monoaminergic function, whereas monoamine depleters such as reserpine altered mood (Davies and Shepherd, 1955, Freis, 1954, Shore *et al.*, 1955). Indeed, most currently available antidepressants enhance some aspect of monoaminergic function.

The noradrenergic system projects from the brainstem extensively throughout cortical and subcortical structures (Rinaman, 2011). Cell bodies containing noradrenaline are found within the relatively discrete locus coeruleus and within the lateral tegmental nuclei that are more loosely scattered throughout the ventral pons and medulla. Noradrenergic neurotransmission in the brain plays a key role in general cognitive

processes (Sara, 2009). The actions of noradrenaline are mediated by the family of G protein-coupled receptors known as the adrenergic receptors, and levels of extracellular noradrenaline are regulated by synaptic clearance via the noradrenaline transporter and modulation of noradrenaline metabolism (Cotecchia, 2010).

The serotonergic system develops early in the course of embryogenesis and has widespread projections from the dorsal raphe nuclei in the brainstem to the forebrain (Kinast *et al.*, 2013). Serotonin is synthesized from the essential amino acid L-tryptophan and the rate limiting step in the pathway is controlled by tryptophan hydroxylase (TPH) which converts tryptophan to 5-hydroxytryptophan. Tryptophan is required for protein synthesis and is the precursor to a number of other bioactives such as melatonin, tryptamine and kynurenines (Ruddick *et al.*, 2006). Preclinical studies have shown serotonin is involved in the modulation of anxiety, conditioned fear, stress responses, and reward (Asan *et al.*, 2013).

There are 15 subtypes of serotonin (5-HT) receptors, which are also found outside of the CNS. Serotonin is produced in the platelets (Berger *et al.*, 2009), with approximately 95% of the body's serotonin located within the gastrointestinal tract, primarily synthesised by enterochromaffin cells (Gershon and Tack, 2007). The presynaptic 5-HT1A autoreceptors detect serotonin in the extracellular space and modulate the activity of the serotonin neuron (Celada *et al.*, 2004). Meta-analyses of positron emission tomography studies reveal widespread reductions of approximately 10% in serotonin reuptake sites in major depression, mainly in the midbrain and amygdala (Gryglewski *et al.*, 2014) and decreased 5-HT1A binding potential in the raphe, medial temporal lobe, and medial prefrontal cortex (Savitz *et al.*, 2009). Indeed, certain patients with low CSF 5-hydroxyindoleacetic acid (5-HIAA) are prone to commit suicide (Mann *et al.*, 1996), though the lower concentrations of 5-HIAA are not specific to depression, and have also been linked to aggressive behaviour (Moore *et al.*, 2002).

Although the monoamine theory has enjoyed considerable support, it is overly simplistic. This complex brain disorder requires a systems level approach, encompassing an understanding of the interaction of the environment on genes, molecules, cells, circuits and physiology. Indeed, depression involves a combination of abnormalities in genetic (Hyde *et al.*, 2016, Karg *et al.*, 2011), reward circuitry (Russo and Nestler, 2013),

neuroendocrine (Stetler and Miller, 2011), neuroimmune (Dinan, 2009b, Dowlati *et al.*, 2010), and metabolic systems (Jokela *et al.*, 2014, Perez-Cornago *et al.*, 2014). At the cellular level deficits in synaptic plasticity (Duman and Aghajanian, 2012) and impaired neurogenesis (Eisch and Petrik, 2012) have been demonstrated. We propose here that the microbiome is an additional system that needs to be considered when understanding the neurobiology of depression.

1.4.1 Hypothalamic-pituitary-adrenal (HPA) axis in Depression

Stress can be physical or psychological and has been defined as a state where homeostasis is threatened or perceived to be threatened (Bradley and Dinan, 2010). The HPA axis is the core endocrine stress system, and when the brain perceives a threat, the HPA, in conjunction with the Sympathetic Adrenal Medullary (SAM) axis, is activated. The paraventricular nucleus (PVN) in the hypothalamus regulates the neuroendocrine response whereas the amygdala regulates the majority of the autonomic and behavioural stress reactions in the brain (Kovacs, 2013). At the behavioural level, the HPA functions to mobilize adaptive behaviours and peripheral functions while inhibiting biologically costly behaviours (e.g. feeding, reproduction, growth) thus priming the body to adapt successfully to the environmental perturbation.

At the molecular level corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) regulate HPA activity, both of which are synthesized in the PVN. These hormones act on the anterior pituitary gland and cause the release of adrenocorticotropic hormone (ACTH). ACTH stimulates the release of glucocorticoids (cortisol) from the adrenal cortex which bind with specific intracellular receptors called the mineralocorticoid (Type I) and glucocorticoid receptors (Type II) which are located in multiple tissues throughout the body. The system works as a negative feedback loop, whereby glucocorticoids modulate their own secretion by acting at various levels of the HPA axis, such as the hippocampus, hypothalamus and pituitary. Cortisol is the main glucocorticoid (GR) in humans and high doses of exogenous steroids and high concentrations of endogenous glucocorticoids, as occurs in Cushing's syndrome, can evoke depressive symptoms in some individuals (Marques *et al.*, 2009, Pereira *et al.*, 2010).

Stressful life events are strongly associated with depression and the vast majority of first episodes are preceded by such triggers (Kendler and Gardner, 2010). A meta-analysis of

361 studies, including 18,454 individuals demonstrated that depressed individuals had increased cortisol and adrenocorticotropic hormone levels but not corticotropin-releasing hormone (Stetler and Miller, 2011). Different subtypes of depression may have different HPA axis profiles and this meta-analysis showed that there were cortisol differences between sub-groups of depression, with more pronounced differences in older inpatients who display melancholic or psychotic forms of depression. However, linking HPA profiles to depression sub-groups has been inconsistent. A study from the Netherlands Study of Depression and Anxiety cohort (Vreeburg *et al.*, 2009) comprising 308 control subjects without psychiatric disorders, 579 persons with remitted major depressive disorder (MDD), and 701 persons with a current MDD diagnosis showed a significantly higher cortisol awakening response (CAR) in both the remitted and current MDD groups. Sub-group analysis revealed no association between CAR and atypical depression, though found higher CAR in those with co-morbid anxiety disorders.

Chronic stress exposure may contribute to HPA hyperactivity by disrupting the glucocorticoid negative feedback system. Rodent models of chronic stress indicate that GRs are down regulated in several stress-sensitive brain regions such as the prefrontal cortex (PFC) and hippocampus (Mizoguchi *et al.*, 2003). Early traumatic events, are of particular importance as they shape the development of neuroendocrine and neuro-inflammatory response systems (DeSantis *et al.*, 2011, Franklin *et al.*, 2010, Pena *et al.*, 2013) and increase the risk of depression later in life (Banyard *et al.*, 2001, Dube *et al.*, 2001, Faravelli *et al.*, 2012, Weber *et al.*, 2013). These traumatic events in adulthood do not have the same impact on the HPA axis, suggesting that traumatic events during the period of brain development may result in persistent changes in the reactivity of the HPA axis (Klaassens, 2010, Klaassens *et al.*, 2009).

1.4.2 Cytokine Hypothesis of Depression

Stress activates the innate immune system (Glaser and Kiecolt-Glaser, 2005). For example, stress induces an enhanced expression of proinflammatory factors cytokines (IL-1, IL-6, Interferon (IFN)-α, IFN-μ), macrophage migration inhibitory factor (MIF) (Bacher *et al.*, 1998) and cyclooxygenase-2 (Madrigal *et al.*, 2003). In clinical studies depression is associated with low grade inflammation with most consistent findings for elevations in IL-6, Tumour Necrosis Factor-α (TNF-α), and CRP (Dowlati *et al.*, 2010, Howren *et al.*, 2009), whereas negative acute-phase proteins (e.g. albumin, retinol

binding protein) are decreased (Maes, 1993). Increased levels of peripheral blood chemokines and cellular adhesion molecules, stress induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and T cells bearing T cell activation markers, such as CD2+CD25+, CD3+CD25+, and HLA-DR+ have also been described in depression (Maes, 2011). Notwithstanding the fact that meta-analyses consistently demonstrate increased peripheral inflammatory biomarkers at the group level in depression, it is likely that these biomarkers relate to sub groups of depressed patients, making up approximately one third of the total depressed group (Dinan, 2009a, Raison and Miller, 2013).

Some studies demonstrate that increased inflammation is a state dependent phenomena, with a return of inflammatory biomarkers to control levels post antidepressant treatment (Dahl *et al.*, 2014, Hannestad *et al.*, 2011, Miller *et al.*, 2009a, O'Brien *et al.*, 2006). Indeed, failure to respond to antidepressant treatment is associated with persistent elevations in inflammatory biomarkers (Fitzgerald *et al.*, 2006, Harley *et al.*, 2010, O'Brien *et al.*, 2007). In longitudinal studies, raised CRP and IL-6 are associated with the development of depressive symptoms (Valkanova *et al.*, 2013). In a prospective study, participants in the top third of IL-6 values compared with the bottom third at age 9 years were more likely to be depressed at age 18 years (Khandaker *et al.*, 2014).

Several studies demonstrate that depressed patients with suicidal ideation may differ from those who are not depressed (Janelidze *et al.*, 2011). A study of 76 depressed patients and 48 healthy controls using an inflammatory index composed of IL-6, TNF-α, IL-10, and CRP showed a significantly higher inflammatory index score in depressed patients with high suicidal ideation compared to depressed patients with lower suicidal ideation and controls (O'Donovan *et al.*, 2013). In a post mortem study using quantitative reverse transcriptase (RT)-PCR to measure mRNA expression in 34 completed suicide victims and 17 comparison subjects, there were higher levels of mRNA expression in the orbitofrontal cortex for IL-4 in females and IL-13 in male suicide victims compared to those who died by other causes (Tonelli *et al.*, 2008). Although this study should be interpreted with caution as protein levels were not measured, other post-mortem studies point to evidence of microglial activation in individuals who were depressed and died by suicide (Steiner *et al.*, 2008). Cytokines can induce depressive symptoms in non-depressed individuals (Raison *et al.*, 2005). A meta-analysis of 26 studies investigating

the administration of IFN-α in Hepatitis C patients demonstrated a 25% incidence of depression (Udina *et al.*, 2012) and this IFN-α induced depressive effect can be prevented and treated by antidepressants (Hauser *et al.*, 2002, Kraus *et al.*, 2008).

1.4.3 Sickness behaviour

Peripheral infection results in the activation of immune, endocrine, autonomic and behavioural changes in the host (Dantzer *et al.*, 2008). The adaptive sickness behaviour that ensues to conserve energy to combat acute inflammation comprises decreased motor activity, social withdrawal, reduced food and water intake, increased slow-wave sleep and altered cognition (Dantzer, 2001). Many of these factors are evident in depression (including behavioral inhibition, anorexia and weight loss, anhedonia, fatigue, hyperalgesia, malaise, anxiety and neurocognitive symptoms), however, whereas sickness behaviour is adaptive, the chronic inflammatory processes in depression, result in neural tissue damage and consequent functional and cognitive sequelae (Maes *et al.*, 2012a).

In both human and animal studies cytokines play a vital role in this process (for reviews see; (Dantzer and Kelley, 2007, McCusker and Kelley, 2013b). Peripheral or central administration of IL-1β or TNF-α to rodents induces the full spectrum of behavioural signs of sickness (Yirmiya, 1996) which occurs in a time and dose dependent manner. In contrast to IL-1β and TNF-α, IL-6 administered peripherally or centrally has no behavioural effect despite its ability to induce a fever response (Dantzer, 2001). Central administration of anti-inflammatory cytokines IL-10 or insulin-like growth factor I (IGF-I), a growth factor that behaves like an anti-inflammatory cytokine in the brain, attenuates behavioural signs of sickness induced by centrally injected lipopolysaccharide (LPS) (Bluthe *et al.*, 1991). Gram-negative bacteria have an asymmetric outer membrane, in which the inner leaflet consists of phospholipid and the outer leaflet is comprised of lipopolysaccharide (LPS) (Raetz and Whitfield, 2002). Peripheral administration of LPS induces the expression of IL-1β (van Dam *et al.*, 1992) and other pro-inflammatory cytokine mRNAs and proteins centrally (Quan *et al.*, 1999). Importantly, this expression occurs at doses of LPS that do not cause sepsis. The subclinical low-dose LPS skews macrophages into a mild proinflammatory state through cell surface TLR4, IL-1R-associated kinase-1, and the Toll-interacting protein, and induces activating transcription factor 2 through Toll-interacting protein-mediated generation of mitochondrial reactive

oxygen species, allowing mild induction of proinflammatory mediators (Maitra *et al.*, 2012). Indeed, both acute and chronic peripheral administration of LPS acting via TLRs increased the transcription of central IFN stimulated genes (Thomson *et al.*, 2014).

Furthermore, peripherally administered LPS stimulates brain 5-HT transporter (SERT) activity, and is associated with increased depressive like behaviours and this was dependent on IL-1R and p38 MAPK pathways (Zhu *et al.*, 2010). Antidepressants can attenuate the effects of LPS (Yirmiya *et al.*, 2001) and inhibit microglial TNF-α, nitric oxide production (Tynan *et al.*, 2012) and IL-6 production (Hashioka *et al.*, 2007). In humans it has been shown that low-dose endotoxemia can modulate emotional and cognitive functioning. In a double-blind, crossover study, 20 healthy male volunteers underwent an intravenous injection of Salmonella abortus equi endotoxin or saline in two experimental sessions and completed psychological and neuropsychological tests at 1, 3, and 9 hours post injection. The Endotoxin increased the circulating levels of TNF-α, soluble TNF receptors, IL-6, IL-1 receptor antagonist, and cortisol. After endotoxin administration, the subjects showed a transient significant increase in the levels of anxiety and depressed mood, verbal and nonverbal memory functions were significantly decreased. There were significant positive correlations between cytokine secretion and endotoxin-induced anxiety, depressed mood and decreases in memory performance (Reichenberg *et al.*, 2001). The endotoxin had no effects on physical sickness symptoms. Endotoxin induced increases in IL-6 and TNF-α levels have been shown to be associated with feelings of social disconnection, depressed mood (Eisenberger *et al.*, 2010a) and memory disturbance (Carroll *et al.*, 2011b, Cohen *et al.*, 2003, Krabbe *et al.*, 2005).

1.4.4 Mechanisms of cytokine effects on the brain

There are a number of possible mechanisms by which increased levels of cytokines in the periphery can reach and affect the brain. These have been summarized by Haroon et al and include passage through leaky regions in the BBB such as circumventricular organs, active transport through transport molecules, activation of cells lining the cerebral vasculature (endothelial cells and perivascular macrophages) binding to cytokine receptors associated with the vagus nerve, stimulating the HPA axis at the anterior pituitary or hypothalamus and recruitment of activated cells such as monocytes/macrophages from the periphery to the brain (Haroon *et al.*, 2012). More recently functional lymphatic vessels lining the dural sinuses have been discovered,

which serve as an additional route by which immune cells can communicate with the CNS (Louveau *et al.*, 2015).

Through activation of the intracellular signalling pathway mitogen-activated protein kinase, cytokines can increase the number and function of the reuptake pumps for serotonin, noradrenaline, and dopamine, which in turn can reduce the availability of these neurotransmitters within the synaptic cleft (Miller *et al.*, 2013). Preclinical studies have demonstrated that increased inflammatory cytokines reduce central levels of brain derived neurotrophic factor (BDNF) and neurogenesis, leading to depressive-like behaviour (Koo and Duman, 2008). However, the relationship between peripheral and central inflammatory markers and antidepressants is complex (Warner-Schmidt *et al.*, 2011) and it remains unclear which pathways are most relevant for cytokine signal transmission in stress related disorders such as depression.

There is some evidence, albeit from small studies of short duration, suggesting that anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDS) and cytokine inhibitors reduce depressive symptoms (Kohler *et al.*, 2014). For those depressed patients with raised inflammatory markers, this raises the prospect of whether reducing the low grade inflammation could reduce depressive symptoms. Although, a randomized controlled trial of the monoclonal antibody infliximab, a TNF-α antagonist, was not superior to placebo in reducing depressive symptoms overall, in those patients with high baseline CRP levels there were greater reductions in depressive symptoms than those with low CRP levels (Raison *et al.*, 2013). Another study showed that CRP level at baseline differentially predicted treatment outcome with escitalopram or nortriptyline (Uher *et al.*, 2014). These studies provide the impetus for stratification of depressed patients based on inflammatory profiles to advance personalized medicine. Though development of more nuanced profiles of inflammatory proteins and gene expression, as well as cellular immune parameters, likely represent the future for predictors and targets of response to anti-inflammatory therapies (Miller and Raison, 2015, Miller and Raison, 2016).

The brain regions most reliably identified as being most affected by administration of inflammatory stimuli include the basal ganglia and the dorsal anterior cingulate cortex (dACC). The dACC part of the brain's limbic system is involved in cognitive and

emotional processing. Cytokines can induce increases in neural activity most strongly in either the subgenual or the dorsal area of the dACC and have been associated with the development of mood and anxiety symptoms (Harrison *et al.*, 2009, Miller *et al.*, 2013, Slavich *et al.*, 2010). Cytokines can impair basal ganglia functioning by known inhibitory effects of cytokines on dopamine signalling in the CNS (Felger and Miller, 2012). Reductions in basal ganglia activity have been noted in more posterior regions, where they associate with fatigue, and in more ventral regions (such as the nucleus accumbens), where they have been associated with the development of anhedonia (Capuron *et al.*, 2012, Felger *et al.*, 2013).

1.4.5 Microglia

Microglia are central to the inflammatory process and a source of cytokines (Facci *et al.*, 2014). These phagocytic innate immune cells account for approximately 10% of cells in the brain (Prinz *et al.*, 2014) and contribute to the plasticity of neural circuits by modulating synaptic architecture and function (Graeber and Streit, 2010). Microglial process motility can be modulated by glutamatergic and GABAergic neurotransmission (Fontainhas *et al.*, 2011). Preclinical studies have shown that acute stress results in microglia activation and increased levels of proinflammatory cytokines in areas such as the hippocampus (Frank *et al.*, 2007) and hypothalamus (Blandino *et al.*, 2009, Sugama *et al.*, 2011).

Most studies show increases in activated microglia in response to chronic stress (Bollinger *et al.*, 2016, Hinwood *et al.*, 2011, Hinwood *et al.*, 2012, Tynan *et al.*, 2010). Preliminary changes in the microenvironment of the microglial may result in a susceptibility to a secondary inflammatory stimulus (Perry and Holmes, 2014). This concept of microglia priming may be of relevance to depression, which often requires multiple environmental “hits” (Fenn *et al.*, 2014). In an environmental two-hit rodent model in which the first experimental manipulation targeted pregnant dams, and the second manipulation was given to the resulting offspring, exposure to prenatal immune challenge and peripubertal stress synergistically induced pathological effects on adult behavioural functions and neurochemistry (Giovanoli *et al.*, 2013, Giovanoli *et al.*, 2015). Thus, early-life stress primes microglia, leading to a potentiated response to subsequent stress (Calcia *et al.*, 2016). Interestingly, the microbiota regulates microglia maturation and function (Erny *et al.*, 2015). Clinically, microglial activation in the PFC,

anterior cingulate cortex (ACC), and insula in medication free depressed patients has been demonstrated using translocator protein density measured by distribution volume in a positron emission tomography study (Setiawan *et al.*, 2015).

1.4.6 Tryptophan/kynurenine pathway

Tryptophan/kynurenine metabolism is one of the indirect mechanisms by which either cytokines or elevated cortisol can lead to depression. The enzyme indoleamine 2,3-dioxygenase (IDO) found in macrophages and microglia cells is the first and rate limiting step in the kynurenine pathway of tryptophan catabolism. The expression of tryptophan-2,3-dioxygenase (TDO) can be induced by circulating glucocorticoids (O'Connor *et al.*, 2009) and has been reported to be regulated by the gut microbiota during colonization (El Aidy *et al.*, 2014). Under normal physiological conditions, approximately 99% of tryptophan is metabolized to kynurenine in the liver by TDO. However proinflammatory cytokines such as IFN-γ, CRP, IL-1, IL-6 and TNF-α can induce IDO resulting in the metabolism of tryptophan to kynurenine. Kynurenine pathway metabolites such as hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid are neurotoxic while kynurenic acid is neuroprotective (Stone, 2000). Additionally, this process diverts tryptophan from the synthesis of serotonin, and could potentially result in decreased availability of serotonin (Halaris, 2013).

1.4.7 Compartmentalization of Central Kynurenine pathways

In the brain, kynurenine metabolism occurs in all cells, though the two kynurenine pathway branches are physically segregated into distinct cell types. Astrocytes contain kynurenine aminotransferases (KATs), not kynurenine 3-monooxygenase (KMO) and therefore cannot produce 3-hydroxykynurenine (3-HK) from KYN (Guidetti *et al.*, 2007). The end result of the metabolic pathway in astrocytes is Kynurenic acid (KYNA) (Gramsbergen *et al.*, 1997), whereas, in microglia due to the enzymatic machinery, it is quinolinic acid (Alberati-Giani *et al.*, 1996). Tryptophan and 3-HK can also cross the BBB and tryptophan’s conversion to kynurenine and 3-HK in the peripheral circulation can therefore contribute significantly to CNS levels, due to the low levels of brain IDO and TDO (Schwarcz 2012).

The kynurenine pathway has important implications for depression, as kynurenine can cross the BBB to increase central levels. Indeed, most of the CNS kynurenine is drawn

from the periphery and when metabolized further, as outlined above could result in excess production of neurotoxic metabolites (Myint and Kim, 2013). Metabolites, such as 3-hydroxykynurenine and quinolinic acid can directly or indirectly modulate several neurotransmitter systems, such as glutamatergic, GABAergic, dopaminergic and noradrenergic neurotransmissions, which in turn induce changes in neuronal-glial network (Myint and Kim, 2014). It has been postulated that depression may be related to an imbalance between quinolinic acid and kynurenic acid in the brain, with a relative abundance of quinolinic acid resulting in enhanced glutamatergic activity and symptoms of depression (Schwarcz and Stone, 2016).

There is an association between cerebrospinal fluid levels of kynurenine and quinolinic acid, and the development of depression during treatment with IFN-α (Raison *et al.*, 2010b). Moreover, increased quinolinic acid has been found in activated microglia in the ACC of suicide victims who were depressed. However, a recent study with 1042 subjects with current major depressive disorder from the Netherlands Study of Depression and Anxiety (NESDA) cohort, found that tryptophan/kynurenine did not mediate the relationship between CRP, IL-6 and depressive symptoms (Quak *et al.*, 2014).

1.4.8 Stress, HPA axis and Inflammation

Inflammatory pathways interact with the HPA axis (Leonard, 2000). In pre-clinical studies, administration of cytokines induces the production of CRH, ACTH and cortisol (Miller *et al.*, 2009a). In a clinical study of malignant melanoma patients, the first IFN-α dose induced the acute ACTH and cortisol response and correlated with the development of depressive symptoms (Capuron *et al.*, 2003). In addition, in the same study, all subjects exhibited significant increases in kynurenine, and the kynurenine/tryptophan ratio during IFN-α therapy. In the antidepressant free patients, decreases in tryptophan correlated with depressive, anxious, and cognitive symptoms suggesting that reduced tryptophan availability plays a role in IFN-α induced depressive symptoms. Chronic IFN-α administration is associated with flattening of the diurnal curve and increased evening cortisol concentration, which correlate with the development of depression and fatigue (Raison *et al.*, 2010a, Raison *et al.*, 2010b).

Stress, both acute and chronic, activates peripheral inflammatory pathways such as NF-κfB in humans. The Trier Social Stress Test (TSST) has been an important test in

determining the relationship between acute stress and inflammation. The TSST is a fifteen minute psychosocial stress protocol involving five minutes of anticipatory stress, five minutes of public speaking and five minutes of mental arithmetic performed in front of a panel of evaluators. The TSST is associated with a significant increase in the DNA binding of the inflammatory transcription factor NF-κB in peripheral blood mononuclear cells (PBMCs) compared with subjects who were spectators of the task (Bierhaus *et al.*, 2003). Early life traumatic events are associated with an exaggerated inflammatory response to the TSST. In adult subjects who were not depressed though had a history of early life traumatic events, there was an increased IL-6 response to acute stress (Carpenter *et al.*, 2010). There was also an increase in NF-κβ B DNA binding in PBMCs and IL-6 levels in depressed male patients with a history of early life stress compared to healthy controls (Pace *et al.*, 2006). The socially evaluated cold pressor test (SECPT) is a combined psychological (social-evaluative threat) and physiological (cold pressor) acute stressor procedure (Schwabe *et al.*, 2008) which also activates the sympathetic nervous system (SNS) and the HPA axis, and has been useful in elucidating the stress response in humans (Minkley *et al.*, 2014).

It is important to note, that in the context of chronic stress or depression, the immune system can become glucocorticoid (GR) resistant (Camilleri *et al.*, 2008, Cohen *et al.*, 2012). This GR resistance may account for the discrepancy between HPA activation and elevated inflammatory markers found in depression. Indeed, using a social defeat rodent model, GR resistance has been hypothesized as an adaptive mechanism that allows the inflammatory component of wound healing to occur in the presence of high levels of corticosterone (Avitsur *et al.*, 2001).

A study using data from 776 subjects from the Netherlands Study of Depression and Anxiety, including 111 chronic depressed subjects with melancholic depression, 122 with atypical depression and 543 controls found higher saliva cortisol awakening curves (area under the curve with respect to the ground (AUCg) and higher diurnal slope) in melancholic depression compared with atypical depression and controls. In the same study subjects with atypical depression had significantly higher levels of CRP, IL-6, TNF-α, body mass index (BMI), waist circumference and triglycerides, and lower high-density lipid cholesterol than persons with melancholic depression and controls (Lamers *et al.*, 2013). However, it is unclear why increased levels of cytokines in the melancholic

group are associated with higher saliva cortisol awakening curves, as cytokines are potent activators of the HPA axis (Dunn, 2000, Miller *et al.*, 2009b).

1.5 Current treatment options and limitations in Depression

As discussed above, the pharmacologic therapy for treatment of depression has focused on modulating concentrations of brain monoamines, namely noradrenaline, serotonin, and dopamine. As is common in drug discovery in psychiatry (Ban, 2006), the monoamine oxidase inhibitors (MAOIs) and the Tricyclic antidepressants (TCAs) were discovered by serendipity. Iproniazid, a non-selective, irreversible monoamine oxidase inhibitor (MAOI) of the hydrazine class was reported to produce euphoria and hyperactive behaviour in some patients treated for tuberculosis. MAOIs inhibit the breakdown of serotonin into 5-hydroxyindoleacetic acid (5-HIAA). However, the irreversible MAOIs had serious side effects, and their use was problematic because of the strict diet people needed to follow in order to prevent hypertensive reactions induced by food rich in tyramine, the so called “cheese reaction” (Youdim *et al.*, 2006).

The discovery of the TCAs is also interesting. Chlorpromazine was first synthesized in 1951 by Paul Charpentier, in the laboratories of Rhône-Poulenc, as an antihistamine and possible potentiator of general anaesthesia (Charpentier P *et al.*, 1952). The French surgeon Henri Laborit, used the new drug to lower body temperature before general anaesthesia and noted it sedative properties (Laborit *et al.*, 1952). Jean Delay and Pierre Deniker, tested it on agitated psychotic patients and it was noted to decrease psychotic symptoms (Delay *et al.*, 1952). Imipramine, the first Tricyclic antidepressant (TCA) was derived from chlorpromazine, but instead of reducing psychotic symptoms, it was noted to cause hypomanic symptoms in some of the schizophrenia patients (Kuhn, 1957).

TCAs block the serotonin transporter (SERT) and the noradrenaline transporter (NET) (Gillman, 2007). The selective serotonin reuptake inhibitors (SSRIs) were first discovered in 1972 (Carlsson et al., 1972) and fluoxetine was introduced clinically in 1987. As mentioned the serotonin transporter (SERT) reuptakes 5-HT from the synaptic cleft into the presynaptic neuron, and SSRI antidepressants act by inhibiting the SERT, resulting in an increase in 5-HT in the extracellular space. A recent X-ray crystallographic study has further defined the structure of the human SERT (Coleman *et al.*, 2016).

Acutely depleting tryptophan the precursor of serotonin, induces depressive symptoms in 50-60% of selective SSRI treated, and recovered depressed patients (Booij *et al.*, 2002). An extensive meta-analysis of 5HT depletion carried out in 2007, demonstrated that acute tryptophan depletion (ATD) resulted in a moderate decrease in mood in drug-free patients with MDD in remission and induced relapse in patients with MDD in remission who used serotonergic antidepressants (Ruhe *et al.*, 2007). Mood in healthy controls was unaffected, apart from those with a family history of MDD. Although, a meta-analysis of 24 studies involving 744 patients and 793 controls demonstrated a significant decrease in tryptophan in MDD patients, with unmedicated patients showing a greater decrease compared to medicated patients (Ogawa *et al.*, 2014) , though there is insufficient evidence for tryptophan as an augmenting strategy in the treatment of depression (Sarris *et al.*, 2016).

The serotonin-noradrenaline reuptake inhibitors (SNRIs), such as venlafaxine and duloxetine, enhance monoaminergic function by inhibiting neuronal reuptake of serotonin and noradrenaline to prolong its concentration and time in the synaptic cleft. Whereas bupropion inhibits neuronal reuptake of noradrenaline and dopamine. Other antidepressants enhance noradrenaline and serotonin release by blocking presynaptic α2 receptors (eg, mirtazapine) or blocking serotonin-2 receptors (eg, trazodone). Agomelatine has agonist properties at M1 and M2 receptors and antagonist properties at 5HT2C receptors (Pringle *et al.*, 2015) and has been shown to be superior to placebo in the treatment of depression (Taylor *et al.*, 2014). See **Table 1.1** for mode of action of Antidepressants.

Antidepressants are effective for certain groups, and can reduce risk of relapse (Geddes *et al.*, 2003). However, a significant percentage of depressed patients either do not respond or partially respond to treatment (Fava, 2003). The landmark STAR*D pragmatic effectiveness trial found that 30% of patients met criteria for remission during first line treatment with a selective serotonin reuptake inhibitor (citalopram) and 30% of patients did not respond after four treatment levels (Huynh and McIntyre, 2008, Trivedi *et al.*, 2006). However, as this trial did not contain a placebo group, remission rates that would have occurred without treatment cannot be accounted for and considering placebo

rates in depression studies are approximately 30% (Walsh *et al.*, 2002) suggests that response rates could be even lower.

Apart from suboptimal efficacy, currently available antidepressants are also limited by slow onset of therapeutic effect (Machado-Vieira *et al.*, 2008) and side effects (Anderson and Tomenson, 1995, Andrews *et al.*, 2015). The glutamatergic system has received recent focus (Caddy *et al.*, 2014, Duman, 2014, Swanson *et al.*, 2005). Several studies have demonstrated rapid antidepressant effects with the N-methyl-D-aspartate glutamate receptor (NMDAR) antagonist ketamine (Allen *et al.*, 2015, Murrough *et al.*, 2013, Price *et al.*, 2014). However, use of ketamine is restricted by dissociative and psychotomimetic adverse effects. A study using the low-trapping NMDA channel blocker lanicemine demonstrated rapid antidepressant effects with less dissociative and psychotomimetic adverse effects though with reduced efficacy compared to ketamine (Sanacora *et al.*, 2013). Recent evidence suggest that the antidepressant properties of ketamine may be mediated via metabolites acting through AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) rather than inhibition of NMDAR (Zanos *et al.*, 2016). Moreover, hallucinogens, such as psilocybin have recently been shown to be efficacious in treatment resistant depression (Carhart-Harris *et al.*, Carhart-Harris *et al.*, 2016). Additionally, antidepressant exploration has extended to cholinergic and opioid systems (Papakostas and Ionescu, 2015).

There is an imperative need for the development of conceptually novel therapeutic strategies for treating depression, with safe side effect profiles and limited abuse potential. This advance in therapeutics will be contingent on further elucidating the pathophysiological mechanisms underlying depression. Concurrently, identification of clinically useful biomarkers to facilitate selection of the most appropriate treatment option and to monitor the impact of treatment will undoubtedly improve patient outcomes (Gururajan *et al.*, 2016a).

Table 1.1 Antidepressants

Antidepressant Class	Drugs	Mode of Action
SSRIs	Citalopram, Escitalopram, Fluoxetine, Paroxetine, Sertraline	Enhance monoaminergic transmission by selectively preventing the reuptake of serotonin
SNRIs	Duloxetine, Venlafaxine	Enhance monoaminergic transmission by selectively preventing the reuptake of serotonin & noradrenaline
NaSSA	Mirtazepine	Increases noradrenergic & serotonergic neurotransmission by inhibition of presynaptic α2 and 5-HT2C receptors
NDRI	Bupropion	Inhibits reuptake of noradrenaline & dopamine
SARI	Trazodone	Inhibits the serotonin transporter & 5-HT2A and 5-HT2C receptor antagonism
TCAs	Amitriptyline, Clomipramine, Imipramine, Lofepramine, Nortriptyline, Trimipramine	Enhance monoaminergic transmission by non-selectively inhibiting the reuptake of serotonin & noradrenaline
MAOIs	Irreversible: phenelzine, tranylcypromine Reversible: moclobemide	Enhance monoaminergic transmission by selectively preventing the breakdown of monoamines by inhibiting the monoamine oxidase enzyme
Melatonergic agonist	Agomelatine	Agonist at M1 & M2 receptors and 5HT2C antagonist

SSRI: selective serotonin reuptake inhibitor, SNRI: serotonin-noradrenaline reuptake inhibitor, NaSSA: noradrenergic and specific serotonergic antidepressant, NDRI: noradrenergic–dopamine reuptake inhibitor, SARI: serotonin antagonist and reuptake inhibitor, TCA: tricyclic antidepressant, MAOI: monoamine oxidase inhibitors

1.6 Structure of the Gut Microbiota

Emerging evidence, mainly from animal studies, indicates that some of the same pathways, discussed above, that are dysregulated in depression are modulated by the microbes that inhabit the gut – the gut microbiota. Indeed, many of the proposed targets of current and novel antidepressants and anxiolytics can potentially be manipulated via the gut microbiota. It is increasingly recognized that the gut microbiota might influence the core symptoms of stress-related psychiatric disorders and that it might be a tractable target for symptom alleviation.

The mutualistic co-evolution of microbes and the human body, composed of more than 90% microbial cells and 10 million microbial genes has led to the collective being described as a "superorganism" (Nicholson *et al.*, 2005). The most heavily colonized area of the human body is the gut, with bacterial concentrations ranging from 10^1 - 10^3 cells per gram in the upper intestines to 10^{11} - 10^{12} bacteria per gram in the colon (Derrien and van Hylckama Vlieg, 2015, O'Hara and Shanahan, 2006). Although the functional significance of the microbiome has yet to be fully determined (Franzosa *et al.*, 2014), it is clear that an interlinked symbiotic relationship exists between host and microbe (Ley *et al.*, 2008b). In terms of bacterial phyla found in the gut, *Firmicutes* (species such as *Lactobacillus, Clostridium, Enterococcus*) and *Bacteroidetes* (species such as *Bacteroides*) account for the majority (Dethlefsen *et al.*, 2007), though the other phyla such as *Actinobacteria (Bifidobacteria), Proteobacteria (Escherichia coli), Fusobacteria, Verrucomicrobia* and *Cyanobacteria* are also present (Eckburg *et al.*, 2005, Qin *et al.*, 2010).

Differences exist between the microbiota composition between the gut lumen and the microbiota composition which lies in close proximity to the mucus layer. For instance, gram negative *Proteobacteria* and *Akkermansia muciniphila* (Verrucomicrobia), which use mucus as a carbon and nitrogen source, adhere and reside within the mucus layer (van Passel *et al.*, 2011). This gradient can be differentially regulated by factors such as stress (Johansson *et al.*, 2014, Johansson *et al.*, 2011, Rozee *et al.*, 1982, Swidsinski *et al.*, 2005).

Studies using different, but complementary, gut-microbiota directed interventions (Germ Free (GF) rodents, antibiotics, probiotics, gastrointestinal infection studies, and fecal

microbiota transplantation studies) have all suggested a number of possible brain-gut signalling pathways under the influence of the gut microbiota and capable of modulating brain and behaviour (Collins and Bercik, 2013, Cryan and Dinan, 2012b, Cryan and Dinan, 2015a, Dinan and Cryan, 2013, Grenham *et al.*, 2011, Mayer *et al.*, 2014, McVey Neufeld *et al.*, 2013, Rhee *et al.*, 2009).

1.7 Development of the Gut Microbiota across the lifespan

The developmental trajectory of the gut microbiota is compatible with concepts in psychiatry of the early-life period as a vulnerable phase for the subsequent emergence of psychopathology in adulthood (O'Mahony *et al.*, 2015b). In the initial days of life, the gut microbiota is unstable and of low diversity, shifting in composition over the first few years to resemble an adult like profile by age three (Voreades *et al.*, 2014). The effect of mode of delivery and the implications for CNS host development has attracted recent attention (Adlerberth and Wold, 2009, Clarke *et al.*, 2014a, Dominguez-Bello *et al.*, 2010). Vaginally delivered infants are colonized by the fecal and vaginal bacteria of the mother, most notably *Lactobacilli*, whereas infants delivered by Caesarean section (C-section) are colonized by other bacteria from the skin of the mother and from environmental sources including health-care workers, air, medical equipment and other newborns (Borre *et al.*, 2014). Other factors such as gestational age (Barrett *et al.*, 2013), feeding mode (Khanna *et al.*, 2014, Koenig *et al.*, 2011), antibiotic use, (Persaud *et al.*, 2014) and exposure to family members and pets (Dominguez-Bello *et al.*, 2010, Fujimura *et al.*, 2010, Marques *et al.*, 2010, Penders *et al.*, 2006) also influence the trajectory of microbiota acquisition. The relative importance of these factors in determining the eventual stable microbiota profile has not been fully elucidated.

A critical function of the microbiota is to prime the development of the neuroimmune system (Chistiakov *et al.*, 2014, Francino, 2014, Olszak *et al.*, 2012, Round and Mazmanian, 2009). The luminal surface of the gut is a key interface in this process (O'Hara and Shanahan, 2006). Alterations in the gut microbiota signature early in life can predispose to immune disorders (Penders *et al.*, 2007). Interestingly, emerging evidence suggests that infants integrate an initial microbiome profile prior to birth, and that both prenatal and postnatal maternal transmission is pivotal in shaping the structure of the microbiome (Funkhouser and Bordenstein, 2013, Gilbert, 2014, Jimenez *et al.*, 2008, Mueller *et al.*, 2015, Prince *et al.*, 2014, Rautava *et al.*, 2012). This challenge to the

prevailing sterile womb paradigm brings into focus the possible role of maternal microbiome transmission in the modulation of immune activation associated with maternal stress and the implications of this on fetal programming (Howerton and Bale, 2012).

Thus, it is plausible that subtle alterations in microbiota acquisition and maintenance, perhaps as early as the prenatal phase by influencing neuroimmune signalling pathways, may act as additional vulnerability factors that predispose to stress related disorders in adulthood. However, the precise microbiota signatures have yet to be determined. This concept is an extension of the hygiene hypothesis first proposed in the late 1980's (Strachan, 1989) and more recently reconceptualised as the "old friends hypothesis" (Rook *et al.*, 2003, Williamson *et al.*, 2015). This proposes that encountering less microbial biodiversity may contribute to the increase in chronic inflammatory disorders including subtypes of depression (Guarner *et al.*, 2006, Hidaka, 2012, Klerman and Weissman, 1989, Rook and Lowry, 2008, Rook *et al.*, 2013, Rook *et al.*, 2014, Stein *et al.*, 2016, 1992).

The gut microbiota patterns change as we age. There is a decrease in microbial diversity during this period and, in conjunction with diet, is associated with a poorer health status in this group (Claesson *et al.*, 2011, Claesson *et al.*, 2012). Furthermore, aging is characterized by chronic low-grade inflammation (termed "inflammaging") as evidenced by increased circulating levels of TNF-α, IL-6 and CRP; known to affect mood and cognition (Frasca and Blomberg, 2015). The fact that the gut microbiota are key regulators of immune function and inflammatory responses, it is likely that a change in the composition of the gut microbiota during ageing plays a role in the gradual activation of the immune system and consequently inflammaging (Prenderville *et al.*, 2015). The ELDERMET consortium demonstrated that the elderly have a distinct microbiota profile, characterised by greater inter-individual variation compared to younger adults (Claesson *et al.*, 2011). Of note, differences in microbiota composition were more pronounced between frail elderly subjects and healthy elderly subjects. Moreover, certain gut microbiota signatures were linked to measures of frailty, co-morbidity, nutritional status, and markers of inflammation (Claesson *et al.*, 2012).

1.8 Factors which influence composition and stability: diet, exercise, antibiotics

As mentioned above, a multitude of factors influence the composition of the microbiota, including genetics (Hufeldt *et al.*, 2010), diet (David *et al.*, 2014, De Filippo *et al.*, 2010, Turnbaugh *et al.*, 2009b), medication (Davey *et al.*, 2012), geography (Yatsunenko *et al.*, 2012), antibiotic use (Blaser, 2011) and stool consistency (Falony *et al.*, 2016). Although the composition of the gut microbiota, in the absence of insults remains relatively stable during adulthood, there are significant interpersonal differences (Hamady and Knight, 2009, Lozupone *et al.*, 2012a). Two recent population-based cohort studies investigating the gut microbiota have confirmed this remarkably high degree of inter-individual variation (Falony *et al.*, 2016). Consequently, there are multiple possible configurations for a healthy microbiome and it is also likely that some stable configurations are associated with disease (Relman, 2015). This concept of an entire ecosystem as a potential pathogen is a somewhat unfamiliar concept in clinical psychiatry. It is important also to appreciate that the functional output of multiple microbiome configurations may in fact be equivalent, given that concepts of redundancy and pleiotropy can also be applied to specific microbial members of the overall consortium.

It is important to state that the relevant features of dysbiosis that might be implicated in stress-related disorders have yet to be fully determined. However, like any ecosystem, diversity and stability brings resilience and these are some of the key indices for the overall health of a particular gut microbiome. For example, a deficiency in *Christensenellaceae* has been associated with obesity and a *C. minuta* amendment was sufficient to reduced weight gain in an animal study (Goodrich *et al.*, 2014). Thus, obesity for example, has been associated with a reduced diversity (Turnbaugh *et al.*, 2009a). Recently, a Mediterranean diet, suggested as protective for depression, has been associated with beneficial microbiome-related metabolomic profiles (De Filippis *et al.*, 2015) and there is increasing awareness of the role of a healthy diet in reducing the risk of depression (Opie *et al.*, 2015).

Indeed, diet is one of the most important modifiable determinants of human health which can profoundly alter the composition of the gut microbiota (Ley *et al.*, 2008a). Human gut microbiomes are less diverse that those of wild apes and have become more specialized for animal-based diets (Moeller *et al.*, 2014). The composition of the gut microbiome can change rapidly and converting from a low-fat, plant polysaccharide-rich

diet to a high-fat, high-sugar "Western" diet shifted the structure of the microbiota within a single day (Turnbaugh *et al.*, 2009b). Even during the course of a day, the gut microbiota, in both mice and humans, exhibits diurnal oscillations that are influenced by feeding rhythms, leading to time-specific compositional and functional profiles. Ablation of host molecular clock components or induction of jet lag leads to aberrant microbiota diurnal fluctuations and dysbiosis, driven by impaired feeding rhythmicity (Thaiss *et al.*). Exercise is another factor in the relationship between the gut microbiota, host immunity and host metabolism. GF mice show reduced levels of exercise and have lower liver, muscle, brown adipose and epididymal fat pad weight. In addition this study showed that the antioxidant enzyme system was altered in the GF group (Cerdá *et al.*, 2016, Hsu *et al.*, 2015). A human study investigating the gut microbiota in athletes from the Irish international rugby squad found a higher level of microbiota diversity, representing 22 distinct phyla, compared to 11 in the low BMI control group and to 9 in the high BMI control group (Clarke *et al.*, 2014b). This diversity was positively correlated with a diet rich in protein and creatine kinase. In addition athletes had lower levels of inflammatory markers and better metabolic profiles compared to the controls. *Akkermansia muciniphila* a mucin-degrading bacteria that resides in the mucus layer, shown in previous studies to be inversely correlated with obesity (Everard *et al.*, 2013, Karlsson *et al.*, 2012) was significantly higher in the athletes in the low body mass index (BMI) group compared with the high BMI group. This study highlights the complex interconnected relationship between diet, gut microbiota, exercise and the metabolic and immune system.

Antibiotics alter the composition of the gut microbiota (Dethlefsen and Relman, 2011) and repeated antibiotic exposure in the second or third trimester or in the first 2 years of life is associated with early childhood obesity (Bailey *et al.*, 2014, Mueller *et al.*, 2014). Furthermore, recurrent antibiotic exposure is associated with increased risk for depression (Lurie *et al.*, 2015).

1.9 From Brain-Gut axis to Brain-Gut-Microbiota axis

A recurring question surrounding the impact of the gut microbiome on the CNS and a possible impediment to integration of this research in psychiatry pertains to the uncertainty surrounding the mechanisms through which this influence can be exerted. The prevailing view currently is that the microbiome recruits the scaffolding provided by the brain-gut axis, a bidirectional communication pathway between the gut and brain

(Cryan and Dinan, 2012b). Studies using different but complementary approaches, such as, germ-free (GF) rodents, antibiotics, probiotics, gastrointestinal infection studies, and fecal microbiota transplantation studies have shown that the gut microbiota acting via the brain-gut axis contributes to the regulation of brain and behaviour (Cryan and Dinan, 2015b, Dinan and Cryan, 2013, Mayer *et al.*, 2014). There are several putative mechanisms by which the gut microbiota can achieve this; via modulation of the immune system (Erny *et al.*, 2015), the HPA axis (Sudo *et al.*, 2004), tryptophan metabolism (O'Mahony *et al.*, 2015a), the production of bacterial metabolites (Tan *et al.*, 2014) and via the vagus nerve (Bravo *et al.*, 2011) (see **Figure 1.1**). Interestingly, the epigenetic factors that play a role in shaping stress-related behaviours could arise as a consequence of host-microbe interactions (Dalton *et al.*, 2014, Stilling *et al.*, 2014a, Stilling *et al.*, 2014b).

1.10 Signalling pathways of the Brain-Gut-Microbiota axis

1.10.1 HPA axis

GF mice exhibit reduced levels of anxiety but increased levels of neuroendocrine responses to stress (Sudo *et al.*, 2004). It is evident that the microbiota are required for the normal development of the HPA axis and that there is a certain period in early life where colonisation must occur to ensure normal development of the HPA axis (Cryan and Dinan, 2012a). In a resistant stress animal model, GF mice had higher ACTH and corticosterone responses than specific pathogen free (SPF) mice. This exaggerated HPA stress response was partly corrected by reconstitution with SPF feces at an early stage but not at a later stage of development and completely corrected by reconstitution with *Bifidobacterium infantis* (Sudo *et al.*, 2004).

1.10.2 Toll-like receptors (TLRs)

TLRs are evolutionarily conserved type I transmembrane proteins that function as pattern recognition receptors (PRRs) that recognize microbial components (McCusker and Kelley, 2013a, Mogensen, 2009, Palsson-McDermott and O'Neill, 2007) and play an important role in the host defence system (Jeong and Lee, 2011). TLRs recognize microbe-associated molecular patterns (MAMPs) which are shared by many microorganisms. TLRs are expressed by a number of immune cells, including dendritic cells (DCs), macrophages, neutrophils, T cells, and B cells but are also found on non-immune cells, such as epithelial and endothelial cells (Hopkins and Sriskandan, 2005).

Without the microbiota certain TLR's are not expressed fully in the gut, affecting the proper functioning of the immune and neuroendocrine systems (O'Hara and Shanahan, 2006).

TLR2 recognizes lipoproteins and peptidoglycans from *Gram* positive bacteria whereas TLR4 mediates responses to lipopolysaccharide (LPS) primarily from *Gram* negative bacteria (Marteau and Shanahan, 2003b). It is well established that administration of LPS can result in behavioural changes including depressed mood, fatigue, cognitive dysfunction and feelings of social isolation (Eisenberger *et al.*, 2010b, O'Connor *et al.*, 2008, Reichenberg *et al.*, 2001). TLR signalling consists of at least two distinct pathways: a Myeloid differentiation primary response gene 88 (MYD88) dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the stimulation of IFN-β and the maturation of dendritic cells (Takeda and Akira, 2005). The TLR4 pathway consists of both pathways whereas the TLR2 pathway consists of only the MyD88-dependent pathway (Leclercq *et al.*, 2014a).

Activation of TLRs initiates signal transduction pathways and triggers the expression of genes that control innate immune responses and further guide development of antigen-specific acquired immunity (Akira and Takeda, 2004). Thus, TLRs might serve as a molecular channel between microbiota alterations and immune homeostasis (Rogier *et al.*, 2015). As well as playing a role in maintaining intestinal barrier function (Cario *et al.*, 2004, Rakoff-Nahoum *et al.*, 2004), TLRs also promote epithelial cell proliferation, secretion of IgA into the gut lumen and expression of antimicrobial peptides (Abreu, 2010). Dysregulation of this process, or excessive TLR activation, can result in chronic inflammatory and over-exuberant repair responses. Recent evidence suggests that the TLR3 synthetic agonist, Poly(I:C) not only decreases epithelial resistance in the small intestine but also promoted thinning of the mucosal layer (Moyano-Porcile *et al.*, 2015).

A study investigating the role of inflammatory pathways and gut-derived bacterial products in alcohol dependence syndrome, a disorder commonly co-morbid with depression (Regier *et al.*, 1990), demonstrated that LPS and peptidoglycans (PGN) cross the gut barrier and activate TLR4 and TLR2 in PBMCs (Leclercq *et al.*, 2014a). Chronic alcohol consumption inhibited the NF-kB pathway, but activated protein kinase/activator protein 1 pathway. IL-8 and IL-1ß were positively correlated with alcohol consumption,

whilst short term alcohol withdrawal was associated with the recovery of LPS but not PGN dependent receptors. The same group also demonstrated that increased intestinal permeability occurred in a sub group of alcohol-dependent subjects which was associated with higher depression and anxiety scores as well as an altered gut microbiota profile (Leclercq *et al.*, 2014b). A study showed that depressed patients had significantly elevated expression of TLR4 RNA and protein, NF-κB RNA and 16S rDNA compared to healthy controls, which the authors suggested may be related to bacterial translocation or to the presence of various damage-associated molecular patterns (Keri *et al.*, 2014).

1.10.3 Short Chain Fatty Acids (SCFAs)

The microbiota produce several bioactive metabolic products, including polysaccharides, lycosylceramides, nucleic acids, structural proteins, and SCFAs (Olle, 2013, Russell *et al.*, 2013). SCFAs (butyrate, acetate and propionate) are neurohormonal signalling molecules produced by certain classes of bacteria such as *Bacteroides, Bifidobacterium, Propionibacterium, Eubacterium, Lactobacillus, Clostridium, Roseburia, and Prevotella* (Macfarlane and Macfarlane, 2012). SCFAs are transported by monocarboxylate transporters, which notably are expressed at the BBB (Steele, 1986, Vijay and Morris, 2014). Indeed, a preclinical imaging study demonstrated that microbiota-derived acetate can cross the BBB where it can subsequently alter hypothalamic gene expression (Frost *et al.*, 2014). Furthermore, butyrate, has been shown to reduce depression and anxiety-related behaviours (Schroeder *et al.*, 2007, Wei *et al.*, 2015).

SCFAs are also pivotal in the maintenance of the intestinal barrier (Peng *et al.*, 2007, Ploger *et al.*, 2012, Suzuki *et al.*, 2008). Butyrate has also been shown to facilitate the association between transcription factors and the claudin-1 promoter (Wang *et al.*, 2012a), increase AMP-activated protein kinase (AMPK) activity (Peng *et al.*, 2009) and to reduce bacterial translocation (Lewis *et al.*, 2010). Interestingly, given the importance of butyrate in the maintenance of the intestinal barrier, IBS has been associated with a reduction in butyrate producing gut micro-organisms. It has, however, proven difficult thus far to demarcate the CNS consequences of SCFA-mediated effects on intestinal barrier function from a direct action in the brain. It is also notable that there is still considerable debate surrounding the ability of physiological levels of SCFAs to impact

substantially on relevant behaviours via central mechanisms, albeit that higher doses do have clear behavioural consequences (Macfabe, 2012, MacFabe *et al.*, 2007).

Other mechanisms by which the microbiota may signal to the underlying mucosa, or mucosal immune system, include delivery to an underlying subset of dendritic cells via small intestine goblet cells (Artis, 2008, McDole *et al.*, 2012). It has also been postulated that bacterial components can cross the intestinal barrier in small lipoprotein vesicles called exosomes which contain protein, nucleic acids, sugars and lipids. These exosomes can then transfer from dendritic cells to T cells in the draining lymph nodes and enter the circulation (Smythies and Smythies, 2014b). Consequently, T cells may receive epigenetic material from gut bacteria, either by direct endocytosis, or via afferent exosomes (Smythies and Smythies, 2014a). More recently, identification of "neuropods" as a pathway by which bacteria can communicate via intestinal enterochromaffin cells to the nervous system provides further insight into pathways responsible for gut to brain communication (Bohorquez *et al.*, 2015).

1.10.4 Neuroactives

Certain bacteria can produce neuroactive metabolites, including neurotransmitters and neuromodulators (Lyte, 2011, 2013, Wikoff *et al.*, 2009). The gut microbiome also appears to have a role in developmental programming of the brain, specifically, synapse maturation and synaptogenesis (Diaz Heijtz *et al.*, 2011). Synaptophysin, a marker of synaptogenesis, and PSD 95, a marker of excitatory synapse maturation, were decreased in the striatum in GF animals compared to SPF animals. This suggests that the microbiota may programme certain brain circuits when colonized by maternal microbiota, though the authors point out that exposure to gut microbiota metabolites during embryogenesis may also be a possible mechanism.

1.10.4.1 GABA

γ-Aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the CNS and implicated in the pathophysiology of depression and anxiety disorders. (Kumar *et al.*, 2013, Olivier *et al.*, 2013). Certain strains of *Lactobacillus* and *Bifidobacteria* can produce GABA by metabolizing dietary glutamate (Barrett *et al.*, 2012) although this is not viewed as a source of central GABA. Indeed, *Lactobacillus rhamnosus* was shown to reduce anxiety and depression related behaviour in mice and increase GABA levels in

the hippocampus (Bravo *et al.*, 2011). Interestingly, in vagotomised mice, these effects were not found, further supporting the concept that the vagus nerve is an important neural signalling pathway between the microbiota and brain. A recent preclinical magnetic resonance spectroscopy study adds further evidence to support the concept that oral *Lactobacillus rhamnosus* can increase central GABA levels (Janik *et al.*, 2015).

1.10.4.2 BDNF

A key regulator of synaptic plasticity and neurogenesis in the brain is the neurotrophin, BDNF (Monteggia *et al.*, 2004). Several studies have demonstrated that clinical depression is associated with reduced peripheral levels of BDNF (Allen *et al.*, 2015, Bocchio-Chiavetto *et al.*, 2010, Bus *et al.*, 2015, Molendijk *et al.*, 2014). In GF rodents levels of BDNF were reduced in the cortex and hippocampus in GF mice (Sudo *et al.*, 2004). In a study by Clarke and colleagues (2013) this finding was replicated, but in male mice only (Clarke *et al.*, 2013). However, not all studies are consistent. (Neufeld *et al.*, 2011) confirmed a decrease level of anxiety like behaviour in GF animals, but found an increase in BDNF mRNA in female mice. In addition there was a decrease in the NMDAR subunit NR2B mRNA expression in the amygdala and decreased serotonin receptor 1A in the hippocampus (Neufeld *et al.*, 2011).

1.10.5 Tryptophan metabolites with a focus on serotonin, kynurenine pathway metabolites and indoles

Serotonin is a critical signalling molecule in the brain-gut-microbiota axis (O'Mahony *et al.*, 2015a) and is involved in a wide range of physiological functions. In the gastrointestinal tract it plays an important role in secretion, sensing and signalling (Mawe and Hoffman, 2013). The largest reserve of 5-HT is located in enterochromaffin cells (Berger *et al.*, 2009). Emerging evidence also suggests that the serotonergic system may be under the influence of gut microbiota, especially, but not limited to, periods prior to the emergence of a stable adult-like gut microbiota (Clarke *et al.*, 2013, Desbonnet *et al.*, 2008, El Aidy *et al.*, 2012). A metabolomics study demonstrated that the gut microbiota has a significant impact on blood metabolites and showed a 2.8 fold increase in plasma serotonin levels when GF mice are colonized by gut microbiota (Wikoff et al., 2009).

Mucosal 5-HT has been demonstrated to play a direct role in the regulation of intestinal permeability. 5-hydroxytryptophan (5-HTP), a precursor of 5HT, significantly decreased

intestinal permeability in healthy control subjects and this was associated with a redistribution of ZO-1. Whilst in IBS patients 5-HTP resulted in a further decrease in occludin expression (Keszthelyi *et al.*, 2014). The gut microbiota itself is also an important, but frequently overlooked, regulator of 5-HT synthesis and secretion. For example, colonic tryptophan hydroxylase 1 (Tph1) mRNA and protein were increased in humanized germ-free and conventionally raised mice. Bacterial metabolites have also been demonstrated to influence Tph1 transcription in a human enterochromaffin cell model (Reigstad *et al.*, 2015). Others have demonstrated that distinct microbial metabolites produced by spore forming bacteria increase colonic and blood 5-HT in chromaffin cell cultures (Yano *et al.*, 2015).

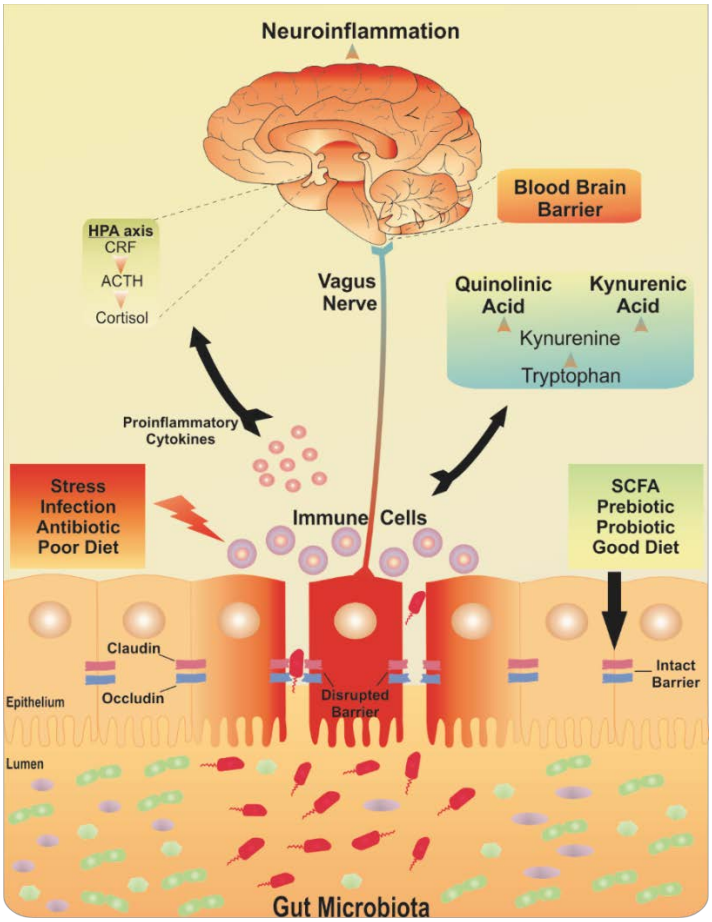


Figure 1.1 The brain-gut-microbiota axis. Postulated signalling pathways between the gut microbiota, the intestinal barrier and the brain.

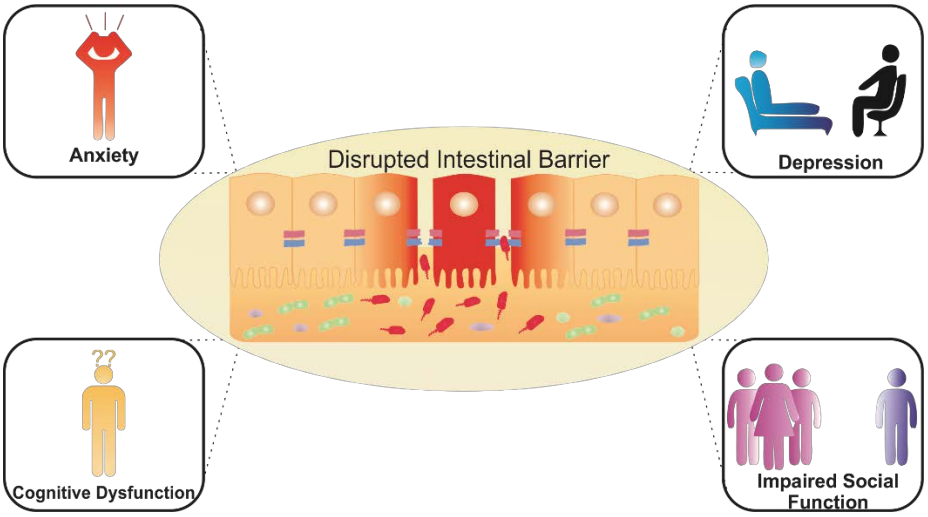


Figure 1.2: Potential neuropsychiatric consequences of a dysregulated intestinal barrier. Activation of brain-gut-microbiota Axis signalling pathways via a compromised intestinal barrier with potential effects on mood, anxiety, cognition and social interaction.

1.11 Stress and the Gut Microbiota

Stressful life events, particularly early life events are associated with the development of depression later in life (Bremner *et al.*, 2003, Heim *et al.*, 2000). The HPA is the core endocrine stress system and the interaction between stress, the HPA and the immune system is well established (Baes *et al.*, 2014, Hueston and Deak, 2014). Evidence suggests the gut microbiota mediates this interaction. Stress can reshape gut microbiota composition (Galley *et al.*, 2014a, Galley *et al.*, 2014b, O'Mahony *et al.*, 2009, Wang and Wu, 2005). For example, early life maternal separation resulted in a significant decrease in fecal *Lactobacillus* numbers on day 3 post separation which was correlated with stress related behaviours in rhesus monkeys (Bailey and Coe, 1999). In a mouse model of social disruption, stress altered the gut microbial profile and increased the levels of the pro-inflammatory cytokine IL-6 (Bailey *et al.*, 2011).

As previously mentioned, the most influential effect of the gut microbiota may occur early in life during critical neurodevelopmental stages (Borre *et al.*, 2014). Preclinical evidence suggests that the gut microbiota signature acquired and maintained during these pivotal stages may affect stress reactivity. GF rodents demonstrate abnormal behavioural and neuroendocrine responses to stress (Crumeyrolle-Arias *et al.*, 2014, Moloney *et al.*, 2014, Nishino *et al.*, 2013, Sudo *et al.*, 2004) and the normal development of the HPA

axis is contingent on microbiota colonisation at specific neurodevelopmental time points (Sudo *et al.*, 2004). The expression of anxiety-like behaviour in a mouse model of early life stress is partially dependent on the gut microbiota (De Palma *et al.*, 2015). Furthermore, using an olfactory bulbectomy model of chronic depression, elevated central CRH expression occurred concomitantly with changes in the gut microbiota (Park *et al.*, 2013). Collectively, these preclinical studies, establish a relationship between the gut microbiota, stress, and depressive and anxiety-related behaviours.

Recent evidence suggests that prenatal stress also impacts the gut microbiota with implications for physiological outcomes in the offspring (Golubeva *et al.*, 2015). In a mouse model of prenatal stress, maternal stress decreased the abundance of vaginal *Lactobacillus*, resulting in decreased transmission of this bacterium to offspring, which corresponded with changes in metabolite profiles involved in energy balance, and with disruptions of amino acid profiles in the developing brain (Jasarevic *et al.*, 2015a, Jasarevic *et al.*, 2015b). Clinical studies examining the relationship between prenatal stress and the gut microbiota are starting to emerge but are far from definitive. In one such study, infants of mother's with high self-reported stress and high salivary cortisol concentrations during pregnancy had significantly higher relative abundances of *Proteobacterial* groups known to contain pathogens and lower relative abundances of lactic acid bacteria (*Lactobacillus*) and *Bifidobacteria* (Zijlmans *et al.*, 2015). It is currently unclear whether this effect was mediated via maternal microbial transmission or through cortisol-specific effects on the developing gastrointestinal tract. Whatever the mechanism, those infants with altered microbiota composition, exhibited a higher level of maternally reported infant gastrointestinal symptoms and allergic reactions, highlighting the functional consequences of aberrant colonisation patterns.

1.12 Fecal Microbiota Transplantation (FMT)

A number of studies have shown that it is possible to transfer characteristics from donor to recipient via fecal microbiota transplantation. For example, the transfer of microbiota from obese mice to lean mice can result in weight gain and obesity associated metabolic profiles (Ridaura *et al.*, 2013). Furthermore, transfer of the human maternal gut microbiota in pregnancy to GF rodents, induces metabolic changes such as greater adiposity and insulin insensitivity, resembling the metabolic changes that occur in

pregnancy (Koren *et al.*, 2012). This has been extended beyond metabolic system, as phenotype transfer via microbiota has also been demonstrated for behaviour.

When the microbiota from BALB/c mice was transferred into adult GF NIH Swiss mice, the result was a decrease in exploratory behaviour and when the NIH Swiss microbiota was transferred into the BALB/c GF mice there was an increase in exploratory behaviour, associated with an increase in hippocampal BDNF levels (Bercik *et al.*, 2011b). Thus, the anxiety-like behavioural phenotype could be transferred via the microbiota, independent of the autonomic nervous system, gastrointestinal specific neurotransmitters or inflammation. The reverse situation has also been demonstrated, as restoration of normal gut microbiota normalised anxiety like behaviour in GF mice (Clarke *et al.*, 2013). More recently, it has been shown that mice that received an obesity associated microbiota exhibit more anxiety-like behaviours associated with increased evidence of neuroinflammation compared to controls (Bruce-Keller *et al.*, 2015).

1.13 Stress and Intestinal Permeability

Stress can impact on the developmental trajectory of the intestinal barrier (Lennon *et al.*, 2013, Smith *et al.*, 2010) and has been associated with an increase in gut permeability (Soderholm *et al.*, 2002). The effects of stress on intestinal permeability are complex and likely involve both the gut and the brain. Corticotrophin releasing hormone (CRH) and its receptors, CRF R1 and CRF R2, play a key role in stress-induced gut permeability dysfunction (Overman *et al.*, 2012, Rodino-Janeiro *et al.*, 2015, Taché and Million, 2015). In response to an acute stressor, colonic paracellular permeability increases and has been associated with the development of visceral hypersensitivity (Ait-Belgnaoui *et al.*, 2005). Early life stress has also been demonstrated to enhance plasma corticosterone in rat pups and is associated with an increase in intestinal permeability and bacterial translocation to liver and spleen. This effect appeared to predominate in the colon (Moussaoui *et al.*, 2014). Human studies further confirm that acute-stress paradigms can affect intestinal permeability. In a public speaking based stressor, small intestinal permeability was significantly increased, however, this was only observed in those subjects who also responded with a significant elevation of cortisol. In a different acute stress model using a cold pain stressor, albumin permeability increased, though in females only (Alonso *et al.*, 2012).

Of note, stress-induced changes in the HPA axis and autonomic nervous system display sensitivity to probiotic intervention (*L. helveticus* R0052 and *Bifidobacterium longum* R0175); (Ait-Belgnaoui *et al.*, 2014). Moreover, *L. helveticus* R0052 and *B. longum* R0175 also restored colonic TJ integrity in stressed mice (Ait-Belgnaoui *et al.*, 2014). Probiotics have also been demonstrated to influence bacterial adhesion and translocation to mesenteric lymph nodes in response to stress (Zareie *et al.*, 2006). *L. farciminis* in particular not only suppresses stress-induced changes in permeability, HPA axis activity, endotoxaemia and neuroinflammation (Ait-Belgnaoui *et al.*, 2012), but also beneficially influences the mucus barrier (Da Silva *et al.*, 2014).

1.14 Leaky gut hypothesis

A dysfunctional intestinal barrier could permit a microbiota-driven proinflammatory state with implications for the brain (see **Figure 1.2**). The sequence of this process is not yet clear. An increase in gut permeability could precede mucosal inflammation to induce the inflammatory response and thus culminate in a feed forward cycle between inflammatory responses and barrier dysfunction. This could subsequently maintain and exacerbate the low grade inflammatory response. Alternatively, systemic inflammation could increase intestinal barrier permeability and thus allow translocation of commensal bacteria with further implications for systemic inflammation. Irrespective of the sequence, both processes could engage the gut microbiota.

The source of the low grade inflammation which has been consistently demonstrated in sub groups of depressed patients has not been isolated to a particular source (Berk *et al.*, 2013, Dinan, 2009b, Jokela *et al.*, 2015, Raison and Miller, 2013). The concept that a dysfunctional intestinal barrier, or “leaky gut” could permit a microbiota-driven proinflammatory state has gained traction (Julio-Pieper *et al.*, 2014, Kelly *et al.*, 2015, Maes, 2008, Maes *et al.*, 2008). Under normal conditions, immune cells are separated from gram negative bacteria in the gut. However, a permeable intestinal barrier could allow certain gram negative bacteria such as *Enterobacteriaceae* to translocate and activate inflammatory pathways (Berg and Garlington, 1979, Lucas and Maes, 2013, Wiest and Garcia-Tsao, 2005).

Higher IgA and IgM-mediated immune responses directed against LPS of certain commensal gram negative gut bacteria have been shown in depressed patients (Maes *et al.*, 2008, Maes *et al.*, 2012c, Maes *et al.*, 2013). The implication being that the presence of such responses may have occurred subsequent to disruption of the intestinal barrier. Moreover, bacterial DNA has been detected in whole serum from depressed patients who also displayed increased TLR4 expression on peripheral mononuclear blood cells compared to healthy controls, which may be related to bacterial translocation (Keri *et al.*, 2014).

1.15 Microbiota and the Blood Brain Barrier

Structural similarities exist between the intestinal, the placental and the BBB (Doran *et al.*, 2013). The BBB is a complex neurovascular unit (Bauer *et al.*, 2014) consisting of CNS endothelial cells which separate the lumen of blood vessels from the CNS parenchyma. Tight Junctions, astrocytes and pericytes seal the capillary endothelial cells of the BBB (Daneman and Rescigno, 2009). The TJ transmembrane proteins claudins, tricellulin and occludin restrict paracellular diffusion of water-soluble substances from blood to the brain (Hawkins and Davis, 2005). Preclinical evidence from GF mice suggests that the microbiota can modulate the BBB. Exposure of GF adult mice to the fecal microbiota from pathogen-free donors decreased BBB permeability and increased the expression of TJ proteins (Braniste *et al.*, 2014). Moreover, monocolonization of the intestine of GF adult mice with SCFA producing bacterial strains normalised BBB permeability whilst sodium butyrate was associated with increased expression of occludin in the frontal cortex and hippocampus (Braniste *et al.*, 2014). This study strengthens the hypothesis that the BBB may also be vulnerable to changes in the gut microbiota (Frohlich *et al.*, 2016).

1.16 Gut Microbiota in Irritable Bowel Syndrome and Inflammatory Bowel Disease

Irritable bowel syndrome (IBS) is a stress related functional brain-gut-microbiota axis disorder associated with an altered gut microbiota profile (Carroll *et al.*, 2011a, Collins, 2014, De Palma *et al.*, 2014, Jeffery *et al.*, 2012b, Rajilic-Stojanovic *et al.*, 2015, Simrén, 2014) and increased intestinal permeability (Camilleri *et al.*, 2012, Dunlop *et al.*, 2006, Rao *et al.*, 2011). Moreover, a significant proportion of IBS patients also suffer from depressive and anxiety symptoms (Lucas *et al.*, 2014, Singh *et al.*, 2012) and this increases with the frequency and severity of gastrointestinal symptoms (Pinto-Sanchez

et al., 2015). In addition, alterations in brain circuits involved in attention, emotion, pain (Blankstein *et al.*, 2010, Labus *et al.*, 2009, Tillisch *et al.*, 2013a) together with deficits in hippocampal-mediated visuospatial memory (Kennedy *et al.*, 2014a, Kennedy *et al.*, 2012) have been noted the disorder. In particular, an altered *Firmicutes: Bacteroidetes* ratio has been linked to IBS and an association between *Firmicutes, Proteobacteria* and IBS symptom scores has been demonstrated (Rajilic-Stojanovic *et al.*, 2011).

Traditionally, there have been a number of different diagnosis methods which are now unified within the Rome framework, at least for research purposes (Soares, 2014). A recent meta-analysis of clinical studies to identify and assess the various diagnostic tests indicated that a combination of intestinal permeability, Rome I criteria and fecal calprotectin (see **Table 1.2** for brief description of Intestinal Permeability markers) provided the highest positive likelihood ratio for predicting IBS (Sood *et al.*, 2015). Although not captured in this meta-analysis, and not part of routine clinical practice, the addition of gut microbiota profiling may deliver further diagnostic accuracy (Casen *et al.*, 2015). For example, IBS subtypes have been stratified according to their microbiota profiles, specifically those with an increased *Firmicutes: Bacteroidetes* ratio (Jeffery *et al.*, 2012a). Furthermore, depression was the most robust clinical discriminator between a high *Firmicutes: Bacteroidetes* ratio in IBS patients relative to IBS patients with a healthy-like microbiota signature (Jeffery *et al.*, 2012a). In addition, the order *Actinomycetales* and the family *Actinomycetaceae* were inversely associated with clinically significant depression (Jeffery *et al.*, 2012a).

Although the microbiota varies along the length of the gastrointestinal tract, the majority of studies use fecal microbiota sampling as a representative of global changes, however site specific changes may influence the ensuing immune consequences. For example mucosal jejunal tissue from diarrhoea-predominant IBS patients is also associated with increased activation of mucosal B lymphocytes, plasma cells and mucosal IgG production. (Vicario *et al.*, 2014). Of note in this study, humoral activity markers positively correlated with depressive symptoms (Vicario *et al.*, 2014). Further studies need to be conducted to disentangle the contributing role of an exaggerated or aberrant immune response, changes in intestinal permeability and psychiatric co-morbidities in IBS. However, individuals with a pre-existing psychological disorders are known to be

at an increased risk of developing post-infectious IBS (PI-IBS) in particular (Thabane and Marshall, 2009).

Variations in several genes associated with bacterial recognition, the inflammatory response and epithelial integrity including TLR9, IL-6 and cadherin 1 genes have been identified as risk factors for the development of PI-IBS. A longitudinal study which examined the rate of IBS development following an accidental outbreak of *E. coli* 0157:H7 into a town's (Walkerton) water supply identified an increased rate of IBS two years after the outbreak (Marshall, 2009). A subsequent study further identified a modest increase in intestinal permeability amongst this IBS cohort (Marshall *et al.*, 2004). An association between this outbreak and depression was also identified (Garg *et al.*, 2006). Similarly, an outbreak of shiga toxin-producing *E. coli* O104 in Germany increased self-reported depressive and anxiety symptoms measured six months after the infection (Lowe *et al.*, 2014).

Inflammatory bowel disease (IBD), a gastrointestinal disorder with overt inflammation is also associated with intestinal barrier dysfunction (Antoni *et al.*, 2014, Laukoetter *et al.*, 2008, Marchiando *et al.*, 2010) increased intestinal permeability (Gerova *et al.*, 2011) immune dysregulation and an altered gut microbiota (Sartor and Mazmanian, 2012). IBD is also associated with a higher prevalence of anxiety and depressive disorders (Walker *et al.*, 2008). Moreover, stress can adversely affect the course of IBD (Mawdsley and Rampton, 2005, Mittermaier *et al.*, 2004). Both Crohn's disease and Ulcerative colitis exhibit alterations in the expression of the TJ proteins, claudin and occludin (Heller *et al.*, 2005, Zeissig *et al.*, 2007). Interestingly, recent preclinical evidence suggests that chronic intestinal inflammation alters hippocampal neurogenesis (Zonis *et al.*, 2015) which itself has recently been reported to be under the influence of the gut microbiota (Ogbonnaya *et al.*, 2015)

Table 1.2: Markers of Intestinal Permeability

Permeability Test	Sample	Measures	Clinical/Preclinical	Representative citation
Challenge Tests				
Lactulose/mannitol	urine	small intestine permeability	clinical & preclinical	(Vanuytsel *et al.*, 2014)
Lactulose/L-rhamnose	urine	small intestine permeability	clinical & preclinical	(Keszthelyi *et al.*, 2014)
Sucrose	urine	gastric permeability	clinical & preclinical	(Mujagic *et al.*, 2014)
Sucralose	urine	colonic permeability	clinical & preclinical	(Anderson *et al.*, 2004)
Polyethylene glycols	urine	entire intestine permeability	clinical & preclinical	(Rao *et al.*, 2011)
^{51}Cr-EDTA	urine	entire intestine permeability	clinical & preclinical	(Leclercq *et al.*, 2014b)
Circulating Markers				
Zonulin	plasma	small intestine epithelial cell damage	clinical & preclinical	(Fasano, 2011)
Intestinal Fatty acid binding protein (I-FABP)	plasma	small intestine permeability	clinical & preclinical	(Derikx *et al.*, 2009)
Citrulline	plasma	small intestine epithelial cell damage	clinical & preclinical	(Crenn *et al.*, 2000)
αGlutathione S-transferase (αGST)	plasma	epithelial cell damage	clinical & preclinical	(McMonagle *et al.*, 2006)
Claudin-3	urine	epithelial cell damage	clinical & preclinical	(Patel *et al.*, 2012a)
Lipopolysacharide (LPS) Binding Protein (LBP)	plasma	indirect evidence of permeability deficit	clinical & preclinical	(Pasternak *et al.*, 2010)
Endotoxin core antibodies (EndoCAb)	plasma	entire intestine permeability	clinical & preclinical	(Ammori *et al.*, 2003)
D-Lactate	plasma	entire intestine permeability	clinical & preclinical	(Poeze *et al.*, 1998)
Fluorescein isothiocyanate–dextran (FITC-Dextran 4)	plasma	entire intestine permeability	preclinical	(Moussaoui *et al.*, 2014)

Fecal Markers				
Calprotectin	faeces	nonspecific marker of gut inflammation	clinical & preclinical	(de Magistris *et al.*, 2010)
Zonulin	faeces	marker of intestinal permeability	clinical	(Lamprecht *et al.*, 2012)
Ex-Vivo				
Ussing chamber	ex vivo biopsies	epithelial ion transport	clinical & preclinical	(Piche *et al.*, 2009)

1.17 Gut Microbiota in Depression: State of Knowledge

To date three clinical studies have investigated the microbiota composition in depressed patients. In the first study, a significant increase in the order *Bacteroidales* and a decrease in *Lachnospiraceae* family was demonstrated in the depressed group (Naseribafrouei *et al.*, 2014). At the genus level there was an increase in *Oscillibacter* and *Alistipes* species. There were no differences in species richness or diversity between the groups. In the second study the depressed group had increased levels of *Enterobacteriaceae* and *Alistipes* and reduced levels of *Fecalibacterium* which negatively correlated with severity of depressive symptoms. They also found no significant differences in richness (Jiang *et al.*, 2015). The most recent study, sequenced the fecal sample from 58 depressed (39 drug free) and 63 healthy controls. At the phylum level they found an increase in *Actinobacteria* and a decrease in *Bacteroidetes* in the depressed group. Using random forests classifier, 29 operational taxonomic units (OTUs) were overrepresented in the depressed subjects and 25 OTUs were overrepresented in healthy control subjects. There were no significant differences in alpha diversity (shannon, simpson, phylogenetic diversity, observed species) while beta diversity, measured by unweighted unifrac, separated the groups to account for 19% of the difference.

In the preclincal arm of the study they compared GF to SPF mice and found that the GF mice exhibited reduced anxiety-like behaviour in the open field test (OFT) and decreased depressive like behaviour in the forced swim test (FST). Preparing an FMT from a pooled sample of 5 antidepressant free male donors and 5 matched controls they found no differences in the OFT, FST or tail suspension test (TST), whereas at two weeks post FMT the mice that received the depressed FMT displayed an increase in depressive and anxiety-like behaviour. Then using multiplex shotgun metagenomic analysis on caecal samples they identified several carbohydrate metabolites that were increased in the mice that received the depressed FMT (α-glucose, β-glucose, fructose and succinate) relative to control mice and this was verified in fecal, serum and hippocampal samples. Dysregulation of amino acid metabolism was also evident in the mice that received the depressed FMT. However, this finding, unlike the carbohydrate metabolites, was not consistent across different samples (Zheng *et al.*, 2016a).

1.18 Psychobiotics

Although diet may be a key factor shaping the gut microbiota, it may prove difficult as a first-line option in a population which may not be sufficiently motivated to radically alter eating habits. Consequently, alternative options need to be considered. A probiotic is defined as a live bacteria which when administered in adequate amounts confers a health benefit on the host (Petschow *et al.*, 2013, WHO, 2001). Previous probiotic health claims have been exaggerated (Hoffmann *et al.*, 2013, Sanders, 2003, Shanahan, 2002) and only certain probiotics are viable in the gastro-intestinal tract (Fredua-Agyeman and Gaisford, 2015). Other strains demonstrate beneficial effects on the intestinal barrier across the lifespan via a number of mechanisms and mediators (Eutamene and Bueno, 2007, Mennigen and Bruewer, 2009) (see **Table 1.3**).

Some of the strongest evidence for the clinical role of probiotics comes from studies in patients with the brain-gut disorder, IBS (Orel and Kamhi Trop, 2014, Whelan and Quigley, 2013). A number of probiotics and commensal organisms, primarily lactic acid bacteria, have been shown to ameliorate certain IBS symptoms (Clarke *et al.*, 2012, Didari *et al.*, 2015, Hoveyda *et al.*, 2009, Ortiz-Lucas *et al.*, 2013, Yoon *et al.*, 2013). Some of these beneficial effects may, at least, relate to the anti-inflammatory effects of particular organisms (O'Mahony *et al.*, 2005). Moreover, probiotics in accordance with preclinical evidence can improve intestinal barrier function under pathological conditions in human populations. In a randomized single blind placebo controlled study a fermented milk drink containing *Streptococcus thermophilus, L. bulgaricus, L.acidophilus and B.longum* decreased small intestinal permeability, though colonic permeability was unaltered (Zeng *et al.*, 2008).

The term "*psychobiotics*" has been recently conceived to encompass the sub-types of probiotics predominately *lactobacillus* and *bifidobacteria* species, that may be capable of modulating the brain-gut-microbiota axis to have a beneficial effect on mood, anxiety and cognition (Dinan *et al.*, 2013). A growing number of studies (see **Table 1.4**) with healthy individuals suggest that prolonged pre and probiotic consumption can positively affect aspects of mood and anxiety in healthy controls (Messaoudi *et al.*, 2011, Mohammadi *et al.*, 2015a, Steenbergen *et al.*, 2015b), modulate HPA axis function (Messaoudi *et al.*, 2011, Schmidt *et al.*, 2015) and alter functional brain activity (Tillisch *et al.*, 2013a). However, a recent systematic review indicated that the impact of

probiotic supplementation on gut microbiota structure in healthy controls was minimal (Kristensen *et al.*, 2016).

Logan and Katzman initially proposed an augmenting role for probiotics in the treatment of depression (Logan, 2015, Logan and Katzman, 2005). Ten years later, the first clinical trial was conducted. This randomized placebo controlled trial (n = 40) of a multispecies probiotic, showed that 8 weeks of *L. acidophilus, L. casei and B. bifidum* reduced Beck depression scores compared to placebo (Akkasheh *et al.*, 2016). Although, diet was recorded in this study, microbiota analysis was not reported.

Table 1.3: Preclinical studies of Probiotics & Intestinal barrier

Probiotic	Effects	Reference
VSL#3	normalization of colonic physiologic function and barrier integrity reduction in mucosal secretion of TNFα and IFNγ & an improvement in histologic disease. Decreased ileal paracellular permeability, decrease claudin-2 and increase occludin in a mouse model of ileitis	(Corridoni *et al.*, 2012)
VSL#3 (protein soluble factor)	enhanced barrier function and resistance to *Salmonella* invasion	(Madsen *et al.*, 2001)
VSL#3	Prevented the increase in epithelial permeability in DSS induced acute colitis & prevented the decreased expression & redistribution of the occludin, zonula occludens-1, and claudin-1, -3, -4, and -5	(Mennigen *et al.*, 2009)
VSL#3	VSL#3 can attenuate intestinal barrier damage and reduced bacterial translocation in a LPS induced mouse model of sepsis	(Ewaschuk *et al.*, 2007)
Lactobacillus rhamnosus & *L. acidophilus*	attenuated the damage caused by *Shigella dysenteriae*	(Moorthy *et al.*, 2009)
Lactobacillus plantarum	prevented the rearrangement of claudin-1, occludin, JAM-1 and ZO-1 proteins induced by *Escherichia coli*	(Qin *et al.*, 2009)
Lactobacillus reuteri	reduced levels of colonic mucosal adherent and translocated bacteria and attenuated the development of the colitis in interleukin IL-10 gene deficient mice	(Madsen *et al.*, 1999)
Lactobacillus rhamnosus GG culture supernatant (LGG-s)	pretreatment significantly inhibited alcohol-induced intestinal permeability, endotoxemia and subsequently liver injury	(Wang *et al.*, 2012b)
Lactobacillus rhamnosus OLL2838 (live & heat-killed)	Administration to DSS-treated animals protected against the increase in mucosal permeability associated with DSS-induced colitis. Increased expression of ZO-1 and myosin light-chain kinase in intestinal epithelial cells isolated from mice of the heat-killed OLL2838 group	(Miyauchi *et al.*, 2009)

Lactobacillus casei DN-114 001 (lysate - Lc)	increased the numbers of CD4(+)FoxP3(+) Tregs in mesenteric lymph nodes, decreased the production of TNFα and IFNγ, and anti-inflammatory IL-10 in peyer's patches and large intestine changed the gut microbiota composition in an DSS colitis BALB/c model Lc also resulted in a significant protection against increased intestinal permeability and barrier dysfunction shown by preserved ZO-1 expression Lc treatment prevented LPS induced TNFα expression in RAW 264.7 cell line by down-regulating the NF-κβ B signalling pathway	(Zakostelska *et al.*, 2011)
Lactobacillus brevis SBC8803 polyphosphate (poly P)	suppressed the oxidant-induced intestinal permeability in the mouse small intestine and its protective effect was prevented by pharmacological inhibitors of p38 MAPK and integrins daily intrarectal administration of poly P improved the inflammatory profile and survival rate when administered to DSS mice.	(Segawa *et al.*, 2011)
Lactobacillus rhamnosus GG, soluble secretory proteins p40 and p75	prevented hydrogen peroxide induced redistribution of occludin, ZO-1, E-cadherin, and beta-catenin from the intercellular junctions	(Seth *et al.*, 2008)
Lactobacillus plantarum DSM 2648 *Lactobacillus* plantarum *MB452*	attenuated the negative effect of enteropathic Escherichia coli (EPEC) O127:H6 (E2348/69) on transepithelial electrical resistance and adherence to intestinal cells. 19 TJ related genes had altered expression levels including those encoding occludin and its associated plaque proteins that anchor it to the cytoskeleton. L. plantarum MB452 altered tubulin and proteasome gene expression levels	(Anderson *et al.*, 2010a) (Anderson *et al.*, 2010b)
Lactobacillus acidophilus protects TJ from aspirin damage in HT-29 cells	protects TJ from aspirin damage in HT-29 cells	(Montalto *et al.*, 2004)
Lactobacillus rhamnosus GG (LGG) - modified lipoteichoic acid (LTA)	correlated with a significant down-regulation of TRL2 expression and downstream proinflammatory cytokine expression in DSS mouse model	(Claes *et al.*, 2010)

Lactobacillus plantarum 299v	1 week of pretreatment the drinking water abolished E. coli-induced increase in permeability	(Mangell *et al.*, 2002)
Lactobacillus helveticus and Lactobacillus rhamnosus	Administration one week prior to and concurrently with Citrobacter rodentium attenuated C. rodentium induced barrier dysfunction, epithelial hyperplasia, and binding of the pathogen to host colonocytes	(Rodrigues *et al.*, 2012)
Lactobacillus rhamnosus LOCK0900, L. rhamnosus LOCK0908 and *L. casei LOCK0919*	Colonization of GF mice enhanced the integrity of gut mucosa and ameliorated allergic sensitization	(Kozakova *et al.*, 2015)
Lactobacillus fermentum AGR1487 - cell surface structures & supernatant	live and deadAGR1487 decreased TEER across Caco-2 cells. only live AGR1487 increased the rate of passage of mannitol	(Sengupta *et al.*, 2015)
Lactobacillus rhamnosus GG (live or heat-killed)	Enteral administration accelerated intestinal barrier maturation and induced claudin 3	(Patel *et al.*, 2012a)
Lactobacillus helveticus R0052 & *Bifidobacterium longum R0175*	reversed the increased intestinal permeability and depressive like behaviours post MI.	(Arseneault-Breard *et al.*, 2012)
Bifidobacterium lactis CNCM I-2494	prevented the increase in intestinal permeability induced by PRS and restored occludin and JAM-A expressions to control levels	(Agostini *et al.*, 2012)
Bifidobacteria infantis	In T84 cells increased TEER, decreased claudin-2, and increased ZO-1 and occludin expression, associated with enhanced levels of phospho-ERK and decreased levels of phospho-p38. prevented TNFα & IFNγ induced decrease in TEER & rearrangement of TJ proteins. Oral administration acutely reduced colonic permeability in mice whereas long-term BiCM treatment in IL-10-deficient mice attenuated inflammation, normalized colonic permeability & decreased colonic and splenic IFN-gamma secretion	(Ewaschuk *et al.*, 2008)
Bacteroides thetaiotaomicron	modulated the expression of genes involved in several important intestinal functions, including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation	(Hooper *et al.*, 2001)
Escherichia coli Nissle 1917	altered the expression, and distribution of ZO-2 protein	(Zyrek *et al.*, 2007)

Escherichia coli Nissle 1917	Colonization of GF mice resulted in an up-regulation of ZO-1 in intestinal epithelial cells at both mRNA and protein levels	(Ukena *et al.*, 2007)

Table 1.4: Probiotics & Clinical Stress Studies

Probiotic	Duration	N	Subjects	Design	Clinical Measures	Biological Measures	Results	Reference
Lactobacillus casei Shirota (milk drink)	21 days	124	Healthy (average age was 61.8 yrs)	randomized double blind placebo controlled	Mood: Profile of Mood States (POMS), at baseline, 10 days & 20 days Cognition: Episodic memory Semantic memory Verbal fluency	N/a	no general effect on mood of taking the probiotic small improvement in mood when post hoc analysis of the lowest tertile mood scores were considered decreased performance on semantic memory	(Benton *et al.*, 2007)
L. helveticus R0052 & B. Longum R0175	30 days	30	Healthy	double blind placebo controlled	Hopkins Symptoms Checklist (HSCL-90) Hospital Anxiety & Depression Scale (HADS) Perceived Stress Scale (PSS) Coping Checklist (CCL)	24 hour urinary free cortisol (UFC)	HSCL-90 scale (global severity index, somatisation, depression, and anger–hostility), the HADS (HADS global score, and HADS anxiety) & by the CCL (problem solving) Decrease in UFC	(Messaoudi *et al.*, 2011)
Lactobacillus casei Shirota	60 days	35	Chronic Fatigue Syndrome	Randomized double blind placebo controlled	Beck Anxiety & Depression Inventories	Fecal	Decrease in Anxiety symptoms Increase in *Lactobacillus* and *Bifidobacteria* in Fecal samples	(Rao *et al.*, 2009)
Clostridium Butyricum	14 days (twice daily)	30 CFS 20 Healthy controls	pre-op laryngectomy	Randomized, placebo controlled	Hamilton Anxiety Scale	Serum CRF Heart rate (HR)	reduced anxiety levels from 19.8 to 10.2 attenuated the increase in CRF & HR pre op	(Yang *et al.*, 2014)

Bifidobacterium animalis, Streptococcus thermophiles, Lactobacillus bulgaricus, & Lactobacillus lactis (fermented milk)	28 days	12	Healthy Females	Randomized placebo controlled parallel-arm design		fMRI: emotional faces attention task	reduced task related response of a distributed functional network containing affective, viscerosensory and somatosensory cortices independent of self reposted GI symptoms	(Tillisch *et al.*, 2013a)

1.19 Prebiotics

A prebiotic effect is defined as the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host (Gibson, 2004, Roberfroid *et al.*, 2010). Preclinical and clinical studies demonstrate that certain prebiotics alter the gut microbiota, can reduce low grade inflammation and improve metabolic function (Bindels *et al.*, 2015, da Silva *et al.*, 2013, Dewulf *et al.*, 2013, Everard *et al.*, 2011, Greiner and Backhed, 2011). Evidence also suggests that prebiotic galacto-oligosaccharides (GOS) can improve intestinal barrier function in a rats (Zhong *et al.*, 2009). Moreover, mice treated with prebiotics exhibit improvements in intestinal permeability, TJ integrity, decreased plasma LPS and cytokine levels in addition to decreased hepatic expression of inflammatory and oxidative stress markers (Cani *et al.*, 2009).

Prebiotics have also been shown to influence brain neurochemistry and behaviour. GOS, for example, increased hippocampal neurotrophin levels and the expression N-methyl-d-aspartate receptor subunits in the rat frontal cortex (Savignac *et al.*, 2013). Behaviourally, a combination of GOS and polydextrose attenuated anxiety like behaviour rats and induced alterations in the gut microbiota composition (Mika *et al.*, 2014). In the context of IBS, GOS also appeared to influence the gut microbiota and improved anxiety scores (Silk *et al.*, 2009). Furthermore, in healthy adults, administration of GOS significantly decreased the cortisol awakening response and decreased attentional vigilance to negative versus positive information in a dot-probe task compared to placebo (Schmidt *et al.*, 2015).

1.20 Minocycline

As discussed above antibiotics have a profound impact on the gut microbiota (Dethlefsen and Relman, 2011). One potential effect of microbiota disruption, is the alteration of its metabolizing capabilities. Alteration of the microbiota, could potentially modulate the efficacy or toxicity of certain drugs, though little is known about these interactions. Although there is a shift towards more targeted narrow spectrum antibiotics, which minimize the disruption to the gut microbiota, (“microbiome sparing”), the broad spectrum antibiotic minocycline has received much attention recently due to its neuroprotective effects (Borre *et al.*, 2012, Plane *et al.*, 2010, Stirling *et al.*, 2005, Stock *et al.*, 2013). This second generation tetracycline antibiotic targets aerobic and anaerobic

gram positive and gram negative bacteria, and as it is highly lipophilic with a bioavailability of 95%, has good penetration into the central nervous system. Minocycline is commonly used in the treatment of acne vulgaris (Garner *et al.*, 2012) and occasionally used in the treatment of sexually transmitted diseases (Rosen *et al.*, 2009).

There are several mechanisms proposed to account for minocycline's neuroprotective effects. Minocycline can suppress microglial activation, reduce the expression of pro-inflammatory cytokines (IL 1ß, IL 6, IL 2, TNFα, IFN-γ), matrix metalloproteinases, and up-regulate the anti-inflammatory cytokine IL-10 (Hinwood *et al.*, 2013, Soczynska *et al.*, 2012, Tikka *et al.*, 2001). It can inhibit free radical generation (Lin *et al.*, 2003) and may indirectly modulate glutamatergic transmission (Chaves *et al.*, 2009, Jin *et al.*, 2012). Minocycline has also been investigated in animal models of depression. Minocycline reduced immobility and increased climbing in the forced swim test (FST) (Molina-Hernandez *et al.*, 2008). It resulted in less escape failures and shorter latency to escape in a learned helplessness model (Arakawa *et al.*, 2012). In LPS experiments, minocycline attenuated the LPS induced expression of pro-inflammatory cytokines (Henry *et al.*, 2008) and attenuated LPS associated increases in the duration of immobility in the FST and TST (O'Connor *et al.*, 2009). Another tetracycline antibiotic, doxycycline, similar to imipramine prevented and reduced LPS alteration in the FST, and also prevented and reversed LPS induced increase in IL 1ß and reversed LPS induced alterations in nitrite content and oxidative stress parameters in the striatum, hippocampus and PFC (Ferreira Mello *et al.*, 2013).

The majority of clinical studies investigating minocycline in psychiatry have involved its evaluation as an augmenting agent in the treatment of the negative symptoms in schizophrenia (Chaudhry *et al.*, 2012, Levkovitz *et al.*, 2010, Liu *et al.*, 2014), though several studies have been conducted in depressed patients. A case report showed mild nonspecific inflammation without lymphocytic infiltration and lipofuscin granule accumulation within the neurovascular endothelium in a brain biopsy of a depressed patient with recurrent treatment resistant depression. After a nine month course of intravenous immunoglobulin and a concurrent six month course of intravenous minocycline, a repeat Single-photon emission computed tomography (SPECT) scan showed normalization of frontal lobe hypoperfusion with a reduction in depression and

anxiety symptoms as measured by the Montgomery-Asberg depression rating scale and the Beck depression and anxiety inventory (Najjar *et al.*, 2013b).

In an open label study involving 25 patients diagnosed with unipolar psychotic depression, minocycline in combination with SSRIs for six weeks resulted in significant reductions in depression scores in the Hamilton Rating Scale for Depression and the Brief Psychiatric Rating Scale (BPRS) (Miyaoka *et al.*, 2012). A number of ongoing trials are investigating minocycline's effect in unipolar (NCT01574742), bipolar (NCT01403663, NCT01514422) and old age depression (NCT 01659320). One such trial is a trial of minocycline and aspirin in the depressed phase of bipolar (Savitz *et al.*, 2012). Another ongoing trial is investing the effect of minocycline in patients that have relapsed into depression after successful treatment with intravenous ketamine (NCT01809340).

An intriguing additional possible mechanism whereby minocycline could exert beneficial effects in neuropsychiatric disorders could be via modulation of the gut microbiota. In a preclinical study, stressed mice treated with minocycline, exhibited increases in the relative abundances of *Akkermansia* and *Blautia* species compatible with beneficial effects of attenuated inflammation and an increase in *Lachnospiracea* consistent with changes of caspase-1 deficiency (Wong *et al.*, 2016).

1.21 Primary hypothesis and aims of thesis

We hypothesise that the gut microbiota may have a causal role in symptom generation in depression and that targeting the gut microbiota with psychobiotics might represent a viable strategy for altering mood and cognitive processes. This hypothesis will be tested via the following objectives:

1. To determine the composition, richness and diversity of the gut microbiota in depressed patients compared to healthy controls and its relationship to: immune activity (plasma cytokines), HPA-axis function, tryptophan metabolism, intestinal permeability and SCFAs.
2. To determine a potential causal role for the gut microbiota, the behavioral & physiological effects of a Fecal Microbiota Transplantation from depressed patients and health controls to a microbiota depleted antibiotic rat model will be assessed.

3. To determine the effect of a putative psychobiotic, *Lactobacillus Rhamnosus*, on mood, stress response, cognition and relevant brain activity patterns in healthy males.

Chapter 2: Gut Microbiota & Physiological profiles in Depression

2.1 Abstract

Background: Major Depressive Disorder (MDD) is a common, often recurrent, heterogeneous disorder responsible for significant disability and economic burden worldwide. The biological mechanisms underlying the pathophysiology of MDD involve immune, endocrine and neurotransmitter dysregulation. Pre-clinical findings suggest that the complex and dynamic gut microbiota can modulate brain development, function and behaviour by recruiting the same neuroimmune, neuroendocrine and neural pathways of the brain-gut-axis which are dysfunctional in MDD. We tested the hypothesis that an altered gut microbiota, contributing to a dysregulated brain-gut-microbiota axis, could contribute to the pathophysiology of MDD**.**

Methods: Thirty four patients with major depression and 33 matched healthy controls were recruited. Cytokines, CRP, Salivary Cortisol and plasma Lipopolysaccharide binding protein (LBP) were determined by ELISA. Plasma tryptophan and kynurenine were determined by High Performance Liquid Chromatography (HPLC). Fecal samples were collected for 16s rRNA sequencing. Fecal Short chain fatty acids (SCFAs) were determined by Gas Chromatography. Fecal metabolites were analysed using Gas Chromatography Mass Spectometry (GC-MS).

Results: Depression is associated with altered gut microbiota composition and decreased gut microbiota richness and diversity. In parallel, patients with depression showed significantly higher levels of IL-6 ($p = 0.01$), IL-8 ($p = 0.02$), TNF-α ($p = 0.02$), CRP ($p = 0.001$) and a Kynurenine/tryptophan ratio ($p = 0.05$) compared to healthy controls. There was a greater cortisol output as measured by the Area under the Curve with respect to ground (AUCg) ($p = 0.05$) in the depressed group. There were no significant differences in LBP levels or fecal SCFAs. A Principal Component Analysis (PCA) model of fecal metabolites did not reveal distinct clusters.

Conclusions: We confirm that depression is associated with HPA axis, immune system and tryptophan dysregulation. Moreover, we show that depression is characterised by alterations in the gut microbiota at the family and genus level. Given that the metabolic output of this altered composition was not compromised and gut barrier function remained intact, further exploration of alternative mechanisms explaining the contribution of an altered gut microbiota profile to the pathophysiology of depression is warranted.

2.2 Introduction

Depression is a common, often recurrent (Eaton *et al.*, 2008) heterogeneous disorder responsible for significant disability worldwide (WHO, 2008). The complex aetiology involves dysregulated neuroendocrine (Stetler and Miller, 2011) neuroimmune (Dowlati *et al.*, 2010), metabolic (Jokela *et al.*, 2014) and neurotransmitter systems (Berton and Nestler, 2006). Current pharmacological interventions are suboptimal (Fava, 2003) and there has been little progress in the identification of biomarkers.

Accumulating evidence from preclinical studies suggests that the gut microbiota can modulate brain activity and behaviour via neuroendocrine, neuroimmune, neural and humoral pathways (Cryan and Dinan, 2012a, Dinan and Cryan, 2013). This emerging link between the gut microbiota and the central nervous system suggests that gut microbiota modification may have translational applications in the treatment of neuropsychiatric disorders (Cryan and Dinan, 2015b, Desbonnet *et al.*, 2014, Hsiao *et al.*, 2013).

Data from animal studies provides evidence that the gut microbiota may impact on the neurobiological features of depression (Park *et al.*, 2013), such as low-grade immune activation (Bailey *et al.*, 2011), hypothalamic-pituitary-adrenal axis (HPA) activity (Sudo *et al.*, 2004), altered tryptophan metabolism (Clarke *et al.*, 2013, El Aidy *et al.*, 2012, O'Mahony *et al.*, 2015a, Yano *et al.*, 2015), neurotrophic factors (Bercik *et al.*, 2011b), and neurogenesis (Mohle *et al.*, 2016, Ogbonnaya *et al.*, 2015).

Different *lactobacillus* and *bifidobacteria* species have been shown to modulate depression and stress-related behaviours in animal models (Bravo *et al.*, 2011, Desbonnet *et al.*, 2010, Savignac *et al.*, 2015b). Furthermore, a growing number of small studies suggest pre- and probiotic consumption can positively affect aspects of mood and anxiety (Akkasheh *et al.*, 2016, Messaoudi *et al.*, 2011, Steenbergen *et al.*, 2015b), modulate HPA function (Messaoudi *et al.*, 2011, Schmidt *et al.*, 2015) and alter brain activity (Tillisch *et al.*, 2013a). However, there are a paucity of clinical studies in well phenotyped pathological populations (Jiang *et al.*, 2015, Naseribafrouei *et al.*, 2014, Zheng *et al.*, 2016a).

We investigated alterations in the gut microbiota composition in patients with depression compared to healthy controls with respect to signature physiological alterations in HPA axis function, immune activation and tryptophan metabolism. We aimed to identify the functional consequences of the gut microbiota alterations in depression by determining levels of fecal short chain fatty acids. We then assessed gut permeability as a potential mechanism by which gut bacteria may influence brain function (Julio-Pieper *et al.*, 2014, Kelly *et al.*, 2015).

2.3 Methods

Subjects

Approval of the study protocol was granted by the Cork University Hospital ethics committee (Protocol Number: APC045) and written informed consent was obtained from all subjects. Thirty four depressed patients were recruited from outpatient and inpatient psychiatric clinics by a psychiatrist (JK). The Diagnostic and Statistical Manual of Mental Disorders (DSM IV) was used to confirm major depressive disorder diagnosis. Thirty three healthy subjects, screened using the Mini International Neuropsychiatric Interview (MINI), and matched for gender, age and ethnicity were recruited from advertisements directed at staff at Cork University Hospital and University College Cork. Inclusion criteria were as follows: age between 18 and 65 years, Hamilton depression score greater than 17. All patients had been prescribed antidepressant medication before referral to the secondary care service. Exclusion criteria were as follows: the use of probiotics, antibiotics use in the previous 4 weeks, active infections, glucocorticoids, nonsteroidal anti-inflammatory drugs, diabetes, inflammatory bowel disease, irritable bowel syndrome, recent gastrointestinal surgery, arthritis, pregnancy, and active alcohol or substance abuse or dependency. Inpatients were excluded if admission was greater than one week.

Clinical Measures

Anxiety	Beck Anxiety Inventory	(Beck *et al.*, 1988)
	Perceived Stress Scale	(Levenstein *et al.*, 1993)
Mood	Hamilton rating scale for Depression (HAMD 17)	(Hamilton, 1960)
	Beck Depression Inventory	(Beck *et al.*, 1961)
Traumatic Events	Early Life events scale Recent Life events scale	
Sleep	Pittsburgh Sleep Quality Index (PSQI)	(Buysse *et al.*, 1989)
Exercise	International Physical Activity Questionnaire (IPAQ) short version	(Craig *et al.*, 2003)
Diet	The Food Frequency Questionnaire (FFQ)	(Harrington *et al.*, 2010)

Microbiota analysis

Fecal samples were collected in a plastic containers containing an anaerobic generator AnaeroGen Compact Oxoid sachet. The fecal sample was transported to the laboratory and opened in an anaerobic hood. For the preparation of the fecal sample for inoculation, 1g of fecal sample was place into a stool collection tube and 10 mls of reduced PBS containing 15% (v/v) glycerol was added. Fecal slurry was prepared by vortexing each tube until the mixture was homogenous. The sample was frozen at -80^{o}C in cryovials in 1ml aliquots until further use. DNA was either extracted from fresh samples within 24 hours or from frozen samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). DNA was extracted from 21 frozen fecal samples and 43 from fresh samples. This has been shown to have little impact on the integrity of the microbiota (Fouhy *et al.*, 2015) and the Chao1, Simpson, Shannon, phylogenetic diversity and observed species between samples extracted from fresh and frozen were compared, and no significant differences were observed. Extracted DNA was prepared for sequencing on the Illumina Miseq platform using the Illumina recommended protocol: DNA was amplified using 16S Amplicon primers selected from (Klindworth *et al.*, 2012). Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC. The PCR product was purified of free primers and primer-dimer species using AMPure XP beads. Amplicons were tagged using the Illumina Nextera XT Index kit, and purified again using AMPure XP beads. The amplicons were quantified using Qubit fluorometric analysis and then normalised before pooling and sequencing. 300bp paired-end reads were combined using FLASH. Quality filtering removed reads with a quality score of <20 using QIIME. Filtered sample read numbers ranged from 28,057-895,372, with a mean read number of 157,030. Removal of chimeras and clustering of reads into operational taxonomic units (OTUs) was performed with USEARCH v7 (64-bit) with a lower threshold of 97% identity. OTUs were then aligned with PyNAST, and taxonomy was assigned using BLAST against the SILVA SSURef database release 111. The observation data were rarefied using QIIME to remove skew caused by samples containing very low or very high numbers of reads; a subsampling depth of 60,000 was chosen as optimal to maintain the maximum of both observed diversity and sample numbers. This left 23 test and 25 control samples. The Benjamini-Hochberg procedure was used to correct for multiple comparisons where appropriate with a FDR-adjusted p-

value ≤ 0.1 considered significant (Thissen *et al.*, 2002). Statistical tests were performed using R using the Mann-Whitney U test with continuity correction. Using QIIME, Alpha diversity was calculated for Chao1, Simpson, Shannon, observed species and Faith's phylogenetic diversity. Beta diversity was calculated for Bray Curtis (R2 = 0.03444, p = 0.014), weighted (R2 = 0.05515, p = 0.018) and unweighted unifrac (R2 = 0.05515, p = 0.018) using the multivariate ANOVA method "adonis" adapted in QIIME from the R "Vegan" package.

SCFA analysis

A modified version of the method used by (Zhao *et al.*, 2006) was used to analyse SCFA from human fecal samples: 1g of sample was mixed with 5mls of sterile distilled water and vortex for 3 minutes. The pH of the suspension was adjusted to 2-3 with 5M HCL and allowed to sit at room temperature for 10 minutes with occasional shaking. The samples were centrifuged at 4600rpm for 30 minutes. The supernatant was filtered through a 0.2um filter and 900ul of the sample was removed in duplicate into sterile eppendorfs. 100ul of 3.0mM-2-ethylbutyric acid (Sigma) was added as an internal standard to each sample. This was centrifuged for 3 minutes at full speed and transferred to a clear glass vial. Standard solutions containing 10.0, 8.0, 6.0, 4.0, 2.0, 1.0 and 0.5 mmol/l of acetic acid, propionic acid, isobutyric acid and butyric acid (Sigma), respectively, were used for calibration. The concentration of SCFA was measured using a Varian 3800 GC flame ionisation system, fitted with a ZB-FFAP column (30m x 0.32mmx 0.25 mm; Sigma). Helium was used as a carrier gas at a flow rate of 1.3 ml/min. The initial oven temperature was 50ºC and held for 0.5 min, raised to 140ºC at a rate of 10C/min and held for 0.5 min for a total of 10 minutes and finally held at 240ºC at a rate of 20ºC /min and held for 5 minutes to give a total run time of 20 minutes. The temperature of the detector and injector were set at 250ºC and 240ºC, respectively Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain calibration.

Metabolomic analysis

Fecal water was prepared from the fecal matter of 20 depression samples and 18 healthy controls. 200-400 mg of fecal material was placed into sterile 2 ml microcentrifuge tubes. Sterile PBS was added. 400 mg required 800 µl PBS. A slurry was produced by vigorous manual shaking and then centrifuged at 16000g for 30 mins. The supernatant was

centrifuged in 2ml microcentrifuge tubes at 16000 g for 30 mins. The supernatant was filtered through a VectaSpin Micro centrifuge filters 0.2 µm at recommended top speed 10,000 g until clear. The fecal water was stored at –20°C. All samples have been derivatized with methyl chloroformate (MCF). MCF converts amino and nonamino organic acids into volatile carbamates and esters. Although limited to compounds presenting amino and/or carboxyl groups, these include most metabolites of the central carbon metabolism, which are key intermediate of the cell metabolism. The samples were randomised and analysed by Gas Chromatography Mass Spectometry (GC-MS). A mixed pooled sample (QC sample) was created by taking a small aliquot from each sample. Every four-to-five samples, this QC sample was analysed. Testing of matrix effects was performed by spiking/dilution of QC samples. The raw GC-MS data was processed by software developed by MS-Omics and collaborators.

Salivary Cortisol analysis

Participants were instructed to collect three saliva samples using Salivettes (Sarstedt AG and Co, Numbrecht, Germany) at the following time points: (t 0) upon wakening, 30 minutes post wakening (t +30hr), and 150 minutes post wakening (t + 150). As it is not yet established if variable waking times affect the cortisol awakening response, we took the approach which is common in the literature (Hinkelmann *et al.*, 2013) and did not require that participants wake at a specific time, but followed their normal routine as closely as possible. Waking times were recorded for analysis to determine any group differences. Salivettes were centrifuged at 1000 g for 5 min and aliquoted and stored initially at -35ºC then transferred to - 80ºC until analysis. Cortisol concentrations were determined using the Cortisol Enzyme Immunoassay Kit as per manufacturers' instruction (Enzo®, Life Sciences). Assay detection limit was 0.16 nmol/L. Inter and intra-assay % C.Vs were 11.24% and 8.2% respectively.

Kynurenine/Tryptophan analysis

Plasma samples were spiked with internal standard (3-Nitro l-tyrosine) prior to being deproteinised by the addition of 20 µl of 4M perchloric acid to 200 µl of sample. Samples were centrifuged at 21000g on a Hettich Mikro 22R centrifuge (AGB, Dublin, Ireland) for 20 minutes at 4°C and 100 µl of supernatant transferred to a HPLC vial for analysis on the HPLC system (UV and FLD detection). All samples were injected onto a reversed phase Luna 3 µm C18 (2) 150 × 2 mm column (Phenomenex), which was protected by

Krudkatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex). The mobile phase consisted of 50 mM acetic acid, 100 mM zinc acetate with 3% (v/v) acetonitrile and was filtered through Millipore 0.45 μm HV Durapore membrane filters (AGB) and vacuum degassed prior to use. Compounds were eluted isocratically over a 30-minute runtime at a flow rate of 0.3 mls/min after a 20 μl injection. The column was maintained at a temperature of 30°C and samples/standards were kept at 8°C in the cooled autoinjector prior to injection. The fluorescent detector was set at an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The UV detector was set to 330 nm. L-tryptophan and kynurenine were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Analyte: Internal standard peak height rations were measured and compared with standard injections and results were expressed as ng/ml of plasma.

Cytokine sampling & analysis

10ml of whole blood was collected in heparinized tubes. Samples were centrifuged immediately at 1,000 x g for 15 minutes and then aliquoted. Samples were frozen initially at -35^{o}C and then transferred to -80^{o}C until analysis. Plasma levels of IL-6, IL-8, TNF-α, and CRP were assayed in duplicate using high sensitivity commercially available electrochemiluminescence MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, 75USA) as per manufacturer's instructions. The median lower limits of detection for each cytokine are; IL-6; 0.06 pg/ml, IL-8; 0.04 pg/ml, TNF-α 0.04 pg/ml and CRP; 0.1 ng/ml.

Lipopolysaccharide Binding Protein (LBP)

10ml of whole blood was collected in heparinized tubes. Samples were centrifuged immediately at 1,000 x g for 15 minutes and then aliquoted. Samples were frozen initially at -35^{o}C and then transferred to -80^{o}C until analysis. LBP concentrations were determined using the Enzyme Immunoassay Kit for free human LBP as per manufacturers' instruction (Enzo®, Life Sciences). Sensitivity: Range 5-50ng/ml.

Statistical Analysis. Data that were normally distributed according to Shapiro-Wilk test were analyzed using unpaired t tests. Outliers were removed by Grubbs' test. Data that were not normally distributed were transformed by square root transformation.

Microbiota data were analyzed using non parametric tests. Benjamini-Hochberg procedure was used to correct for multiple comparisons with a FDR-adjusted p-value ≤ 0.1 considered significant. Statistical procedures were carried out using IBM SPSS 20.0. Graphs were generated using GraphPad Prism 5. Macronutrient data was generated using Diet Plan 6.

2.4 Results

Demographic data and health indicators

Other than education level, employment status, smoking and alcohol consumption, there were no differences between the groups **(Table 2.1)**. Clinical characteristics of the depressed patients are presented in **(Table 2.2).**

Table 2.1: Comparison of group demographics and health indicators

	Controls (n=33)	**Depression (n=34)**	**p-value**
Age mean (s.d.)	45.8 (11.9)	45.8 (11.5)	0.98
Sex: Male (%)	19 (57.6)	21 (61.8)	0.73
Female (%)	14 (42.4)	13 (32.8)	
Education, degree level (%)	26 (78.8)	8 (23.5)	<0.001***
Relationship status (% yes)	23 (69.7)	20 (58.8)	0.35
Employed (% yes)	31 (93.9)	16 (47.1)	<0.001***
Alcohol units/week mean, (s.d)	9.24 (8.3)	5.29 (5.9)	0.03*
1st degree relative with alcohol use disorder	4 (12.1)	12 (35.3)	0.03*
Smoking (%)			
Current	3 (9.1)	13 (38.2)	
Ex	19 (57.6)	3 (8.8)	
Never	11 (33.3)	18 (52.9)	<0.001***
BMI mean (s.d.)	24.58 (2.7)	26.2 (4.5)	0.07
Dyslipidaemia (%)	4 (12.1)	7 (20.6)	0.51
HTN (%)	3 (9.1)	3 (8.8)	0.97
Physical Activity (IPAQ)			
Low (%)	7 (21.2)	13 (38.2)	
Moderate (%)	16 (48.5)	14 (41.2)	
High (%)	10 (30.3)	6 (17.6)	0.23
Metabolic Equivalent Task Units (MET) median, range	1386 (7287)	693 (7722)	0.1
Hours sitting per day mean, (s.d)	6.03 (2.730)	5.97 (2.456)	0.92

Data are presented as mean and S.D, or median and range. BMI, body mass index; HTN, Hypertension; IPAQ, International Physical Activity Questionnaire. *p<0.05 vs control. **p<0.01 vs control; ***p<0.001 vs control.

Table 2.2: Comparison of group clinical characteristics

	Controls (n=33)	Depression (n=34)	p- value
Diagnosis			
Depression (%)	NA	29 (85.3)	
BPAD II (%)	NA	5 (14.7)	
Co-morbid Anxiety Disorder (%)	NA	4 (11.7)	
Ex-Alcohol abuse (%)	NA	8 (23.5)	
Ex-Substance abuse (%)	NA	3 (8.8)	
Ex-Alcohol & Substance abuse (%)	NA	2 (5.8)	
HAMD 17 median (range)	NA	19.5 (14)	
Beck Depression mean, (s.d)	NA	32.4 (9.92)	
Duration of Depressive sx (months) median, (range)	NA	3.0 (72)	
Number of Depressive episodes median (range)	NA	1.0 (8)	
Positive Family History of Depression (%)	2 (6.1)	21 (61.8)	<0.001***
Beck Anxiety median, (range)	NA	25.5 (45)	
Perceived Stress Scale (PSS) mean, (s.d)	7.5 (4.9)	27.7 (6.0)	<0.001***
Pittsburgh Sleep Quality Index (PSQI) mean, (s.d)	2.8 (1.8)	11.7 (4.3)	<0 .001***

Data are presented as mean and S.D, or median and range. *p<0.05 vs control. **p<0.01 vs control; ***p<0.001 vs control.

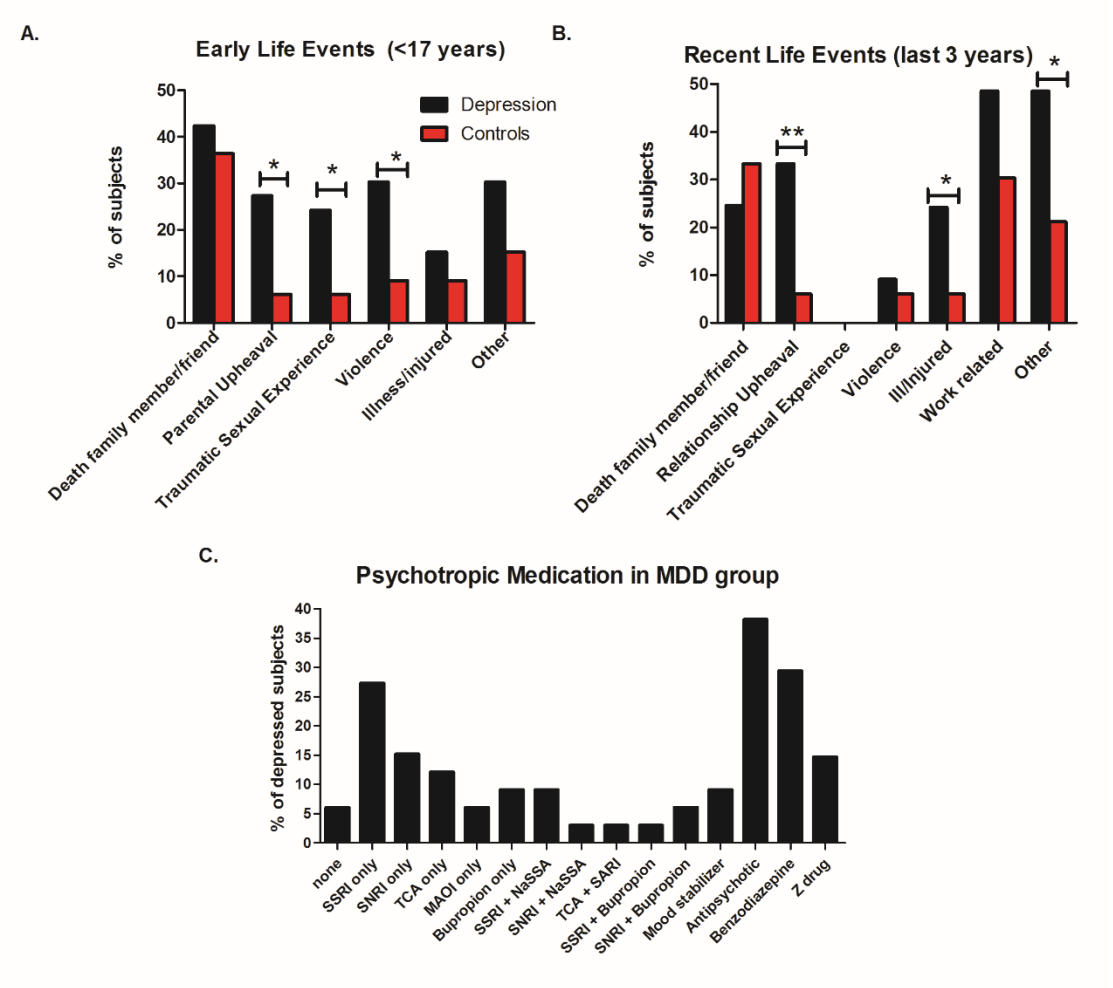


Figure 2.1: Life Events and Medication.

(A) The depressed group (n = 34) experienced significantly more parental upheaval (χ^2 (1) = 5.35, p = 0.02), traumatic sexual experiences (χ^2 (1) = 4.24, p = 0.04) and violence (χ^2 (1) = 4.70, p = 0.03) prior to the age of 17 years and **(B)** more relationship upheaval (χ^2 (1) = 7.76, p = 0.01) and illness/injury (χ^2 (1) = 4.24, p = 0.04) in the last 3 years compared to the control group (n = 33). **(C)** Percentage of depressed subjects prescribed psychotropic medication.

Daily Macronutrient Consumption similar in depressed patients and controls

We assessed Daily Macronutrient Consumption using a food frequency questionnaire **(Table 2.3).** Apart from Trans fats (t (61) = 2.06, p = 0.05) there were no significant differences in diet between the groups. Total fats were not significantly different between the groups (t (61) = 1.19, p = 0.24).

Table 2.3. Daily Macronutrient Consumption.

Macronutrient	Controls (s.d.), N=33	Depression (s.d.), N=30	p
Quantity (g)	2330.53 (1130.1)	2304.9 (1210.67)	0.93
Total Nitrogen (g)	13.36 (4.46)	14.07 (6.59)	0.62
Protein (g)	82.5 (27.61)	87.47 (41.18)	0.57
Fat (g)	75.49 (31.54)	87.70 (50.66)	0.25
CHOm (available carbs) (g)	296.33 (166.6)	288.62 (135.65)	0.84
Energy kcal	2202.09 (827.96)	2234.51 (1064.75)	0.89
Starch (g)	107.55 (44.03)	114.96 (38.75)	0.49
Total Sugar (g)	157.35 (80.71)	151.48 (107.07)	0.81
Non Milk Extrinsic Sugars (g)	23.85 (median), 22.88 (IQR)	29.1 (median), 28.9 (IQR)	0.43
Glucose (g)	28.45 (17.31)	27.78 (21.24)	0.89
Fructose (g)	51.31 (39.36)	48.11 (40.65)	0.75
Sucrose (g)	60.27 (39.62)	59.58 (38.20)	0.94
Maltose (g)	2.07 (1.39)	2.41 (1.53)	0.36
Lactose (g)	11.65 (median), 9.75 (IQR)	8.45 (median), 20.7 (IQR)	0.55
Non-starch polysaccharides (g)	26.96 (12.53)	24.91 (12.43)	0.52
Fibre (AOAC method) (g)	34.2 (16.21)	31.53 (16.32)	0.52
Sat.fats (g)	28.96 (14.89)	35.63 (22.19)	0.17
MonoUfats (g)	25.08 (10.24)	29.37 (16.62)	0.22
PolyUfats (g)	14.17 (6.13)	14.57 (8.96)	0.48
Transfats (g)	1.66 (0.87)	2.31 (1.53)	0.05
Cholesterol (mg)	259.85 (122.43)	294.92 (132.50)	0.28
Calories from Protein	329.93 (110.36)	350.58 (164.89)	0.56
Calories from Carbs	1021.16 (367.96)	1082.58 (508.87)	0.59
Calories from Fat	679.43 (283.91)	789.19 (455.72)	0.25
Calories from Fiber	68.41 (32.47)	63.19 (32.65)	0.53
Percentage Protein	14.99 (3.23)	15.6 (3.12)	0.46
Percentage Carbs	47.53 (9.16)	47.55 (7.90)	1.00
Percentage Fat	30.43 (7.81)	33.52 (7.75)	0.12

Percentage Fiber	3.32 (1.41)	2.83 (1.12)	0.14

Macronutrient	**Controls (s.d.), N=33**	**Depression (s.d.), N=30**	**p**
Sodium (mg)	2385.9 (942.86)	2552.3 (1180.20)	0.55
Potassium (mg)	4355.22 (1610.90)	4253.87 (2077.0)	0.83
Calcium (mg)	1002.78 (482.24)	1043.96 (753.06)	0.78
Magnesium (mg)	398.21 (181.9)	352 (180.99)	0.32
Phosphorus (mg)	1615.75 (626.24)	1603.32 (862.58)	0.95
Iron (mg)	13.28 (5.12)	12.45 (5.59)	0.54
Copper (mg)	1.99 (0.93)	1.87 (1.00)	0.64
Zinc (mg)	10.44 (3.68)	11.24 (4.75)	0.45
Chloride (mg)	4021.12 (2483.31)	4231.58 (2423.71)	0.74
Manganese (mg)	4.23 (1.82)	3.95 (1.98)	0.58
Selenium (ug)	49.91 (21.40)	47.53 (26.33)	0.70
Seleno Protein P (mg/ml serum)	3.61 (1.50)	2.85 (1.55)	0.05
Iodine (ug)	187.77 (100)	174.41 (117.77)	0.63
Retinol (ug)	337.87 (193.81)	340.48 (204.72)	0.96
Carotene (ug)	3566.22 (2037.85)	3488.56 (2136.80)	0.89
Vitamin D (ug)	2.87 (1.57)	2.38 (1.57)	0.24
Vitamin E (mg)	12.95 (6.14)	11.64 (7.32)	0.45
Thiamin (mg)	1.62 (0.72)	1.5 (0.59)	0.48
Riboflavin (mg)	1.85 (0.89)	1.79 (1.01)	0.80
Niacin (mg)	21.05 (6.18)	20.66 (9.43)	0.85
Tryptophan divided by 60 (mg)	16.59 (5.76)	17.06 (7.76)	0.78
Vit.B6 (mg)	2.26 (0.8)	2.22 (0.9)	0.83
Vit.B12 (ug)	4.96 (2.78)	6.1 (4.59)	0.24
Folate (ug)	286.12 (142.64)	241.96 (108.96)	0.21
Pantothen (mg)	5.97 (2.8)	5.92 (3.1)	0.95
Biotin (ug)	43.98 (21.1)	40.1 (25.40	0.51
Vitamin C (mg)	154.51 (131.48)	142.19 (111.64)	0.69

Proinflammatory profile in depression

The depressed group had increased levels of IL-6 (t (62) = 2.69, p = 0.009), IL-8 (t (61) = 2.37, p = 0.021), TNF-α, (t (49) = 2.36, p = 0.022) and CRP (t (45) = 3.6, p = 0.001) compared to the healthy controls **(Figure 2.2A-D).**

Activated Kynurenine pathway in depression

The kynurenine/tryptophan ratio was significantly higher in the depressed group compared to the controls (t (61) = 2.01, p =0.049) **(Figure 2.2E).** There were no significant differences in tryptophan (t (58) = 0.92, p=0.362), kynurenine (t (63) = 1.00, p=0.320), kynurenic acid (t (45) = 0.38, p=0.70), or the kynurenic acid/kynurenine ratio (t (44) = 0.40, p=0.685).

Altered HPA axis in depression

AUCg analysis showed that patients with depression exhibited a greater total cortisol output (t (50) = 2.06, p = 0.045) **(Figure 2.2F)** but no difference in the delta cortisol response (t (50) = - 0.40, p = 0.69) or AUCi (t (50) = -0.67, p = 0.51). There were no significant differences between baseline cortisol levels upon wakening (t (50) = 1.88, p = 0.06), 30 minutes post wakening (t (50) = 1.28, p = 0.206), or 150 minutes post wakening (t (50) = 1.73, p = 0.09) **(Figure 2.2G).** Although there was no significant baseline difference between groups, when controlling for baseline cortisol values there was not a significantly elevated AUCg between groups.

No alterations in intestinal permeability in depression

There were no significant differences in plasma LBP levels (Depression versus Healthy controls, 39 ± 2.7, *vs.* 36 ± 2.1; t (63) = 1.05, p = 0.30) between the groups (data not shown).

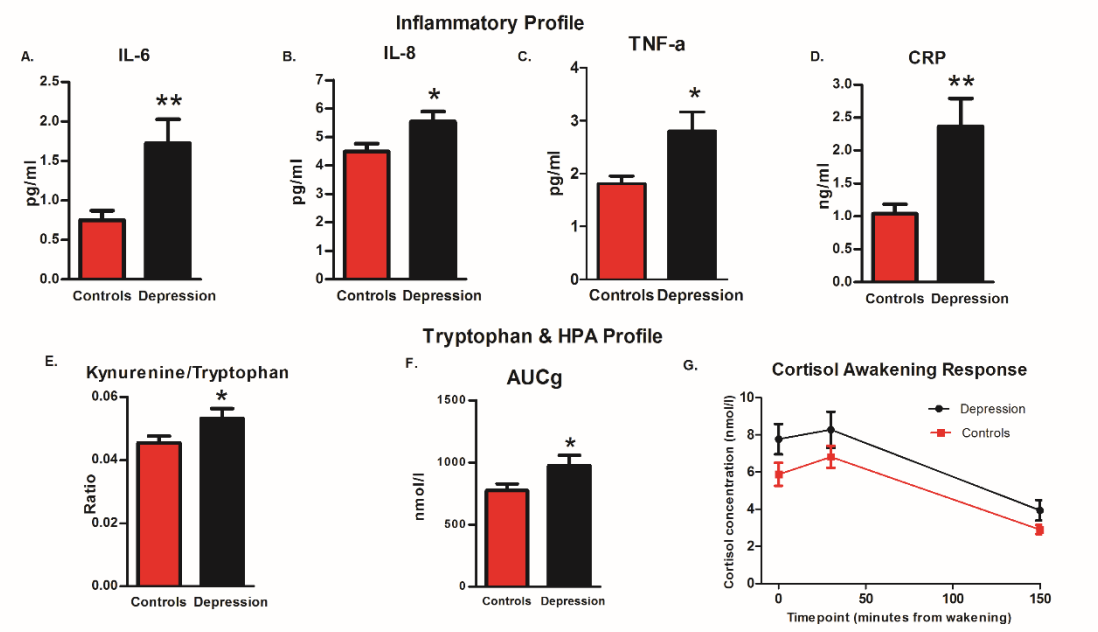


Figure 2.2: Altered Inflammatory, Tryptophan, and HPA profile in depressed patients. The depressed patients (n = 34) had significantly increased levels of **(A)** IL-6 (t (62) = 2.69, p = 0.009) **(B)** IL-8 (t (61) = 2.37, p = 0.021) **(C)** TNF-α (t (49) = 2.36, p = 0.022) and **(D)** CRP (t (45) = 3.6, p = 0.001) compared to the healthy controls (n = 33). The depressed group had a significantly increased **(E)** Kynurenine/tryptophan ratio (t (61) = 2.01, p =0.049) and a greater cortisol output as measured by the **(F)** Area under the Curve with respect to ground (AUCg) (t (50) = 2.06, p = 0.045), though no significant difference in the **(G)** Cortisol Awakening Response (CAR) (p = 0.21).

Altered Gut Microbiota Composition, Alpha Diversity & Richness in depressed patients

Chao1 richness (U = 424, p = 0.005), total observed species (U = 441, p = 0.002) and phylogenetic diversity (U = 447.5, p = 0.001) were decreased in the depressed group (n = 23). There was no difference in Shannon diversity (U = 350, p = 0.197) **(Figure 2.3A-D)**. The difference of the global microbiota composition from the 16S rRNA data of the depressed and control groups (n = 25) was assessed by ordination. Statistics based on random permutations of the redundancy analysis (RDA) showed that the depressed group is significantly separated at genus level (p = 0.03) from the control group **(Figure 2.3E).**

At the phylum level, there were no statistically significant differences in the relative abundances between the depressed group and the healthy controls (data not shown). At the family level, the relative proportions of *Prevotellaceae* (U = 355, p = 0.007) were decreased, whereas *Thermoanaerobacteriaceae* were increased in the depressed group (U = 52.5, p = 0.021) **(Figure 2.3F).** At the genus level, the relative proportions of *Eggerthella* (U = 21.0, p = 0.009), *Holdemania* (U = 146.5, p=0.023), *Gelria* (U = 52.5, p = 0.021), *Turicibacter* (U = 89, p = 0.034), *Paraprevotella* (U = 119, p = 0.041), and *Anaerofilum* (U = 50.5, p = 0.021) were increased in the depressed group, whereas *Prevotella* (U = 324.5, p = 0.022) and *Dialister* (U = 153.5, p = 0.032) were decreased **(Figure 2.3G).**

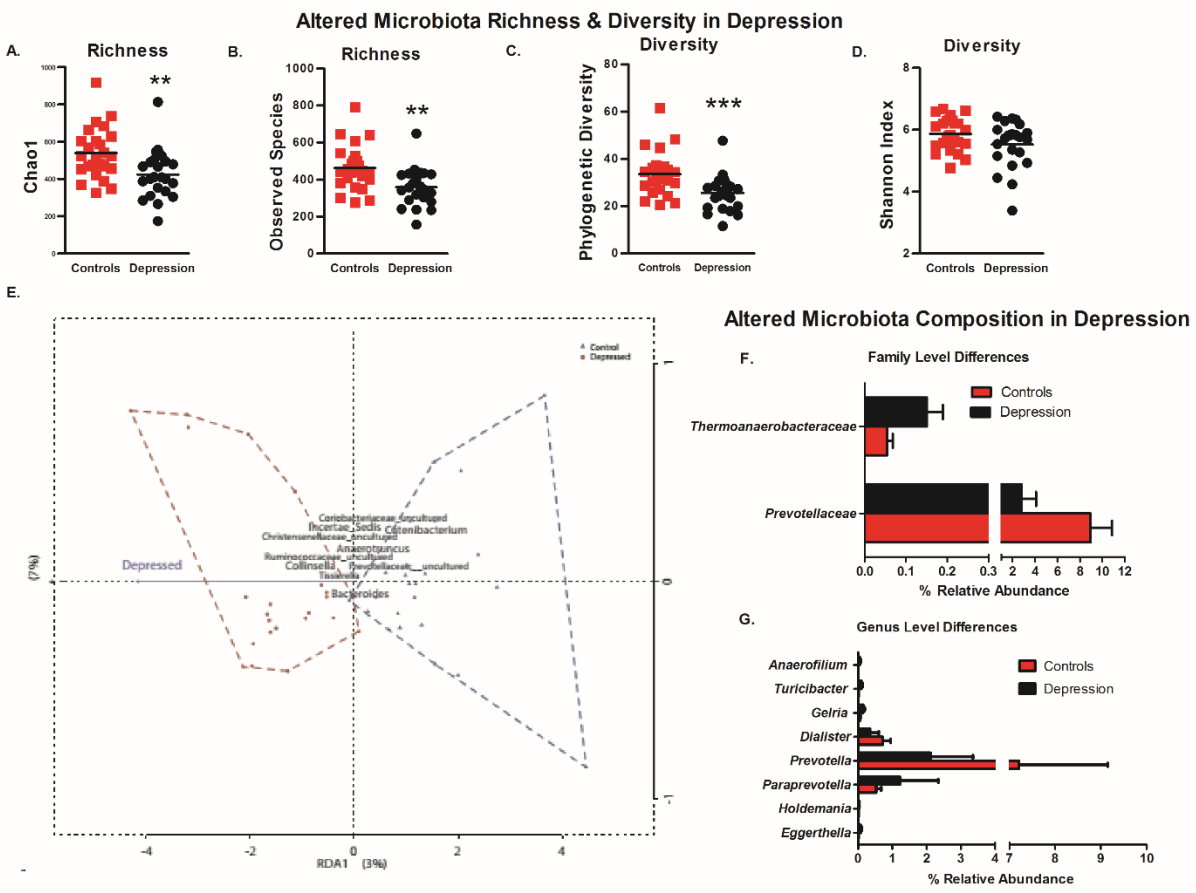


Figure 2.3: Altered Gut Microbiota Composition, Alpha Diversity & Richness in depressed patients. There was a significant reduction in richness as measured by **(A)** Chao1, (U = 424, p = 0.005) and **(B)** Total observed species, (U = 441, p = 0.002). The depressed group (n = 23) showed reduced **(C)** phylogenetic Diversity, (U = 447.5, p = 0.001), but no significant differences in **(D)** Shannon diversity, (U = 350, p = 0.197). **(E)** The significant differences at genus level between the depressed group and the controls (n = 25) which cluster by group in a Redundancy analysis (RDA) plot (p = 0.03). **(F)** Significant Family level differences and **(G)** significant genus level differences between depressed patients and healthy controls in % relative abundances.

Altered Beta Diversity in depressed patients

There were significant differences in beta diversity between the healthy (n = 25) and depressed groups (n = 23) (Bray-Curtis (p = 0.014), unweighted unifrac (p = 0.002) and weighted unifrac (p = 0.018). However, PCoA analysis was unable to separate the groups **(Figure 2.4A-C).**

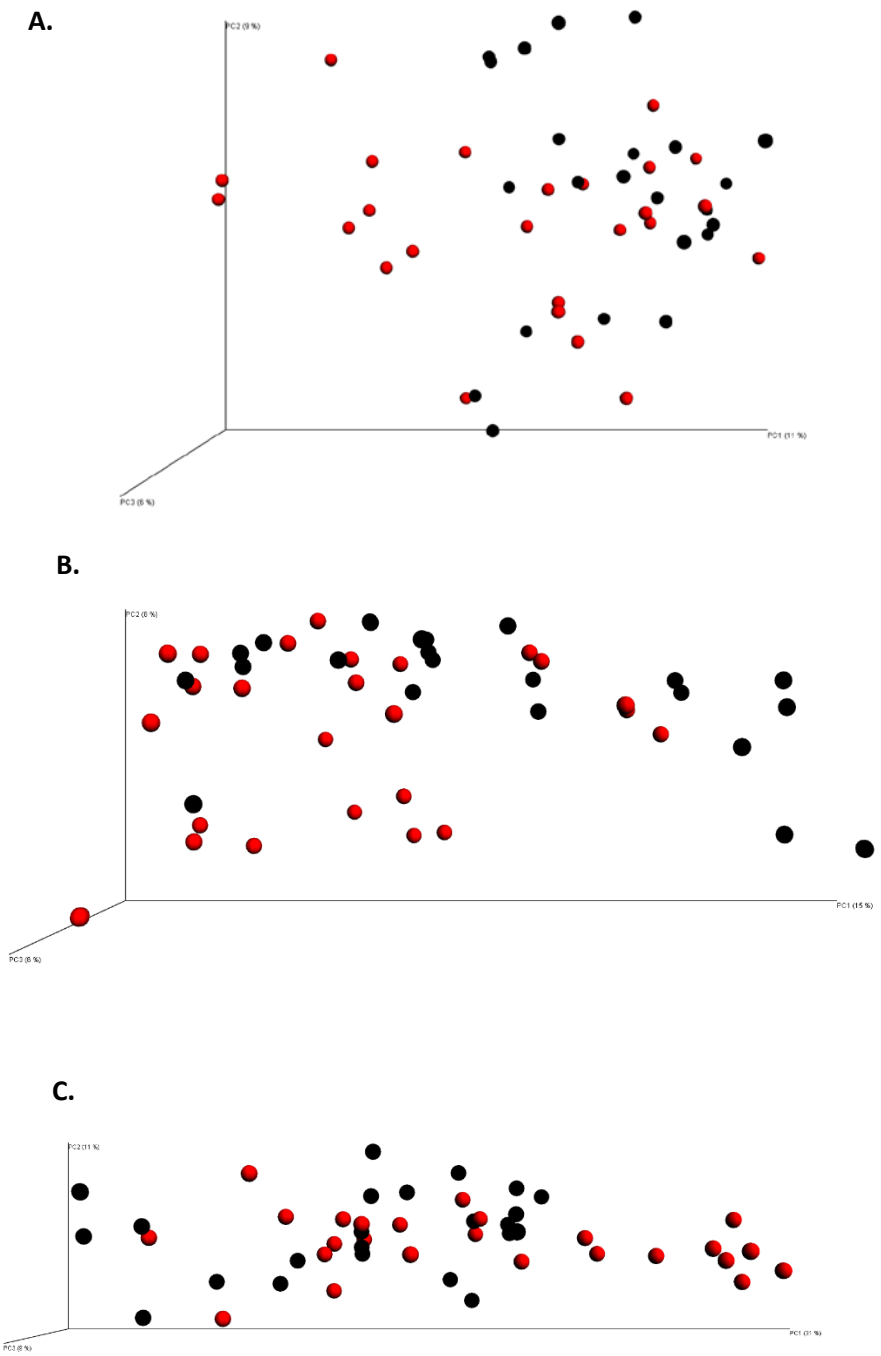


Figure 2.4: Beta Diversity in depressed patients (n = 23) PCoA plots representing **(A)** Bray-Curtis (R2 = 0.03444, p = 0.014), **(B)** unweighted (R2 = 0.05515, p = 0.018) and **(C)** weighted unifrac beta diversity (R2 = 0.05515, p = 0.018). Red represents healthy controls (n = 25) and black represents depressed patients.

Short chain Fatty Acids

There were no significant differences between groups in the fecal SCFAs, acetate (t (49) = 0.457, p = 0.65), propionate (t (49) = 0.103, p = 0.918), iso-Butyrate (t (43) = 0.678, p = 0.501) or butyrate (t (49) = - 0.168, p = 0.867) (**Figure 3A**).

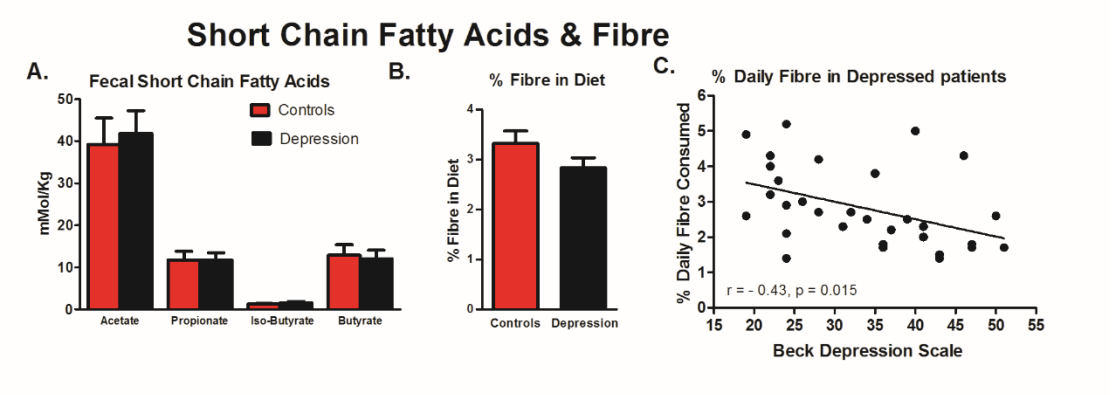
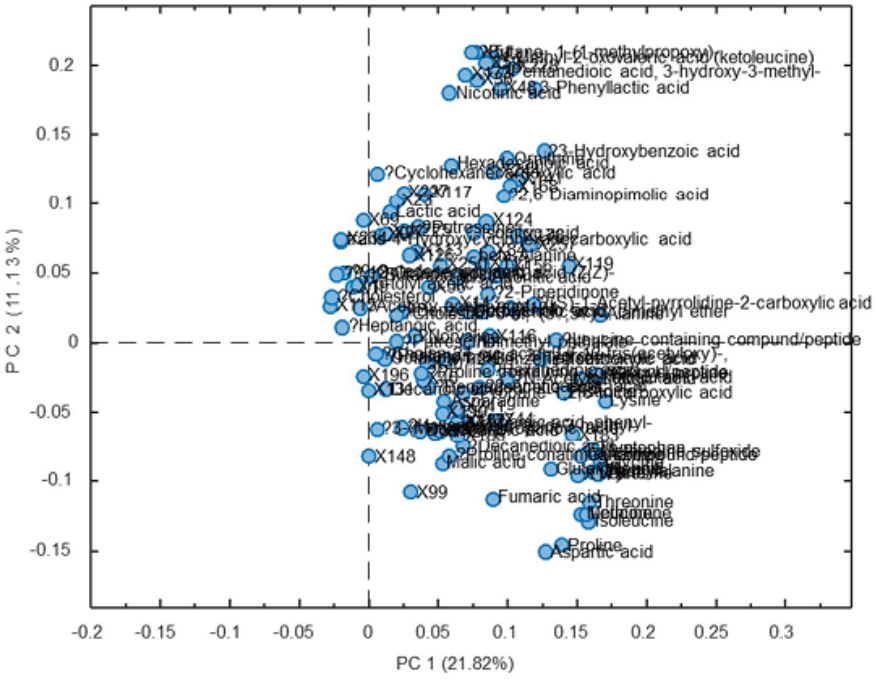


Figure 2.5. Short chain Fatty Acids & Fibre. (A) There were no significant differences in fecal SCFAs. **(B)** There were no significant differences in the daily % fibre consumed between the groups (t (61) = 1.5, p = 0.134) **(C)** though within the depressed group (n = 34) the daily % fibre negatively correlated with the Beck Depression Scale (r = - 0.43, n = 31, p = 0.015).

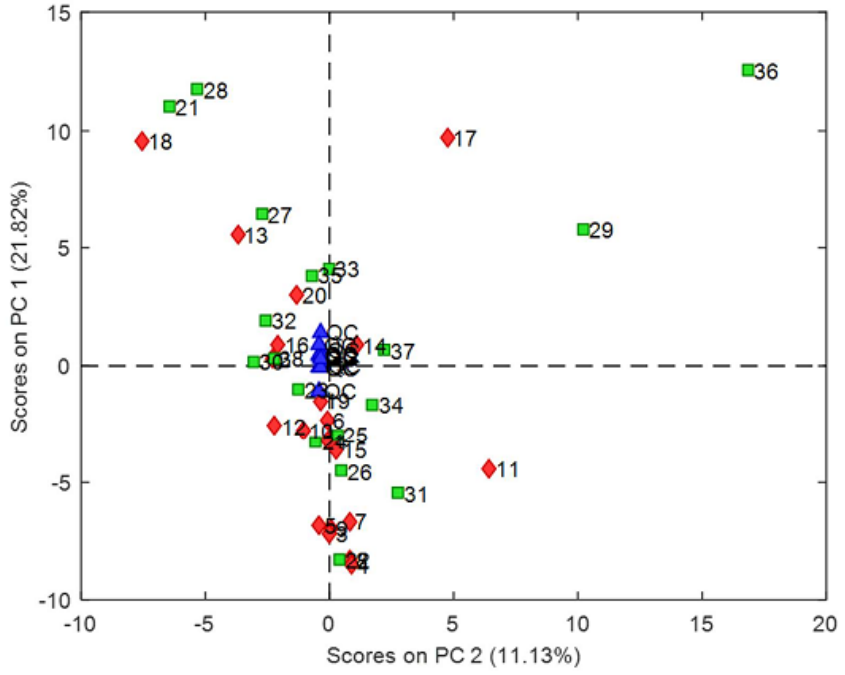
No alterations in Fecal Metabolites.

A Principal Component Analysis (PCA) model did not reveal distinct grouping of the depression (n = 20) samples compared to the healthy control (n = 18) samples **(Figure 2.6). (A)** Loading plot from PCA model calculated on the relative concentrations of the metabolites. **(B)** Score plot from PCA model calculated on the relative concentrations of the fecal metabolites. Red represents depressed patients and green represents healthy controls, blue triangles represents a mixed pooled sample (quality control sample). Data has been autoscaled. **(C)** Heat map demonstrating the change in concentration of fecal metabolites (log2) between depressed patients and healthy controls.

(A)


PC 2 (11.13%)
PC 1 (21.82%)
Nicotinic acid
Phenyllactic acid
3-Hydroxybenzoic acid
2,6-Diaminopimelic acid
Lactic acid
Fumaric acid
Threonine
Isoleucine
Proline
Aspartic acid
Lysine
X148
X99

(B)


Scores on PC 1 (21.82%)
Scores on PC 2 (11.13%)

(C)

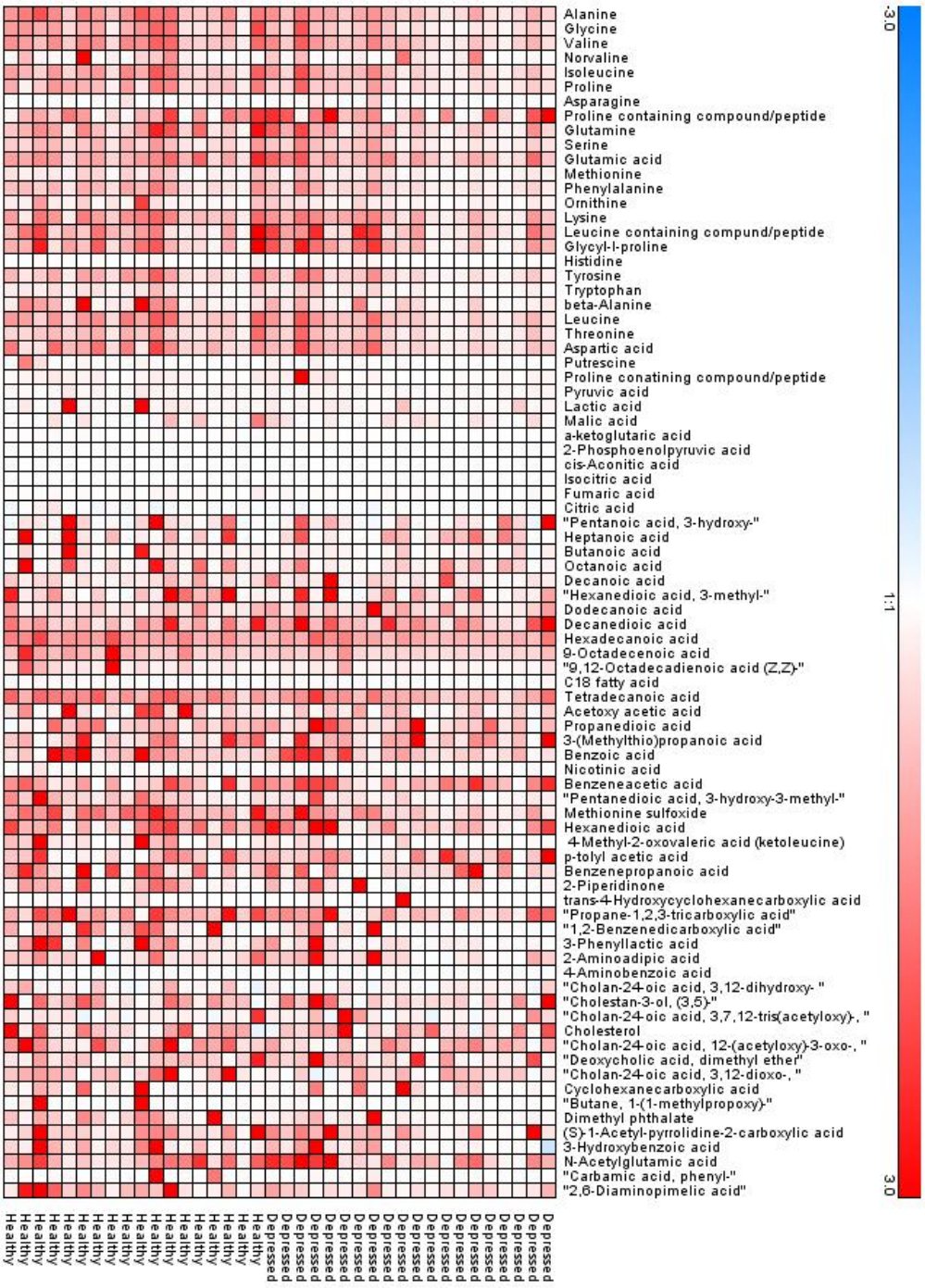


Figure 2.6. Fecal Metabolites. (A) Loading plot from PCA model calculated on the relative concentrations of the metabolites. **(B)** Score plot from PCA model calculated on the relative concentrations of fecal metabolites. Red represents depressed patients, green represents healthy controls and blue represents quality control samples. Data has

been autoscaled. **(C)** Heat map demonstrating the change in concentration of fecal metabolites (log^2) between depressed patients (n = 20) and healthy controls (n = 18).

2.5 Discussion

Our results confirm our main hypothesis and we have demonstrated that depression is associated with an altered gut microbiota composition, richness and diversity. It was possible to differentiate the depressed group from the healthy control group based on differences at the genus level using redundancy plot analysis **(Figure 2.3E).** The most pronounced difference was observed in the reduction of *Prevotellaceae* family and subsequently in the *Prevotella* genus, previously found to be decreased in Parkinson's disease patients (Gustafsson *et al.*, 2015, Scheperjans *et al.*, 2015). A significant association has been noted between the Mediterranean diet, regarded as a preventive strategy in depression, and *Prevotella* (De Filippis *et al.*, 2015, Opie *et al.*, 2015). However, the differences in the gut microbiota profiles did not impact functional readouts as measured by SCFAs or in the fecal metabolomic profile between depressed patients and healthy controls.

A significant increase in the order *Bacteroidales* and a decrease in *Lachnospiraceae* family compared to controls was previously shown in depressed patients (Naseribafrouei *et al.*, 2014). The second study to investigate the gut microbiota in depression separated the depressed group into an actively depressed group and those that had responded to treatment (Jiang *et al.*, 2015). Similar to our study, they demonstrated a decrease in *Prevotellaceae* and *Prevotella* in depressed patients. However, they found a significant increase in Shannon diversity in the actively depressed group compared to controls and no significant differences in richness. They reported no differences in circulating pro-inflammatory cytokines in contrast to low grade inflammation described in depressed patients (Dinan, 2009b). The most recent study, found an increase in *Actinobacteria* and a decrease in *Bacteroidetes* in the depressed group at the phylum level and 54 differences in the operational taxonomic units (OTUs) between the groups. There were no significant differences in alpha diversity (Shannon, Simpson, phylogenetic diversity, observed species). Dietary factors, geography or methodological differences during sampling, processing or analysis may also account for discrepancies (Kelly *et al.*, 2016c).

We show that depression is associated with dysregulated tryptophan metabolism as indicated by an increased plasma kynurenine: tryptophan ratio. Together with the potential impact on serotonin, increased microbiota-mediated degradation of tryptophan along this pathway has a broad range of implications for multiple neurotransmitter systems (Schwarcz *et al.*, 2012). The rate of tryptophan metabolism along the kynurenine pathway is dependent on the activity of indoleamine-2, 3-dioxygenase (IDO), an enzyme induced by cytokines, and tryptophan-2,3-dioxygenase (TDO), the expression of which can be induced by circulating glucocorticoids (O'Connor *et al.*, 2009) and has been reported to be regulated by the gut microbiota during colonization (El Aidy *et al.*, 2014).

The impact of stress on the gut microbiome might also be a factor and we have previously shown that early life stress can remodel the gut microbiota (O'Mahony *et al.*, 2009). It is plausible that subtle alterations in microbiota acquisition or maintenance during this vulnerable early life period may act as to impact on (neuro)endocrine and (neuro)immune signalling pathways of the brain-gut-microbiota axis, disruption of which may subsequently predispose to stress-related disorders in adulthood (Sudo *et al.*, 2004). The depressed group in our study did experience more stressful life events prior to the age of 17 years and in the last 3 years compared to the healthy group **(Figure 2.1A-B).**

Studies have demonstrated higher IgA- and IgM-mediated immune responses directed against LPS of certain commensal gram negative gut bacteria in depression (Maes, 2008, Maes *et al.*, 2012b, Maes *et al.*, 2013). Bacterial DNA has been detected in whole serum from depressed patients who also displayed increased TLR4 expression on peripheral mononuclear blood cells compared to healthy controls (Keri *et al.*, 2014). Although we demonstrated dysregulated inflammatory **(Figure 2.2A-D)** and HPA axis function **(Figure 2.2G)** in the depressed group, we cannot rule out the possibility of glucocorticoid receptor resistance playing a role (Calfa *et al.*, 2003, Pariante and Miller, 2001).

We hypothesized that gut permeability could act as a conduit by which the gut microbiota may impact brain and behaviour. Using LBP as a marker of intestinal permeability (Forsyth *et al.*, 2011) we found no statistically significant differences in intestinal permeability in the depressed patients. It is possible that gut permeability challenge studies or alternative markers to localise the barrier deficit may be required to resolve this uncertainty.

Our study was the first to record diet. Apart from trans-unsaturated fatty acids there were no significant differences in diet between the groups. Although the percentage of daily fibre consumed was the same between our depressed and healthy groups, we found a negative correlation with depressive symptoms in our depressed group suggesting an important role for fibre, and fermentation products of fibre in depression **(Figure 2.5).**

Limitations

Ideally, medication free patients would have been recruited. However, in this study, the majority of depressed patients in this study were prescribed antidepressant medication **(Figure 2.1C)** and there were significant differences in alcohol and cigarette consumption **(Table 2.1).** We acknowledge the possibility of an antidepressant-related and serotonin-driven contribution to the alteration in the gut microbiota. In addition, as this study was cross-sectional, only one time point was assessed.

In conclusion, we confirm that depression is associated with neuroimmune and neuroendocrine dysfunction. Moreover, we show that depression is associated with an altered gut microbiota profile albeit in the absence of a compromised metabolic output or impacting on gut barrier integrity. Further studies exploring the mechanisms by which an altered gut microbiota profile may contribute to the pathophysiology of depression are required.

Chapter 3: Fecal Microbiota Transplantation induces behavioural & physiological changes in Antibiotic treated Rats

3.1 Abstract

Background: Depression is associated with an altered gut microbiota composition, richness and diversity. However, it is not clear whether the gut microbiota plays a causal role in the pathophysiology of depression. A number of preclinical studies have shown that it is possible to transfer characteristics from donor to recipient via fecal microbiota transplantation (FMT). We aimed to further elucidate the mechanistic underpinnings explaining the interaction between depression and the microbiota using a FMT to microbiota-depleted rats. We tested the hypothesis that behavioural and physiological features of depression could be transferred via the gut microbiota.

Methods: FMT was prepared from a sub group of depressed patients and matched healthy controls. The FMT was transferred by oral gavage to a microbiota-depleted rat model. A battery of behavioral tests and physiological measures were carried out post FMT. Plasma cytokines, CRP, cortisol and Lipopolysaccharide binding protein (LBP) were determined by ELISA. Plasma tryptophan and kynurenine were determined by HPLC. Hippocampal BDNF gene expression levels were determined by qRT-PCR. Fecal samples were collected for 16s rRNA sequencing. Fecal Short chain fatty acids (SCFAs) were determined by Gas Chromatography.

Results: Rats that received the FMT from depressed patients demonstrated decreased sucrose intake ($t(12) = 2.628$, $p = 0.022$), decreased visits to the open arms ($t(12) = 2.471$, $p = 0.029$) in the elevated plus maze, and a reduction in time spent in the centre in the open field ($t(24) = 2.662$, $p = 0.013$). There were no significant differences in the Forced swim test. In addition, they had increased plasma kynurenine levels ($t(25) = 2.3$, $p = 0.029$) and an increased plasma kynurenine/tryptophan ratio ($t(25) = 2.9$ $p = 0.008$) and a trend toward increased levels of plasma CRP ($t(25) = 1.803$, $p = 0.083$), though no differences in the levels of plasma IL-6, ($t(23) = -0.173$, $p = 0.864$), TNF-α, ($t(24) = 0.569$, $p = 0.574$) or IL1b ($t(24) = 1.716$, $p = 0.09$). There were no significant differences in plasma LBP levels ($t(22) = 0.15$, $p = 0.878$) or plasma corticosterone levels ($t(26) = 0.063$, $p = 0.949$) There were differences in the gut microbiota composition at the phylum, family and genus level and reduced richness as measured by Chao1 ($p = 0.004$), observed species ($p = 0.006$) and reduced diversity measured by phylogenetic diversity ($p = 0.006$) and Shannon index ($p = 0.002$). Fecal acetate and total SCFAs were increased ($p= 0.011$). There were no significant differences in hippocampal BDNF expression ($t(26) = 0.312$, $p = 0.757$).

Conclusions: FMT from depressed patients to microbiota-depleted rats induced behavioural and physiological features characteristic of depression in the recipient animals, including anhedonia and anxiety-like behaviours, as well as alterations in tryptophan metabolism**.** This data suggests that the gut microbiota may play a causal role in the development of features of depression and may provide a tractable target in the treatment and prevention of this disorder.

3.2 Introduction

The emerging links between our gut microbiome and the central nervous system are regarded as a paradigm shift in neuroscience with possible implications for not only our understanding of the pathophysiology of stress-related psychiatric disorders, but also their treatment. This narrative positions the gut microbiome and its influence on host barrier function as a critical node of the brain-gut axis. Mounting pre-clinical evidence broadly suggests that the gut microbiota can modulate brain development, function and behaviour by immune, endocrine and neural pathways of the brain-gut-axis. Moreover, we and other groups have shown that depression is associated with an altered gut microbiota profile, though the mechanisms underlying the interaction remain underdeveloped.

A number of studies have shown that it is possible to transfer characteristics from donor to recipient via fecal microbiota transplantation (FMT). For example, the transfer of microbiota from obese mice to lean mice can result in weight gain and obesity associated metabolic profiles (Ridaura *et al.*, 2013). Furthermore, transfer of the human maternal gut microbiota in pregnancy to GF rodents, induces metabolic changes such as greater adiposity and insulin insensitivity, resembling the metabolic changes that occur in pregnancy (Koren *et al.*, 2012). This has been extended beyond metabolic system, as phenotype transfer via microbiota has also been demonstrated for behaviour.

When the microbiota from BALB/c mice was transferred into adult GF NIH Swiss mice, the result was a decrease in exploratory behaviour and when the NIH Swiss microbiota was transferred into the BALB/c GF mice there was an increase in exploratory behaviour, associated with an increase in hippocampal BDNF levels (Bercik *et al.*, 2011b). Thus, the anxiety-like behavioural phenotype could be transferred via the microbiota, independent of the autonomic nervous system, gastrointestinal specific neurotransmitters

or inflammation. Restoration of normal gut microbiota normalised anxiety like behaviour in GF mice (Clarke *et al.*, 2013). It has been shown that mice that received an obesity associated microbiota exhibit more anxiety-like behaviours associated with increased evidence of neuroinflammation compared to controls (Bruce-Keller *et al.*, 2015).

More recently, a FMT from depressed patients to GF mice resulted in depression and anxiety like behaviours and disturbances of microbial genes and host metabolites involved in carbohydrate and amino acid metabolism (Zheng et al., 2016). While GF animals are an excellent proof-of principal tool, we and others have shown that these animals have some profound CNS abnormalities in adulthood as a consequence of GF status during critical neurodevelopmental windows, many of which are relevant for the depressive phenotype. The main advantage then of antibiotic-induced microbiota depletion during adulthood is that it avoids these potential confounding influences (Arrieta *et al.*, 2016, Luczynski *et al.*, 2016).

Consequently, to confirm that an altered gut microbiota specifically influences aspects of depressive symptomatology, we carried out an FMT from depressed patients to a microbiota depleted antibiotic rat model to assess if a depressive-like phenotype emerged in the treated animals. In addition, using the same microbiota depleted antibiotic rat model we also determined the impact of the FMT on neurobiology.

3.3 Methods

Animals and Treatments

Twenty-eight male Sprague-Dawley rats obtained from Harlan Laboratories UK, weighing an average of 350 g. Food and water was available *ad libitum* and animals were maintained on a 12:12-h dark–light cycle with temperature at 21 ± 1°C. All experiments were in full accordance with the European Community Council Directive (86/609/EEC). The animals were acclimated to the environment and handling for 14 days. They were divided into control and depressed groups, matched for average body weight. Rats were then given a cocktail of ampicillin and metronidazole (both at 1g/L), vancomycin (500 mg/L), ciprofloxacin HCl (200 mg/L) and imipenem (250 mg/L) once daily for 28 consecutive days in drinking water. Seventy-two hours later, animals were colonized via daily oral gavage of donor microbiota (300 µL) for 3 days. Donor microbiota was acquired from pooled fecal samples from 3 of the most severely depressed male patients

and 3 age and sex matched healthy controls. To offset potential founder and/or cage effects and to reinforce the donor microbiota phenotype, booster inoculations were given biweekly throughout the study. Body weight was measured regularly, and all animals were euthanized following behavioural testing.

Behaviour test battery

Following the FMT and recolonization period, animals underwent a behavioural test battery. For all behavioural tests, animals were habituated to the testing room by placing home-cages in the test room for at least 30 min prior to testing. The same animals were assessed across all behavioural tests with at least 6-7 days of resting in between tests.

Sucrose preference test

Sucrose preference test assesses animal's depressive like behaviour - anhedonia. Animals were single-housed 24 hours prior to and for the duration of the procedure. Twenty-four hours later, animals were presented with 2 bottles of water: one containing plain drinking water, and the second contains a 1% sucrose solution. Animals were then given the free choice of either drinking the 1% sucrose solution or plain water for a period of 24hr hours. The positions of two bottles is switched every 12 hours to reduce any confound produced by a side bias. Water and sucrose solution intake was measured 24 hours after introducing sucrose solution by weighing the bottles. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake. At the end of the procedure, animals were group housed and returned to their home cage.

Elevated plus maze

The elevated plus maze is one of the most commonly used rodent tests of anxiety-like behaviours and was performed as previously described (Cryan *et al.*, 2004). The maze consisted of two open arms (51 × 10 cm) and two enclosed arms (51 × 10 × 41 cm) that all extended from a common central platform (10 × 10 cm). The apparatus was elevated 55 cm above the floor on a central pedestal. Animals were acclimatized to the testing room for 30 min prior experiment. At week 11, animals were placed in the centre of the maze facing an open arm to begin. Animal behaviour was videotaped for the duration of 5 min test. Frequency of open and closed arms entries were scored and percentage visits to the open arms was calculated.

Open field test

At week 12, rats were tested in the open field as described previously (O'Mahony *et al.*, 2014). At the beginning of each trial, animals were placed in the centre of a brightly illuminated white open field arena (1000 lux), measuring 0.9 m in diameter, and observed for 10 min. Animals were acclimatized to the testing room for 30 min prior experiment. The arena was cleaned with 70% ethanol to avoid cue smell between each trial. At the end of each trial, animals were returned to their cages. Total activity and time spent in inner zone were analyzed as recorded using a tracking system (Ethovision XT 8.5, Noldus).

Forced swim test

To assess depression-like behaviours at week 14, a modified rat forced swim test (FST) (Slattery and Cryan, 2012) was used. Briefly, on day one, rats were placed individually in Pyrex cylinders (46 cm tall × 21 cm in diameter) filled with water to 30 cm depth at 25°C for habituation to the test. The rats were removed 15 min later, dried and placed in their home cage. On day two, 24 h after the first exposure, the rats were again placed in the swim apparatus for 5 min and behaviours were monitored from above by video camera for subsequent analysis. Three predominant behaviours were recorded in each 5 s period of the 300 s test. Climbing behaviour was defined as upward movements of the forepaws along the edge of the swim chamber. Swimming behaviour was identified as horizontal movements throughout the cylinder. Immobility was described when no additional activity was observed other than that required to keep the rat's head above the water. Latency to become immobile was defined as the time at which the rat first initiated a stationary posture that did not reflect attempts to escape from the water. In this characteristic posture, the forelimbs are motionless and tucked towards the body. To qualify as immobility, this posture had to be clearly visible and maintained for ≥ 2s.

Rat Intestinal Transit time determination

Animals were single-housed (with a thin layer of bedding) with food and water *ad libitum*. Three hours after being single-housed, animals were given 200 ul of 6% carmin red in 0.5% methylcellulose (in PBS) given by oral gavage. After the gavage, the cages were inspected every 10 minutes, and the appearance of the first red fecal pellet was recorded. After the test, animals were group housed in their home cages.

Rat corticosterone determination

On day one of the FST, blood samples *via* tail incision were collected 15 min after the test. Rats were restrained and the end of the tails was held with two fingers. Using a single edge razor blade a diagonal incision of 2 mm long was made at 15 mm from the end of the tail. Approximately 200 µl blood was collected in a collecting tube containing EDTA to avoid blood coagulation by increasing the pressure of the fingers on the tail above the incision. Blood was mixed with EDTA by gently inverting the tube and centrifuged at 3500 × g at room temperature for 15 min. Plasma was carefully aspirated and stored at −80°C. Corticosterone levels were assayed using a commercially-available ELISA kit (Corticosterone EIA Kit, ADI-900-097, Enzo®, Life Sciences) according to manufacturer instructions. Light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm. Sensitivity: 27.0 pg/ml (range 32 - 20,000 pg/ml).

Rat CRP and cytokine determination

Animals were sacrificed by decapitation and immediately after culling, trunk blood was collected in EDTA-coated tubes and centrifuged at 3500 × g for 15 min. Plasma supernatant was collected and stored on dry ice. All samples were frozen at −80°C for later analysis. CRP was determined using commercially available RayBio® Rat CRP ELISA Kit. Sensitivity 200 pg/ml. Detection Range 0.2 ng/ml - 60 ng/ml. TNF-α and IL-6 were analyzed using a commercially available electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA) according to the manufacturer protocol. The median lower limits of detection for; TNF-α; 0.72 pg/ml, IL-6; 13.8 pg/ml.

Rat Lipopolysaccharide determination

LBP concentrations were determined using the Enzyme Immunoassay Kit as per manufacturers' instruction (Enzo®, Life Sciences). Detection Range 1-50ng/ml.

Rat Short Chain Fatty Acid Caecal Content determination

Caecal content was vortex-mixed with Milli-Q water, incubated at room temperature for 10 min and centrifuged to pellet bacteria and other solids. The supernatant was filtered, transferred to a clear GC vial and 2-ethylbutyric acid (Sigma) was added as internal standard. The concentrations of SCFA were measured using a Varian 3800 GC flame-ionization system, fitted with a ZB-FFAP column (30 m x 0.32 mm x 0.25 um;

Phenomenex, Macclesfield, Cheshire, UK). The initial oven temperature was set at 50°C for 0.5 min, raised to 140°C at 10°C/min and held for 0.5 min, then increased to 240°C at 20°C/min, and finally held at this temperature for 12 min. The temperature of the injector and the detector were set at 240°C and 250°C, respectively. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. A standard curve was built with different concentrations of a standard mix containing acetic acid, propionic acid, n-butyric acid and iso-butyric acid (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software.

Rat BDNF determination

The hippocampus was rapidly hand-dissected and stored in RNAlater at 4°C for 24h followed by removal of RNAlater and storage at -80°C until tissues was processed for RNA extraction. RNA was isolated for the Hippocampus (healthy n=15 and depressed n = 13) using miRVanaTM miRNA Isolation Kit, with Phenol following (Thermo Fisher Scientific, AM1560) manufactures protocol for total RNA. Quantitative real-time PCR (qRT-PCR) reverse transcription was carried out on isolated RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, 4368814) in a G-storm thermocycler (G-storm, Surry, UK). Analyses of gene expression was carried out using TaqMan Gene Expression Assay for brain derived neurotrophic factor (BDNF) on a Roche LightCycler 480 instrument. Gene expression levels for BDNF was calculated as the average ct value of 3 replicates for each biological sample from both groups relative to Gapdh expression. Following this fold change was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). For statistical analysis t-test was carried out.

Statistical Analysis. Data that were normally distributed according to Shapiro-Wilk test were analyzed using unpaired t tests. Outliers were removed by Grubbs' test. Data that were not normally distributed were transformed by square root transformation. Microbiota data were analyzed using non parametric tests. Benjamini-Hochberg procedure was used to correct for multiple comparisons with a FDR-adjusted p-value ≤ 0.1 considered significant. Statistical procedures were carried out using IBM SPSS 20.0. Graphs were generated using GraphPad Prism 5.

3.4 Results

Adoptive transfer of depressive phenotype

Behaviour

Rats that received the FMT from the depressed pool demonstrated anhedonia-like behaviours as assessed in the sucrose preference test ($t(12) = 2.628$, $p = 0.022$) **(Figure 3.1B)** with a significant decrease in sucrose intake without affecting fluid intake. Rats receiving FMT from depressed patients also exhibited anxiety-like behaviours as demonstrated by a significant decrease in visits to the open arms ($t(12) = 2.471$, $p = 0.029$) in the elevated plus maze **(Figure 3.1C)** and a reduction in time spent in the centre in the open field ($t(24) = 2.662$, $p = 0.013$) **(Figure 3.1D).** Importantly, these changes occurred without alterations in overall activity as measured in the total visits to the closed and open arms in the EPM and total activity in the open field. In the forced swim test, there were no significant differences between the groups in immobility time ($t(26) = -0.43$, $p = 0.966$), swimming ($t(26) = 1.164$, $p = 0.255$) or climbing ($t(26)$ 0.629, $p = 0.535$) **(Figure 3.1E).**

Physiology altered following FMT

Rats that received the depression FMT had significantly increased plasma kynurenine levels ($t(25) = 2.3$, $p = 0.029$) and an increased plasma kynurenine/tryptophan ratio ($t(25) = 2.9$ $p = 0.008$) **(Figure 3.1F)** but no differences in plasma tryptophan levels ($t(25) = 0.41$, $p = 0.686$). There was a trend toward increased levels of plasma CRP in rats that received the depression FMT ($t(25) = 1.803$, $p = 0.083$) **(Figure 3.1G)** though no differences in the levels of plasma IL-6, ($t(23) = -0.173$, $p = 0.864$), TNF-α, ($t(24) = 0.569$, $p = 0.574$) or IL1b ($t(24) = 1.716$, $p = 0.09$). There were no significant differences in plasma LBP levels ($t(22) = 0.15$, $p = 0.878$) **(Figure 3.1H)** or plasma corticosterone levels ($t(26) = 0.063$, $p = 0.949$) **(Figure 3.1 I).** Rats receiving the depression FMT demonstrated a significant increase in intestinal transit time ($t(26) = 2.652$, $p = 0.013$) **(Figure 3.1J).**

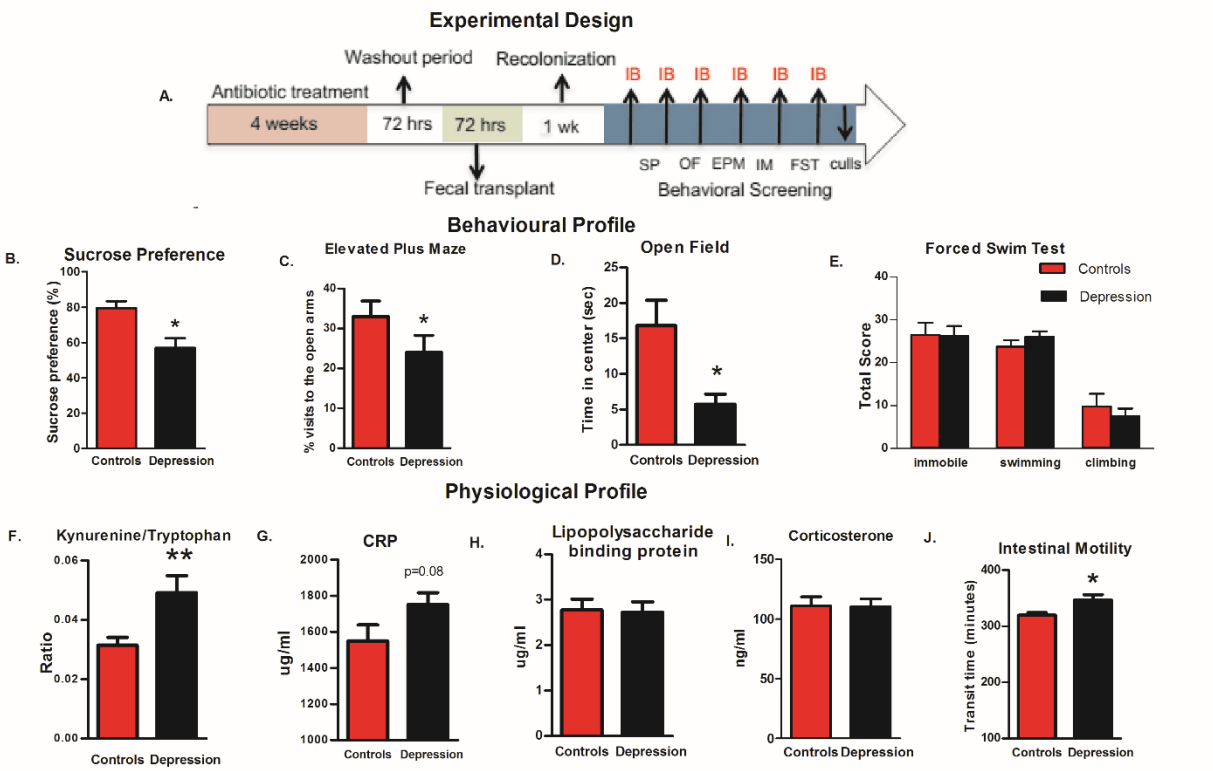


Figure 3.1: Behavioural and physiological changes following fecal transplantation **(A)** Experimental design (Sucrose preference; SP, Open field; OF, Elevated plus maze; EPM, Intestinal motility; IM, Forced swim test FST, Inoculation boost; IB (twice a week). The rats that received the depression FMT (n = 13) exhibited **(B)** anhedonia like behaviour, indicated by a decrease in the 24 hour 1% sucrose preference test ($t(12) = 2.628$, $p = 0.022$) and an increase in anxiety like behaviour measured by **(C)** a decrease in open arm visits in the elevated plus maze (EPM) ($t(12) = 2.471$, $p = 0.029$) and **(D)** a decrease in time spent in the open field ($t(24) = 2.662$, $p = 0.013$) and compared to rats that received the healthy FMT (n = 15). **(E)** There were no differences in the immobility, swimming or climbing time in the Forced Swim Test (FST). **(F)** There was an increase in the plasma Kynurenine/Tryptophan ratio ($t(25) = 2.9$ $p = 0.008$) in the rats that received the FMT from depressed patients. **(G)** There was a trend for increased plasma CRP levels in the rats that received the FMT from depressed patients ($t(25) = 1.803$, $p = 0.083$). There were no significant differences in **(H)** plasma Lipopolysaccharide binding protein (LBP) levels ($t(22) = 0.15$, $p = 0.878$) or **(I)** plasma corticosterone levels 15 minutes post-acute FST stressor ($t(26) = 0.063$, $p = 0.949$). **(J)** Rats that received the FMT from depressed patients demonstrated a significant increase in intestinal transit time ($t(26) = 2.652$, $p = 0.013$).

Neurobiology following FMT

There were no significant differences in hippocampal BDNF expression (t (26) = 0.312, p = 0.757) **(Figure 3.2).**

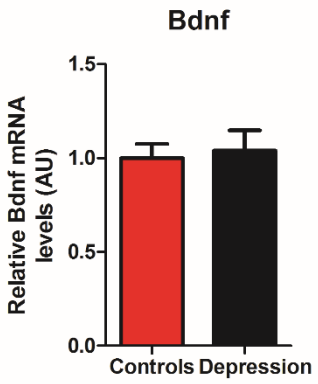


Figure 3.2: Quantitative real-time PCR (qRT-PCR) of BDNF gene expression levels in the rat hippocampus. 15 rats received the healthy FMT and 13 rats received the depression FMT. There were no significant differences in hippocampal BDNF expression (t (26) = 0.312, p = 0.757). Bar graphs indicate average values after Gapdh normalization relative to average control levels.

Altered Gut Microbiota Richness, Diversity, Composition & SCFAs following FMT

Rats that received the FMT from depressed patients had reduced gut microbiota richness as measured by Chao1 (p = 0.004) and observed species (p = 0.006) and reduced diversity measured by phylogenetic diversity (p = 0.006) and Shannon index (p = 0.002) **(Figure 3.3A-D).**

At the phylum level, the relative abundances of *Actinobacteria* (U = 32, p = 0.013) and *Candidate Division TM7* (U = 28.5, p = 0.006) were decreased in rats that received the depression transplantation **(Figure 3.3E).** At the family level, the relative proportions of *Bifidobacteriaceae* (U = 138.5, p = 0.001), *Coriobacteriaceae* (U = 129, p = 0.004), *Porphyromonadaceae* (U = 123, p = 0.012), *Candidate division TM7 uncultured bacterium* (U = 128, p = 0.005), *Caldicoprobacteraceae* (U = 126.5, p = 0.007), *Alcaligenaceae* (U = 144, p = < 0.000) were decreased in rats that received the depression FMT. *Propionibacteriaceae* (U = 27, p = 0.006) was increased in the rats that received the depression FMT **(Figure 3.3F).**

At the genus level, the relative abundances of *Bifidobacterium uncultured* (U = 136, p = 0.001), *Coriobacteriaceae uncultured* (U = 128, p = 0.005), *Caldicoprobacter* (U = 124, p = 0.01), *Roseburia* (U = 132, p = 0.003), *Allobaculum* (U = 126, p = 0.004), *Burkholderiales* (U = 146, p = < 0.000) were decreased in rats that received the depression FMT. *Freudenreichii* (U = 26, p = 0.004), *Staphyloccus* (U = 37, p = 0.013), *Peptococcus* (U = 27.5, p = 0.006) were increased in rats that received the depression transplantation **(Figure 3.3G).**

Fecal acetate and total SCFAs were increased in the rats that received the depression FMT (p= 0.011). There was a trend toward significant increases in the levels of propionate (p = 0.068) and butyrate (p = 0.06) following FMT from depressed patients **(Figure 3.3H).**

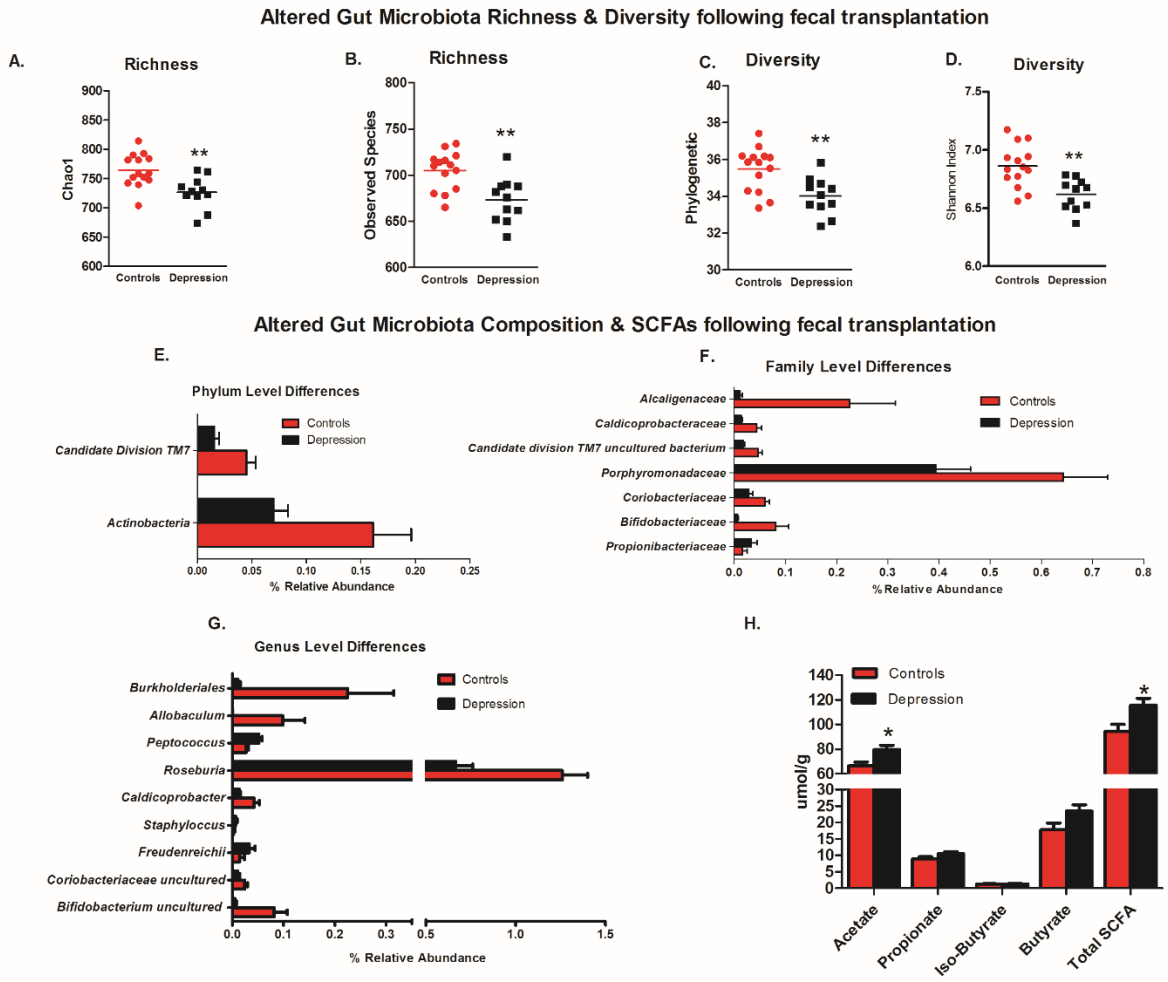


Figure 3.3: Altered Gut Microbiota Richness, Alpha Diversity & SCFAs following fecal transplantation

Rats that received the FMT from depressed patients (n = 13) had reduced gut microbiota richness as measured by **(A)** Chao1 (p = 0.004) and **(B)** observed species (p = 0.006) and reduced diversity measured by **(C)** phylogenetic diversity (p = 0.006) and (**D)** Shannon index (p = 0.002). Significant differences at the phylum level (E), the family level **(F)** and the **(G)** genus level between the rats that received the FMT from the depressed patients compared to the rats that received the healthy FMT (n = 15). **(H)** Levels of fecal acetate and total SCFAs were increased in the rats that received the depression FMT (p= 0.011). There was a trend toward significant increases in the levels of propionate (p = 0.068) and butyrate (p = 0.06) following FMT from depressed patients.

(A)

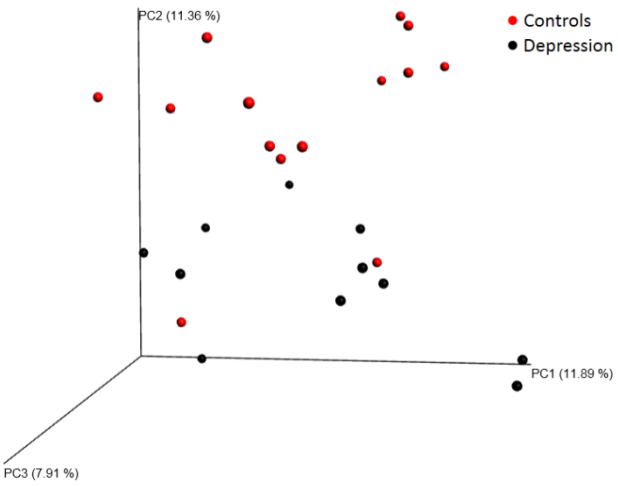


Figure 3.4: Altered Beta Diversity following fecal transplantation

(A) PCoA plot representing unweighted unifrac beta diversity in the rats. Red represents rats that received the healthy FMT (n = 15) and black represents the rats that received the FMT from depressed patients (n = 13).

3.5 Discussion

The present findings represent definitive evidence that depression-associated alterations in the gut microbiome are sufficient to disrupt behavioural and physiological homeostasis. Specifically, transplantation of the perturbed microbiota signature from depressed patients to microbiota-depleted rats induced the development of some of the behavioural and physiological features of the depressive phenotype. Furthermore, this data indicates that a gut microbiota transfer from depressed patients could serve as a novel animal model of depression in the context of microbiome-gut-brain axis dysfunction.

We have demonstrated, that transferring the gut microbiota from depressed patients to rats with a depleted gut microbiota can induce the development of some of the features of the depressive phenotype, such as anhedonia and anxiety-like behaviours, and produce a physiological profile similar to depressed individuals. In contrast to a recent FMT study from depressed patients using germ-free mice (Zheng *et al.*, 2016a), we did not observe alterations in the FST. Our data is consistent with the view that the emergence of only some of the behavioural and neurobiological correlates of depression are contingent on the gut microbiota (De Palma *et al.*, 2015).

Similar to the depressed patients, the rats that received the depression FMT also had an increased kynurenine: tryptophan ratio. The pro-inflammatory profile was also partially transferred, with a trend towards an increase in plasma CRP in the rats that received the depressed FMT. However, there were no differences in the levels of plasma IL-6, TNF-α, or IL1b in the rats that received the depression FMT. A dysregulated HPA axis function was not observed following the FMT, at least in terms of corticosterone output following an acute stressor. However, single time point analysis of HPA axis function can be unreliable (Allen *et al.*, 2014b).

Rats that received the depression FMT demonstrated increased intestinal transit, suggestive of colonic motility dysfunction. Altered gastro-intestinal function is a well-established but often neglected characteristic of depression (Gorard *et al.*, 1996, Haug *et al.*, 2002). Importantly, there were also comparable significant differences in gut microbiota richness and diversity following FMT as between the clinical depression and control groups **(Figure 3.3A-D).** The rats that received the FMT from depressed patients

had altered levels of caecal SCFAs **(Figure 3.3H)** despite this not being noted in the clinical fecal samples **(Figure 2.5A)** suggesting that a depression-associated microbiota can impact SCFA production. Microbial metabolites such as SCFAs can reach the circulation, cross the BBB (Frost *et al.*, 2014, Vijay and Morris, 2014) and activate specific receptors in relevant brain regions underpinning the neurocircuitry pertinent to the expression of depression and anxiety-related behaviours (Schroeder *et al.*, 2007, Wei *et al.*, 2015).

We acknowledge that antidepressant use in the depressed group is a limitation of our study, and that we cannot exclude the possibility of an antidepressant-related serotonin-driven contribution to the alteration in the gut microbiota. However, antidepressants would be expected to reduce the kynurenine/tryptophan ratio rather than increase it via, for example, activity on TDO (Badawy and Morgan, 1991, Badawy *et al.*, 1991) making it unlikely that residual medication in the fecal transplantation from the depressed patients would increase depressive and anxiety like behaviours in the rats that received the depressed FMT. Regardless of the origins of the gut microbiota differences in the transplant, the preclinical data confirms that when a depression-associated microbiota is transferred, neurobiological and behavioural consequences can ensue. Although the gut microbiota can impact transcriptional regulation (Stilling *et al.*, 2015) we found no significant differences in hippocampal BDNF expression in the rats that received the FMT.

In conclusion, we have demonstrated that it is possible to reproduce aspects of depressed behaviour and physiology via a gut microbiota transfer. This suggests that the gut microbiota could play a causal role in the complex mechanisms underlying the development of depression. The profile of depression-like behaviours and physiological alterations noted following FMT suggests that this represents a novel paradigm in behavioural pharmacology to investigate microbiota-associated depression. Ultimately, findings from this study advance the concept that targeting the gut microbiota may be a viable therapeutic strategy for novel antidepressant development in sub groups of depressed patients and may augment depression prevention strategies.

Chapter 4: Targeting the Microbiota-gut-brain axis with *Lactobacillus Rhamnosus* (JB-1): Evaluation of a Candidate Psychobiotic in healthy male volunteers

4.1 Abstract

Background: Preclinical studies have identified certain probiotics as psychobiotics - live microorganisms with a potential mental health benefit. *Lactobacillus rhamnosus* (JB-1) has been shown to reduce stress-related behaviour, corticosterone release and alter central expression of GABA receptors in an anxious mouse strain. However, it is unclear if this single putative psychobiotic strain has psychotropic activity in humans. Consequently, we aimed to examine if these promising preclinical findings could be translated to healthy human volunteers.

Objectives: To determine the impact of *L. rhamnosus* on stress-related behaviours, physiology, inflammatory response, cognitive performance and brain activity patterns in healthy male participants.

Methods: An 8 week, randomized, placebo-controlled, cross-over design was employed. Twenty-nine healthy male volunteers participated. Participants completed self-report stress measures, cognitive assessments and resting electroencephalography (EEG). Plasma IL10, IL1β, IL6, IL8 and TNFα levels and whole blood Toll-like 4 (TLR4) agonist cytokine release were determined by multiplex ELISA. Salivary cortisol was determined by ELISA and subjective stress measures were assessed before, during and after a socially evaluated cold pressor test (SECPT).

Results: There was no overall effect of probiotic treatment on measures of mood, anxiety, stress or sleep quality and no significant effect of probiotic over placebo on subjective stress measures, or the HPA response to the SECPT. Visuospatial memory performance, attention switching, rapid visual information processing, emotion recognition and associated EEG measures did not show improvement over placebo. No significant anti-inflammatory effects were seen as assessed by basal and stimulated cytokine levels.

Conclusions: *L. rhamnosus* was not superior to placebo in modifying stress-related measures, HPA response, inflammation or cognitive performance in healthy male participants. These findings highlight the challenges associated with moving promising preclinical studies, conducted in an anxious mouse strain, to healthy human participants. Future interventional studies investigating the effect of this psychobiotic in populations with stress-related disorders are required.

4.2 Introduction

An abundance of preclinical studies have shown that probiotics acting via the brain-gut-axis can affect brain development, function and behaviour (Cryan and Dinan, 2015b, Desbonnet *et al.*, 2014, Desbonnet *et al.*, 2010, Hsiao *et al.*, 2013). This has prompted a growing interest in the possibility of targeting the gut microbiome to beneficially impact human brain and behaviour. Psychobiotics have been defined as bacteria that ingested in adequate amounts produce a positive mental health benefit (Dinan *et al.*, 2013).

Considering the potential impact of putative psychobiotics upon central nervous system processes, especially stress, mood, anxiety and cognition (Cryan and Dinan, 2012c, Dinan *et al.*, 2015), the prospect of targeting the gut microbiota as a potential modifiable risk factor for stress-related disorders is appealing (Kelly *et al.*, 2015). Preclinical research has indicated that chronic probiotic administration can reduce anxiety-like and depressive-like behaviour, and can normalise associated physiological outputs such as corticosterone, noradrenaline, brain-derived neurotrophic factor (BDNF) and immune function (Bercik *et al.*, 2011a, Bravo *et al.*, 2011, Desbonnet *et al.*, 2010, Janik *et al.*, 2016, Messaoudi *et al.*, 2011). There is a growing appreciation of the need to translate this promising preclinical work to the clinic while at the same time recognising the challenges inherent in this process (Kelly *et al.*, 2016c).

To date, there are indications from a number of sources that highlight the opportunities in this regard, for example, probiotic use in irritable bowel syndrome (IBS) (O'Mahony *et al.*, 2005, Whorwell *et al.*, 2006), a stress-related brain-gut axis disorder associated with high rates of psychopathology (Whitehead *et al.*, 2002) as well as altered hypothalamic-pituitary-adrenal (HPA) axis activity (Kennedy *et al.*, 2014c) and cognition (Kennedy *et al.*, 2015, Kennedy *et al.*, 2014b). A number of proof-of-principle studies in healthy human volunteers have demonstrated that multi-strain probiotics, fermented drinks containing probiotics, or prebiotics, can alter resting brain activity, cognitive performance, baseline physiological stress outputs and self-reported psychological variables (Benton *et al.*, 2007, Chung *et al.*, 2014, Messaoudi *et al.*, 2011, Schmidt *et al.*, 2015, Steenbergen *et al.*, 2015a, Tillisch *et al.*, 2013b). More recently, *Bifidobacterium longum* **1714**, selected based on pre-clinical evidence (Savignac *et al.*, 2014, Savignac *et al.*, 2015a), reported reduce stress levels and a neurocognitive profile associated with enhanced memory in healthy volunteers (Allen *et al.*, 2016).

By utilizing a well-validated preclinical screening platform, developed to inform efficient selection of prospective psychobiotic strains, we identified *L. rhamnosus* (JB-1). In these studies, which were carried out in the stress-sensitive BALB/c mice, ingestion of the JB-1 strain reduced anxiety in the elevated plus maze and despair-like behaviour in the forced swim test. Moreover, there was enhanced learning in a fear conditioning paradigm and reduced stress-induced corticosterone levels. At a brain level there were marked alterations in central GABAA and GABAB receptor levels (Bravo *et al.*, 2011). Furthermore, a magnetic resonance spectroscopy study, also conducted in BALB/c mice showed that treatment with the JB-1 strain significantly elevated central GABA levels by 25% after four weeks of treatment (Janik *et al.*, 2016). In addition, *L. rhamnosus* treatment modulates the immune system (Forsythe *et al.*, 2012, Karimi *et al.*, 2009, Kozakova *et al.*, 2016, Ma *et al.*, 2004), intestinal motility (Wang *et al.*, 2010), gut barrier function (Patel *et al.*, 2012b, Wang *et al.*, 2012b) and enteric nervous system (Kamiya *et al.*, 2006, Ma *et al.*, 2009). Taken together, these preclinical studies identify *L. rhamnosus* as the candidate psychobiotic with the most comprehensive behavioural, physiological and neurobiological profile.

We employed a randomized, placebo-controlled, cross-over, repeated measures design to examine the effects of the JB-1 strain compared to placebo on the psychobiological response to an acute, controlled stressor (Schwabe *et al.*, 2008, Schwabe and Wolf, 2010) and assessed cognitive performance on tests assessing memory, sustained attention, social cognition and emotional processing. In addition, we measured the immune response to this candidate psychobiotic by measuring a panel of cytokines and TLR4 induced cytokine release.

Finally, to ascertain if the JB-1 strain effected brain activity patterns, we assessed brain activity in frontal, parietal and central regions using EEG following 4-week supplementation with the JB-1 strain in comparison to placebo, as these regions have been associated with memory and sustained attention (Coull *et al.*, 1996, Hales *et al.*, 2009) and are sensitive to anxiolytics (Fukami *et al.*, 2010).

4.3 Methods

Subjects

Approval of the study protocol was granted by the Cork University Hospital ethics committee (Protocol Number: APC057) and conducted in accordance with the ICH Guidelines on Good Clinical Practice, and the Declaration of Helsinki. Written informed consent was obtained from all subjects before any study procedures were conducted.

Participants were aged between 20 and 33 years of age. Inclusion criteria were as follows: aged between 18 - 40 years, able to speak English, in good health as determined by the investigator. Male participants were selected to avoid the need to control for menstrual cycle, which can impact upon cortisol output and other readouts. Exclusion criteria were as follows: having a significant acute or chronic illness, following a diet or taking a medication that would interfere with the objectives of the study, pose a safety risk or confound the interpretation of the study results, to include, probiotics, antibiotics, antipsychotics, anxiolytics, laxatives, enemas, anti-coagulants and over-the counter non-steroidal anti-inflammatorys (NSAIDS), antidepressants or any other psychotropic medication, Evidence of immunodeficiency, bleeding disorder or coagulopathy, colour blindness, dyslexia or dyscalculia, or receiving any treatment involving experimental drugs.

Design

A repeated measures crossover design was employed. Participants were screened at an initial visit for psychiatric disorder using the MINI International Neuropsychiatric Interview (MINI)(Sheehan *et al.*, 1998) and demographic and baseline psychological information was collected. Following screening, participants completed neurocognitive visits and acute stress visits utilizing the socially evaluated cold pressor test (SECPT) at baseline, at 4 weeks and at 8 weeks. Participants were administered placebo capsules for four weeks or *L. Rhamnosus* capsules for four weeks in a randomized single blind placebo controlled cross over trial. (See **Table 4.1** for detailed participant characteristics).

Materials

Both active and placebo capsules contained corn starch, magnesium stearate and silicon dioxide. The count for *L. Rhamnosus* (JB-1) in the active capsules was $1x10^9$ colony-

forming units (CFU). Participants were instructed to take one capsule each morning. Compliance was assessed by self-report at each study visit.

Tests from the CANTAB Battery

Tests from the Cambridge Neuropsychological Test Automated Battery (CANTAB) were presented on a touch-screen monitor, Sahara i440D Slate Tablet PC (Sand Dune Ventures, Tablet Kiosk) running CANTABeclipse™ software (Cambridge, UK). The researcher provided verbal instructions to participants from a standardised script, and had full control of a keyboard used to start, pause or abort each test. As a test battery of multiple cognitive tests was employed, test order was counterbalanced, using a Latin square design, to avoid effects of fatigue for tests completed later in the session. The test battery lasted approximately 45 minutes in total. Participants were assessed on the following tests from the battery:

Motor Screening Test (MOT)

The MOT is a screening test and allows participants to get familiar with using the touchscreen. It screens for difficulty with movement, comprehension and vision. Participants are presented with a series of crosses in different locations on the screen and instructed to touch them in turn. The administration time was approximately 2 minutes.

Paired Associates Learning (PAL)

PAL test was used to assess conditional learning of pattern-location associations. Paired associate performance has shown sensitivity to functional changes in the hippocampus (de Rover *et al.*, 2011) and frontal lobes (Hales *et al.*, 2009). The parallel mode (which presents different shapes at each visit) was used in order to avoid practice effects. White boxes are presented on-screen and each opens in a randomized order; in some boxes a pattern is shown. Participants must remember patterns associated with different locations. In the practice phase 2 patterns are presented and after this presentation the participant must touch the boxes the patterns appeared in. Participants then have to remember 2 patterns for 1 trial, 3 for 1 trial, 6 for 1 trial and 8 for 1 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 proceeds to the next stage. If participants do not correctly identify the pattern locations within 10 trials the test terminates. The administration time was approximately 10 minutes. Outcomes

assessed: Total errors per stage (8 shapes), mean trials required to locate all patterns correctly, first trial memory score.

Attention Switching Task (AST)

AST was used to measure the subject's ability to switch attention between the direction of an arrow and its location on the screen and to ignore task-irrelevant information. This test has been designed to measure top-down cognitive control processes involving the prefrontal cortex. The test displays an arrow which can appear on either side of the screen (right or left) and can point in either direction (to the right or to the left). Each trial displays a cue at the top of the screen that indicates to the participant whether they have to press the right or left button according to the "side on which the arrow appeared" or the "direction in which the arrow was pointing". Some trials display congruent stimuli (e.g. arrow on the right side of the screen pointing to the right) whereas other trials display incongruent stimuli which require a higher cognitive demand (e.g. arrow on the right side of the screen pointing to the left). The administration time was approximately 8 minutes. Outcomes assessed: Reaction latency, Reaction latency congruent, Reaction latency incongruent, congruency cost, switching cost, percent correct.

Rapid visual information processing (RVP)

RVP was used to assess sustained attention. Performance on this task activates a fronto-parietal network of brain regions (Coull *et al.*, 1996), and a modified version of this task is sensitive to changes in frontal EEG (Allen *et al.*, 2014a). Subjects are presented with digits appearing on screen one at a time. Participants are required to press the button on the press pad as soon as they detect target sequences of digits (e.g. 3-5-7). The test consists of a practice phase followed by a four-minute testing phase. The administration time was approximately 7 minutes. Outcomes assessed: Targets correctly detected, false alarms and reaction time.

Emotion Recognition Task (ERT)

ERT was used to assess social cognition. fMRI has previously demonstrated probiotic effects on a network of brain regions involved in emotional and viscerosensory processing in healthy controls (Tillisch *et al.*, 2013b). The participant is briefly shown faces displaying distinct emotions; happiness, anger, sadness, fear, surprise and disgust. The participant is then required to identify the emotion as quickly as possible from a list

of possible options. The administration time was approximately 8 minutes. Outcomes assessed: Total correct, total errors, mean speed of response.

Other cognitive tests

Emotional Stroop

In addition to tests from the CANTAB battery, we assessed emotional processing using an Emotional Stroop (Strauss *et al.*, 2006). The Stroop test was presented on the same high-resolution touch screen monitor used with the CANTAB battery. The emotional Stroop is associated with activation in the anterior cingulate cortex (Bush *et al.*, 2000, Etkin *et al.*, 2006). Positively, negatively and neutrally valenced words were presented, matched for length in letters, orthographic neighbourhood size (i.e. the number of words which differ from a given word by only one letter) and frequency of use (Larsen *et al.*, 2008). Participants were asked to name the colour the word was printed in. Administration time was approximately 10 minutes. Outcomes assessed: Positive percent correct, Positive reaction time, Negative percent correct, Negative reaction time, Neutral percent correct, Neutral reaction time.

Acute Stress Procedure

We employed the socially evaluated cold pressor test (SECPT) (Schwabe *et al.*, 2008) as a combined psychological and physiological stressor procedure, which has been shown not to induce HPA axis habituation across repeated exposures (Minkley *et al.*, 2014). Participants were required to avoid alcohol for 24 hours prior to the visit, as well as caffeinated beverages on the day of the stress procedure and strenuous exercise from 2pm the day before, and to fast for 2 hours prior to testing.

After the participant completed the state items from the state-trait anxiety inventory (Spielberger *et al.*, 1970) a baseline saliva sample was taken. The baseline measures were followed by a 5-minute resting phase. Following the 5-minute resting phase, another saliva sample was taken. The participant read the instructions for the SECPT and the experimenter answered any questions. The participant was then lead into a second room in which a confederate, who was dressed in a white lab coat and maintained a neutral demeanour throughout, was seated at a table with a container of ice water (at 0-4°C), and a camera which was directed towards the participant's face during completion of the SECPT. Paper towels were kept beside the container. The experimenter said:

"*Researcher, this is subject number xxx. Subject xxx, remember that your task is to keep your hand submerged in cold water, that your facial expressions will be video recorded for later analysis, and that the researcher is specially trained to monitor non-verbal behaviour. Do you have any questions?*" After answering any questions the experimenter left. The confederate instructed the participant to complete the cold pressor test and started the timer; during the entire procedure the confederate maintained a neutral demeanour. After withdrawing their hand from the water, the participant returned to the previous room, and completed the post-stress state anxiety questionnaire. Further saliva samples were taken 1 minute after the cessation of the stressor, as well as, 10, 20, 30, and 60 minutes post-stressor cessation.

Neurocognitive assessment

Prior to EEG testing participants were asked to refrain from caffeine on the morning of their experimental session, as well as ensuring they got a good night's sleep, to remove any piercings and avoid wearing hair gel. All EEG measurements were made using a Neuroscan®, SynAmps 2 Amplifier and Neuroscan 4.3.1 acquisition software. EEG was recorded at a sampling rate of 1,000Hz. Scalp electrodes were attached at midline positions Fz, Pz, Cz, (see **Figure 4.1**) and F1, F2, F3, F4, F5, F6, F7, F8, according to the international 10/20 system, as well as mastoid electrodes and a reference electrode on the nose. Vertical eye movements were detected using electrodes attached above and below the orbit of the left eye, simultaneously horizontal eye movements were monitored by electrodes at the right and left outer canthi. EEG recordings were made using Neuroscan ® Quick-Cap (containing AgCl sintered electrodes and Neuroscan Quick-Cell technology) therefore ensuring reduced impedance levels for optimized recordings at each electrode. Following a resting EEG recording, the cognitive tasks were completed (see Cognitive tasks).

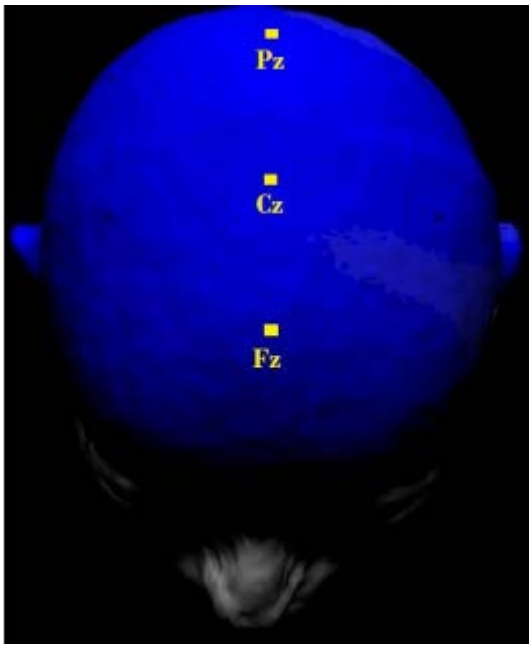


Figure 4.1: Electrode position of Fz, Cz and Pz. Scalp electrodes were attached at midline positions Fz, Pz, Cz according to the international 10/20 system.

Resting EEG

EEG measures of absolute power in the delta (2-4Hz), theta1 (4-6Hz), theta2 (6-8 Hz) alpha1 (8-10Hz), alpha2 (10-12Hz), beta (15-30Hz) frequency bands were taken for five minutes with eyes closed. Participants were requested to relax and sit still with their eyes closed while resting EEG was recorded.

EEG analysis

The EEG signal was down sampled from 1000Hz to 256Hz with an antialiasing filter set at 128Hz. The filtered EEG signal was segmented into 1s windows without overlap. Curve length, root mean squared amplitude, Hjorth parameters (activity, mobility, complexity) (Hjorth, 1970), zero crossings (raw epoch, first and second derivative), autoregressive modelling error (model order 1-9), nonlinear energy, variance (first and second derivative), entropy (Shannon entropy, spectral entropy, singular value decomposition entropy), Fisher information, and wavelet energy (Daubechy 4) were calculated using MATLAB. EEG measures of absolute power were extracted in the delta (2-4Hz), theta1 (4-6Hz), theta2 (6-8 Hz) alpha1 (8-10Hz), alpha2 (10-12Hz), beta (15-30Hz) frequency bands.

Sample analysis

Cortisol sampling & analysis

Salivettes were centrifuged at 1000 g for 5 min and aliquoted and stored at -80ºC until analysis. Cortisol concentrations were determined using the Cortisol Enzyme Immunoassay Kit as per manufacturers' instruction (Enzo®, Life Sciences). Assay detection limit was 0.16 nmol/L. Inter and intra-assay % C.Vs were 11.24% and 8.2% respectively.

Cytokine sampling & analysis

10ml of whole blood was collected in an EDTA tube. Samples were centrifuged at 1000 g for 15 minutes and then aliquoted and stored at -80ºC until analysis. Plasma levels of IL1β, IL6, IL8, IL10 and TNFα were assayed in duplicate using high sensitivity commercially available electrochemiluminescence MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, 75USA) as per manufacturer's instructions. The median lower limits of detection for each cytokine are; IL-1β; 0.04 pg/ml, IL-6; 0.06 pg/ml, IL-8; 0.04 pg/ml, IL-10; 0.03 pg/ml, TNF-α 0.04 pg/ml.

TLR4 cytokine release

TLR cytokine release was determined as previously described (McKernan *et al.*, 2011). Whole blood was collected in lithium heparin tubes and diluted 1:10 with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and 5% penicillin streptomycin. Each blood sample was cultured with and without the TLR4 receptor ligand - lipopolysaccharide (LPS), from the Human TLR agonist kit (InvivoGen, San Diego, CA, USA) for 24 hours. After the 24 hour culture period the supernatant from both untreated and stimulated cells was aspirated and stored at -80°C. Levels of IL1β, IL6, IL8, I-10 and TNFα were assayed in duplicate using high sensitivity commercially available electrochemiluminescence MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, 75USA) as per manufacturer's instructions.

Statistical analysis

With a power of 0.8 for a one-way ANOVA, a minimum sample size of 20 was required to demonstrate an effect sized f = 0.3 at alpha = 0.05 (Buchner *et al.*, 1997) (Allen *et al.*, 2016). Data were analysed using SPSS 21. Repeated measures ANOVA and pairwise t-tests using post-hoc Fisher's least significant difference (LSD) were used to examine

differences between conditions, and non-parametric equivalents (Friedman and Wilcoxon respectively) were used where parametric assumptions were violated. Areas under the curve with respect to ground (AUCg) were also calculated (Pruessner *et al.*, 2003), and analysed in the same manner.

4.3 Results

Demographic data

See **(Table 4.1)** for Participant characteristics.

Table 4.1: Participant characteristics. Means and Standard errors of the mean in parentheses

Participant characteristics	Total sample (n=29)	Placebo / Probiotic (n=15)	Probiotic / placebo (n=14)	p-value
Age (mean)	24.59 (0.75)	23.6 (0.97)	25.64 (1.14)	0.22
Ethnicity				
Caucasian	25	13	12	
Asian	4	2	2	1.00
BMI	24.55 (0.58)	24.8 (0.69)	24.29 (0.96)	0.66
Alcohol (units per week)	10.14 (1.85)	11.85 (2.68)	8.42 (2.56)	0.36
Years of Education	18.45 (0.49)	17.87 (0.71)	19.07 (0.65)	0.22

Subjective stress measures

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that there was no overall effect of treatment phase on the Beck Depression Inventory (p = 0.75), the Beck Anxiety Inventory (p = 0.95), the Perceived Stress Scale (p = 0.053), the State Anxiety Inventory (p = 0.09), the Trait Anxiety Inventory (p = 0.72), the Symptom Checklist-90 (p = 0.87) or the Pittsburgh sleep quality index (p = 0.07). In the coping checklist, there was a reduction in wishful thinking (p = 0.03) in the placebo (p = 0.04) and probiotic phase (p = 0.02), see **(Figure 4.1).** For pairwise comparisons see **(Table 4.2).**

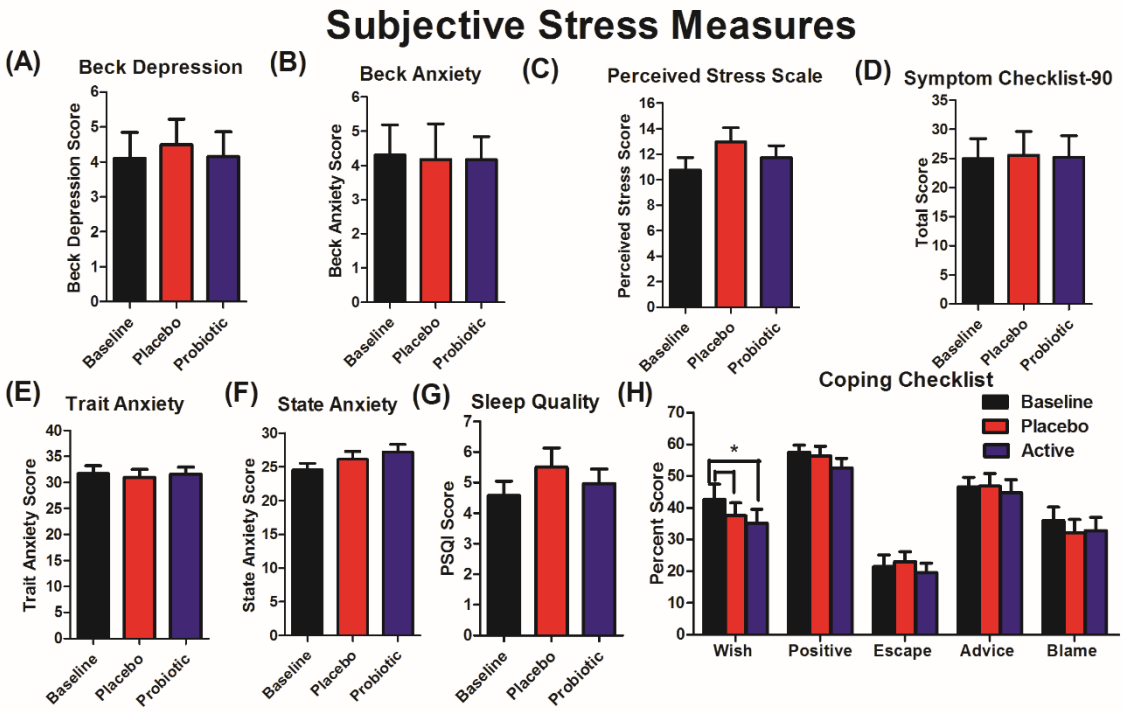


Figure 4.1: Subjective Stress Measures

There was no overall effect of treatment phase in the **(A)** Beck Depression Inventory (F 1.93, 50.36 = 0.27, p = 0.75), **(B)** the Beck Anxiety Inventory (F 1.53, 43.00 = 0.02, p = 0.95) **(C)** the Perceived Stress Scale (F 1.80, 50.38 = 3.23, p = 0.053) **(D),** the State Anxiety Inventory (F 1.72, 48.18 = 2.57, p = 0.09), **(E)** the Trait Anxiety Inventory (F 1.97, 55.19 = 0.31, p = 0.72, **(F)** the Symptom Checklist-90 (F 1.93, 52.11 = 0.12, p = 0.87) or **(G)** the Pittsburgh sleep quality index (F 1.74, 47.16 = 2.80, p = 0.07). **(H)** In the coping checklist, there was a reduction in wishful thinking (F 1.74, 47.08 = 3.96, p = 0.03) in the placebo (p = 0.04) and probiotic phase (p = 0.02).

Table 4.2: Subjective Stress Measures. Means and Standard errors of the mean in parentheses.

	Baseline	Placebo	Probiotic	Baseline V Placebo (p value)	Placebo V Probiotic (p value)	Baseline V Probiotic (p value)	Treatment Effect (Repeated Measures ANOVA)
Beck Depression Inventory	3.92 (0.76)	4.33 (0.72)	3.88 (0.67)	0.57	0.95	0.51	F (1.93, 50.36) = 0.27, p = 0.75
Beck Anxiety Inventory	4.31 (0.88)	4.17 (1.04)	4.17 (0.67)	0.88	1.00	0.84	F (1.53, 43.00) = 0.02, p = 0.95
Perceived Stress Scale	10.75 (0.98)	12.96 (1.12)	11.72 (0.94)	0.03	0.10	0.26	F (1.80, 50.38) = 3.23, p = 0.053
Symptom Checklist							
Total (global severity index)	24.28 (3.47)	25.57 (4.05)	24.35 (3.68)	0.65	0.69	0.97	F (1.93, 52.11) = 0.12, p = 0.87
State Trait Anxiety Inventory							
Trait	31.69 (1.50)	30.96 (1.50)	31.58 (1.36)	0.47	0.54	0.91	F (1.97, 55.19) = 0.31, p = 0.72
State	24.58 (0.95)	26.10 (1.18)	27.20 (1.13)	0.26	0.36	0.008	F (1.72, 48.18) = 2.57, p = 0.09
Pittsburgh Sleep Quality Index (PSQI)	4.57 (0.47)	5.50 (0.62)	4.96 (0.47)	0.008	0.25	0.32	F (1.74, 47.16) = 2.80, p = 0.07
Coping Checklist							
Wish %	44.19 (4.62)	37.50 (3.99)	35.56 (4.45)	0.04	0.48	0.02	F (1.74, 47.08) = 3.96, p = 0.03
Positive %	56.98 (2.24)	56.34 (2.98)	52.54 (3.11)	0.78	0.30	0.18	F (1.63, 44.08) = 1.17, p = 0.30
Escape %	22.09 (3.72)	23.01 (3.13)	19.04 (3.02)	0.74	0.08	0.43	F (1.41, 38.28) = 0.95, p = 0.36
Advice (%)	46.03 (3.15)	46.82 (3.96)	43.45 (3.91)	0.83	0.46	0.47	F (1.84, 49.80) = 0.38, p = 0.66
Blame %	36.60 (4.33)	32.14 (4.10)	32.73 (4.34)	0.22	0.87	0.37	F (1.92, 51.91) = 0.75, p = 0.47

Acute Stress response to the Socially Evaluated Cold Pressor Test (SECPT)

There was no overall effect of treatment phase on subjective stress measures pre or post the SECPT (see **Figure 4.2A-H**), and no significant overall effect of probiotic over placebo in HPA response to the SECPT (**Figure 4.2I-J**). See **(Table 4.3)** for pairwise comparisons.

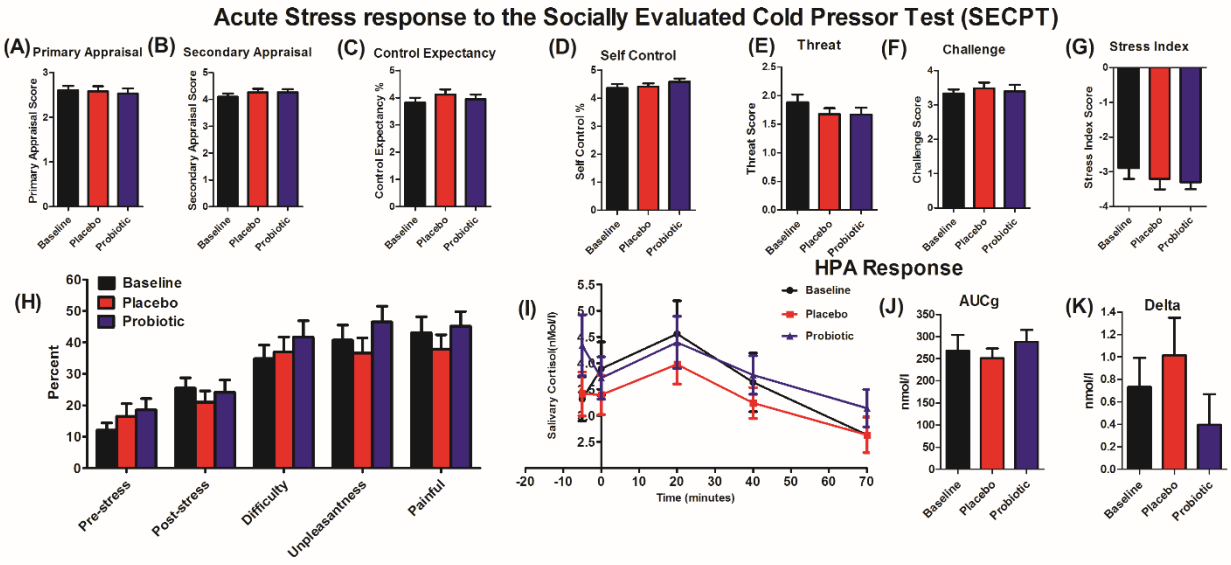


Figure 4.2: Acute Stress response to the Socially Evaluated Cold Pressor Test (SECPT)

In the primary appraisal/secondary appraisal scale, there was no significant effect of probiotic the **(A)** primary appraisal (F 1.52, 42.70 = 0.23, p = 0.73), **(B)** secondary appraisal (F 1.69, 47.56 = 2.10, p = 0.14) in **(C)** control expectancy (F 1.58, 44.39 = 1.91, p = 0.16), **(D)** self-control (F 1.80, 50.62 = 2.21, p = 0.12), **(E)** threat (F 1.45, 40.70) = 1.28, p = 0.27), **(F)** challenge (F 1.83, 51.48 = 0.64, p = 0.51), **(G)** or stress index (F 1.54, 41.65 = 1.01, p = 0.35). **(H)** There was no significant effect of treatment in pre-stress (F 1.66, 46.58 = 2.59, p = 0.09), post-stress, (F 1.89, 52.92 = 0.65, p = 0.51), difficulty (F 1.83, 51.38 = 1.23, p = 0.29), unpleasantness (F 1.91, 53.55 = 2.16, p = 0.12), or pain reports (F 1.60, 45.04 = 1.27, p = 0.28). **(I)** There were no significant differences in the HPA response to the SECPT (F 4.53, 104.22 = 0.80, p = 0.54), the **(J)** Area under the curve with respect to ground (AUCg) (F 1.60, 38.52 = 1.03, p = 0.35) or the **(K)** delta cortisol response (p = 0.28).

Table 4.3: Subjective Stress Measures in the SECPT. Means and Standard errors of the mean in parentheses.

	Baseline	Placebo	Probiotic	Baseline V Placebo (p value)	Placebo V Probiotic (p value)	Baseline V Probiotic (p value)	Treatment Effect (Repeated Measures ANOVA)
Primary Appraisal/Secondary Appraisal							
Threat	1.87 (0.14)	1.67 (0.10)	1.66 (0.12)	0.26	0.92	0.20	F (1.45, 40.70) = 1.28, p = 0.27
Challenge	3.32 (0.12)	3.48 (0.16)	3.39 (0.18)	0.29	0.46	0.64	F (1.83, 51.48) = 0.64, p = 0.51
Self-Control	4.36 (0.13)	4.41 (0.11)	4.58 (0.10)	0.68	0.08	0.04	F (1.80, 50.62) = 2.21, p = 0.12
Control Expectancy	3.81 (0.17)	4.11 (0.19)	3.94 (0.17)	0.11	0.12	0.43	F (1.58, 44.39) = 1.91, p = 0.16
Primary appraisal	2.60 (0.09)	2.57 (0.11)	2.53 (0.11)	0.83	0.51	0.55	F (1.52, 42.70) = 0.23, p = 0.73
Secondary appraisal	4.09 (0.12)	4.26 (0.12)	4.26 (0.11)	0.14	1.00	0.07	F (1.69, 47.56) = 2.10, p = 0.14
Stress index	-2.89 (0.12)	-3.20 (0.29)	-3.30 (0.19)	0.39	0.65	0.18	F (1.54, 41.65) = 1.01, p = 0.35
Socially Evaluated Cold Pressor (SPECT) %							
Prestress	12.50 (2.33)	17.14 (4.04)	18.57 (3.59)	0.16	0.70	0.02	F (1.66, 46.58) = 2.59, p = 0.09
Difficulty	34.82 (4.31)	36.89 (4.86)	41.72 (5.15)	0.63	0.35	0.09	F (1.83, 51.38) = 1.23, p = 0.29
Unpleasantness	40.69 (4.88)	36.55 (4.92)	46.55 (4.94)	0.41	0.06	0.18	F (1.91, 53.55) = 2.16, p = 0.12
Post stress	25.51 (3.27)	21.03 (3.62)	24.13 (3.92)	0.31	0.45	0.69	F (1.89, 52.92) = 0.65, p = 0.51
Painful	43.10 (5.08)	37.93 (4.53)	45.17 (4.64)	0.37	0.09	0.60	F (1.60, 45.04) = 1.27, p = 0.28

Immune response

There was no overall treatment effect on the concentrations of IL10 ($p = 0.32$), IL1β ($p = 0.08$), IL6 ($p = 0.13$) or IL8 ($p = 0.16$) **(Figure 4.3A-D).** The concentration of TNFα increased from baseline during the placebo phase ($p = 0.02$), but there was no significant change in baseline versus probiotic ($p = 0.08$) or placebo versus probiotic ($p = 0.18$) **(Figure 4.3E).** There was no overall treatment effect on the IL1β:IL10 ($p = 0.68$), IL6:IL10 ($p = 0.12$), IL8:IL10 ($p = 0.97$), or TNFα:IL10 ($p = 0.99$) ratios **(Figure 4.3F-I).** Inspection of IL6:IL10 data suggested differences during the probiotic phase so we carried out exploratory analysis by using pairwise comparisons **(Table 4.4)** which indicated a lower IL6:IL10 ratio from baseline ($p = 0.03$) **(Figure 4.3G)**, however this was non-significantly lower than placebo levels ($p = 0.13$).

TLR4 cytokine release

In the TLR4 stimulated cytokines, there was an increase in the level of IL1β ($p = 0.02$) **(Figure 4.3J)** and TNFα ($p = 0.01$) **(Figure 4.3K)** during the placebo phase compared to baseline ($p = 0.01$) but no effect of probiotic ($p = 0.03$). There was no effect of either treatment phase on TLR4 stimulated IL10 ($p = 0.12$), IL6 ($p = 0.22$) or IL8 ($p = 0.25$) cytokine release **(Figure 4.3L-M).**

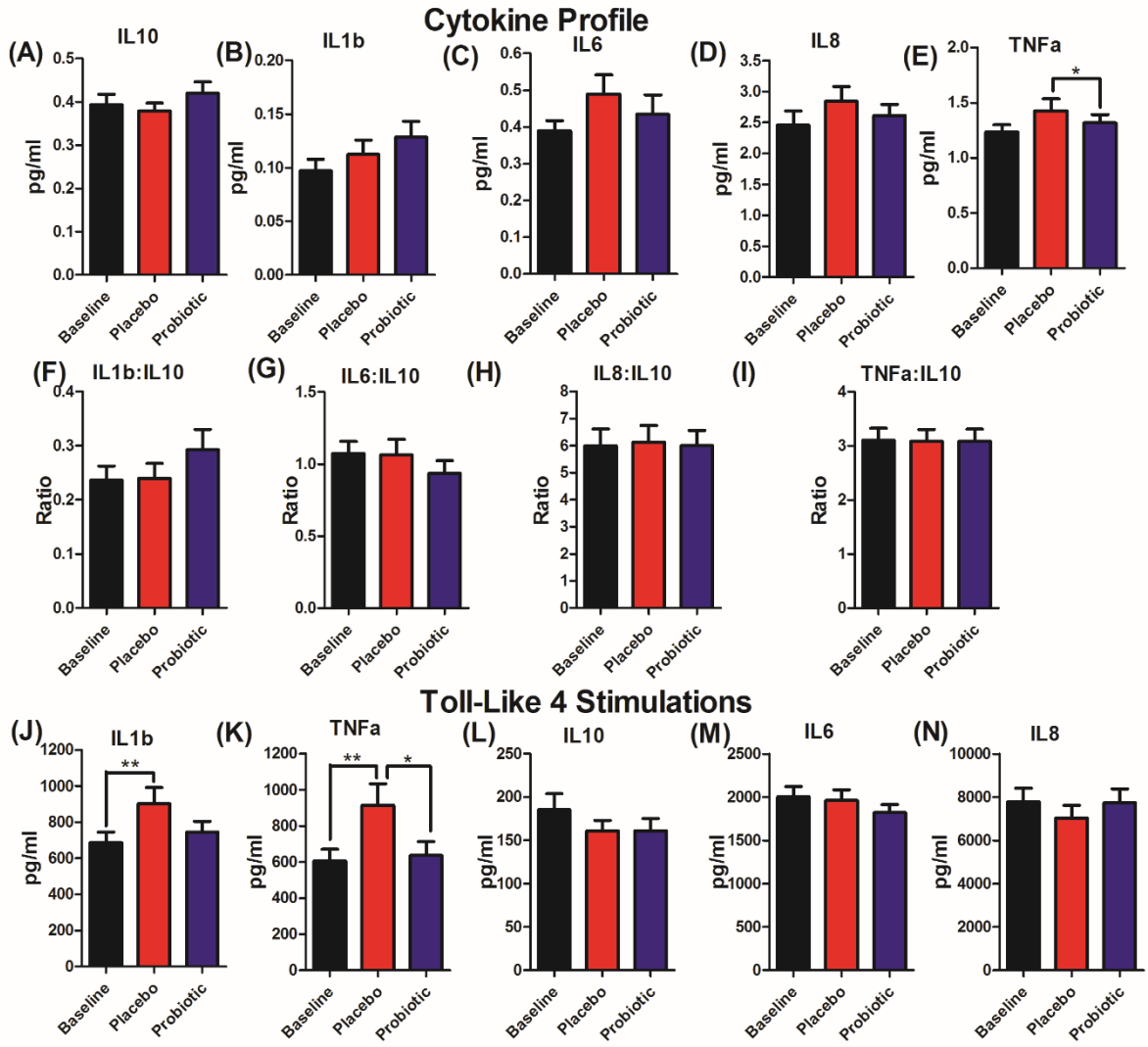


Figure 4.3: Immune response

There was no overall treatment effect on the plasma concentrations of **(A)** IL10 (F 1.64, 34.57 = 1.12, p = 0.32) **(B)** IL1β (F 1.69, 47.46 = 2.72, p = 0.08), **(C)** IL6 (F 1.97, 51.38 = 2.11, p = 0.13) or **(D)** IL8 (F 1.78, 50.00) = 1.87, p = 0.16). **(E)** The concentration of TNFα increased from baseline during the placebo phase (p = 0.02), but there was no significant change in baseline versus probiotic (p = 0.08) or placebo versus probiotic (p = 0.18). There was no overall treatment effect on the **(F)** IL1β:IL10 (F 1.91, 51.81 = 0.36, p = 0.68), **(G)** IL6:IL10 (F 1.84, 48.00 = 2.165, p = 0.12) **(H)** IL8:IL10 (F 1.91, 51.56 = 0.02, p = 0.97), or **(I)** TNFα:IL10 (F 1.96, 54.90 = 0.006, p = 0.99) ratios. Pairwise comparisons showed that the probiotic decreased the IL6:IL10 ratio (p = 0.03), though not significantly over placebo (p = 0.13). In the TLR4 stimulated cytokines, there was an increase in the level of **(J)** IL1β (F 1.77, 40.77 = 4.46, p = 0.02) and **(K)** TNFα (p = 0.01) during the placebo phase compared to baseline (p = 0.01). There was no effect of either

treatment phase on **(L)** IL10 ($F_{1.68, 42.04} = 2.30$, $p = 0.12$) **(M)** IL6 ($F_{1.72, 43.02} = 1.57$, $p = 0.22$) or **(N)** IL8 ($F_{1.17, 29.24} = 1.409$, $p = 0.25$) cytokine release.

Table 4.4: Inflammatory Measures. Means and Standard errors of the mean in parentheses.

	Baseline	Placebo	Active	Baseline V Placebo (p value)	Placebo V Probiotic (p value)	Baseline V Probiotic (p value)	Treatment Effect (Repeated Measures ANOVA)
IL10	0.35 (0.10)	0.36 (0.07)	0.39 (0.13)	0.80	0.30	0.22	F (1.64, 34.57) = 1.12, p = 0.32
IL1b	0.09 (0.01)	0.10 (0.01)	0.11 (0.01)	0.25	0.62	0.11	F (1.69, 47.46) = 2.72, p = 0.08
IL6	0.38(0.03)	0.49 (0.05)	0.44 (0.05)	0.05	0.31	0.31	F (1.97, 51.38) = 2.11, p = 0.13
IL8	2.45 (1.22)	2.84 (1.25)	2.61 (0.96)	0.10	0.25	0.37	F (1.78, 50.00) = 1.87, p = 0.16
TNFα	1.23 (0.07)	1.42 (0.11)	1.31 (0.08)	0.02	0.18	0.08	F (1.50, 42.00) = 3.79, p = 0.04
IL1b:10	0.24 (0.03)	0.24 (0.03)	0.26 (0.03)	0.91	0.52	0.39	F (1.91, 51.81) = 0.36, p = 0.68
IL6:10	1.07 (0.09)	1.05 (0.11)	0.90 (0.09)	0.84	0.13	0.03	F (1.84, 48.00) = 2.165, p = 0.12
IL8:10	5.99 (0.63)	6.12 (0.62)	5.99 (0.57)	0.85	0.87	0.99	F (1.91, 51.56) = 0.02, p = 0.97
TNFα:10	3.10 (0.22)	3.08 (0.22)	3.09 (0.22)	0.91	0.97	0.95	F (1.96, 54.90) = 0.006, p = 0.99
TLR4 IL10	185.26 (18.55)	160.83 (12.16)	161.14 (14.10)	0.09	0.98	0.12	F (1.68, 42.04) = 2.30, p = 0.12
TLR4 IL1b	669.17 (58.91)	903.14 (89.57)	763.95 (59.51)	0.01	0.12	0.15	F (1.77, 40.77) = 4.46, p = 0.02
TLR4 IL6	2002.99 (119.85)	1960.94 (121.74)	1821.67 (92.86)	0.62	0.25	0.13	F (1.72, 43.02) = 1.57, p = 0.22
TLR4 IL8	7781.87 (625.10)	7043.81 (575.93)	7739.74 (644.47)	0.22	0.25	0.83	F (1.17, 29.24) = 1.409, p = 0.25
TLR4 TNFα	612.12 (66.22)	909.65 (124.17)	637.77 (75.06)	0.00	0.03	0.64	F (1.29, 31.04) = 6.717, p = 0.009

Cognitive Measures

Paired Associates Learning (PAL)

There was no overall treatment effect on the total errors made (p = 0.06) **(Figure 4.4A)**, however at the 8 shape stage **(Figure 4.4B)**, there was a significant reduction in errors from baseline in the placebo (p = 0.04) and probiotic phases (p = 0.04), but no significant difference between the placebo and probiotic. There was no significant difference in the mean trials to success (p = 0.13) **(Figure 4.4AC)**.

Attention Switching Task (AST)

There was an increase in the correct response in the placebo (p = 0.03) **(Figure 4.4D)** and probiotic phases (p = 0.01) compared to baseline, and a decrease in the reaction time to correct response **(Figure 4.4E)** in the probiotic phase compared to baseline (p = 0.006), however the differences between placebo and probiotic were not significant.

Rapid visual information processing (RVP)

The placebo and probiotic improved the total correct hits (p = < 0.001) **(Figure 4.4F)**, but there was no overall effect in the total false alarms (p = 0.53) **(Figure 4.4F)**, or the reaction time (p = 0.48) **(Figure 4.4H).**

Emotional Stroop

There was an increase in the percentage of correctly identified neutral words in the probiotic phase of treatment (p = 0.03) **(Figure 4.4I)**, but this was not significantly greater than baseline (p = 0.54). There was no difference in reaction time to identify neutral words (p = 0.85) **(Figure 4.4J).** There were no significant differences in positive percent correct, positive reaction time, negative percent correct, negative reaction time (data not shown).

Emotion Recognition task

The total correctly identified emotions increased in the placebo and probiotic phase compared to baseline (p = < 0.001) **(Figure 4.4K)**, manifest in the disgust (p = 0.02) and fear (p = < 0.001) categories, but no differences between placebo and probiotic. In addition, there was a non-significant decrease in time taken to correctly identify emotions in the placebo and probiotic phases **(Figure 4.4L).**

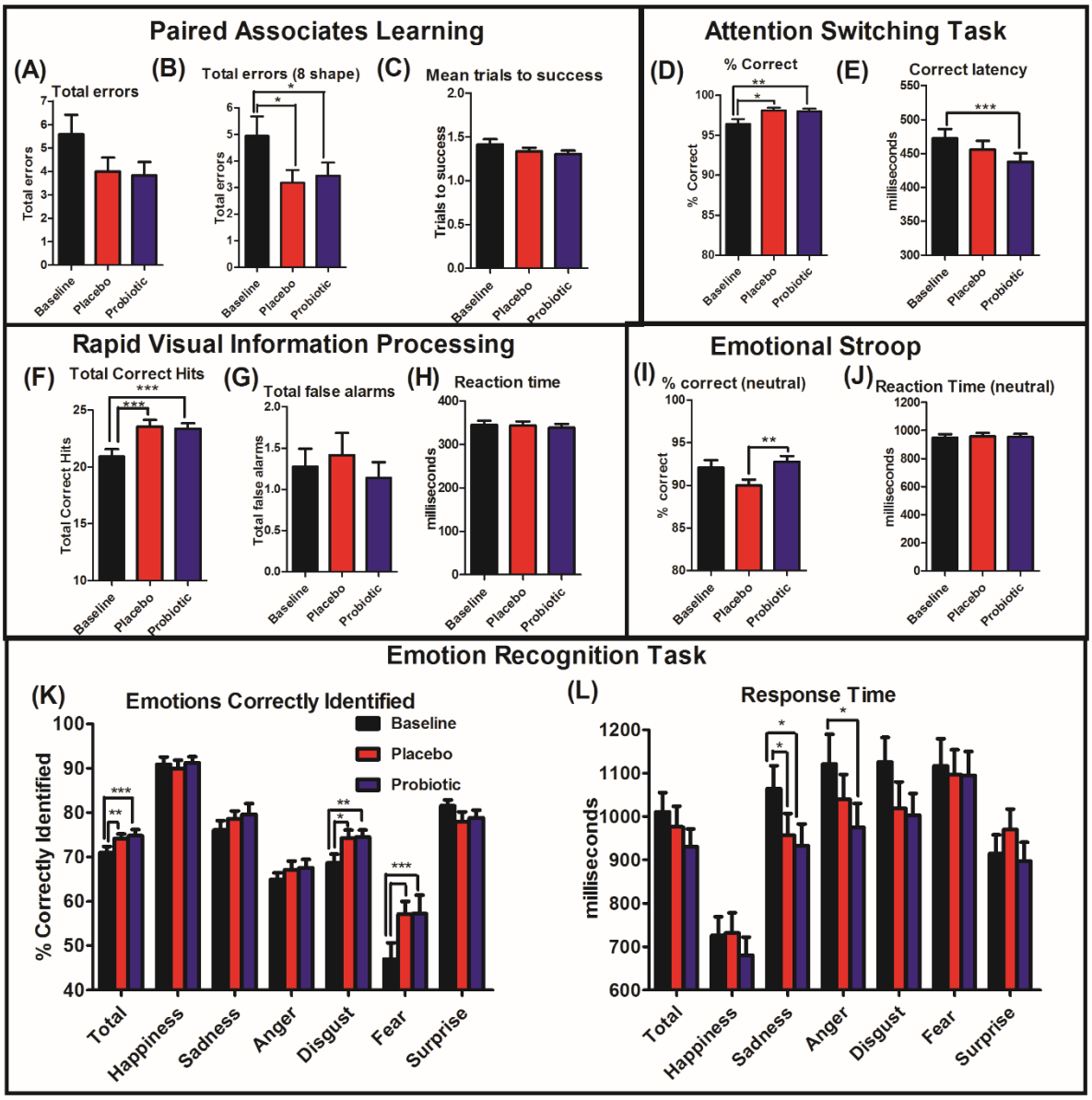


Figure 4.4: Cognitive Measures

In the paired associates learning task, there was no overall treatment effect on the **(A)** total errors made (F 1.71, 48.05 = 3.09, p = 0.06), however at the **(B)** 8 shape stage, there was a significant reduction in errors from baseline in the placebo (p = 0.04) and probiotic group (p = 0.04), but no significant difference between the placebo and probiotic. There was no significant difference in the **(C)** mean trials to success (F 1.55 , 42.10 = 2.21, p = 0.13). **(D)** In the attention switching task, there was an increase in the correct responses in the placebo (p = 0.03) and probiotic phases (p = 0.01) compared to baseline, and a decrease in the **(E)** reaction time to correct response in the probiotic phase compared to baseline (p = 0.006), however the differences between placebo and probiotic were not

significant. In the rapid visual information processing task, the placebo and probiotic improved the **(F)** total correct hits ($p = < 0.001$), but there was no overall effect in the **(G)** total false alarms ($F\ 1.57, 44.06 = 0.55$, $p = 0.53$) or the **(H)** reaction time ($F\ 1.65, 44.55 = 0.68$, $p = 0.48$). In the emotional stroop task there was an increase in the percentage of correctly identified neutral words in the probiotic phase of treatment **(I)** ($p = 0.03$), but this was not significantly greater than baseline ($p = 0.54$). There was no difference in **(J)** reaction time to identify neutral words ($F\ 1.93, 54.09 = 0.15$, $p = 0.85$). In the emotion recognition task, the **(K)** total correctly identified emotions increased in the placebo and probiotic phase compared to baseline ($p = < 0.001$), manifest in the disgust ($p = 0.02$) and fear ($p = < 0.001$) emotion sub-categories. **(L)** In addition, there was a non-significant decrease in time taken to correctly identify emotions in the placebo and probiotic phases.

Table 4.5: Cognitive Measures. Means and Standard errors of the mean in parentheses

	Baseline	Placebo	Active	Baseline V Placebo (p value)	Placebo V Probiotic (p value)	Baseline V Probiotic (p value)	Treatment Effect (Repeated Measures ANOVA)
Paired associate learning (PAL)							
Total errors	5.58 (0.82)	4.00 (0.59)	3.82 (0.57)	0.08	0.78	0.04	F (1.71, 48.05) = 3.09, p = 0.06
Total errors (8 shape)	4.93 (0.74)	3.17 (0.49)	3.44 (0.50)	0.05	0.58	0.04	F (1.51, 42.47) = 3.73, p = 0.04
First trial memory score	17.27 (0.47)	18.10 (0.40)	18.13 (0.37)	0.12	0.94	0.11	F (1.87, 50.653 = 2.47, p = 0.09
Mean trials to success	1.42 (0.06)	1.33 (0.040	1.30 (0.04)	0.22	0.49	0.06	F (1.55 , 42.10) = 2.21, p = 0.13
Attention Switching Task (AST)							
Mean correct latency	472.10 (13.87)	455.39 (13.36)	437.41 (12.740	0.13	0.05	0.00	F (1.90, 53.36) = 6.40, p = 0.004
Correct %	96.75 (0.57)	98.22 (0.34)	98.10 (0.33)	0.03	0.74	0.01	F (1.34, 32.22) = 5.25, p = 0.02
Rapid visual information processing (RVP)							
Total hits	21.17 (0.62)	23.53 (0.60)	23.50 (0.47)	<0.001	0.93	<0.001	F (1.97, 55.19) = 15.21, p = <0.001
Total false alarms	1.27 (0.22)	1.41 (0.27)	1.13 (0.19)	0.67	0.29	0.49	F (1.57, 44.06) = 0.55, p = 0.53
Mean reaction time (ms)	344.69 (9.77)	338.21 (8.68)	334.03 (7.32)	0.55	0.56	0.26	F (1.65, 44.55) = 0.68, p = 0.48
Emotional Stroop							
% correct (positive)	99.04 (0.29)	98.61 (0.37)	99.30 (0.21)	0.35	0.12	0.42	F (1.72, 48.40) = 1.50, p = 0.23
Mean RT (positive)	943.00 (22.72)	944.77 (21.46)	933.77 (20.13)	0.91	0.36	0.56	F (1.81, 49.09) = 0.34, p = 0.69
% correct (negative)	98.70 (0.39)	98.95 (0.27)	98.78 (0.27)	0.57	0.65	0.84	F (1.92, 53.97) = 0.19, p = 0.81
Mean RT (negative)	957.07 (28.42)	952.15 (22.43)	958.02 (22.22)	0.79	0.67	0.96	F (1.81, 50.78) = 0.07, p = 0.91
% correct (neutral)	92.06 (0.87)	90.00 (0.67)	92.75 (0.65)	0.07	0.00	0.54	F (1.86, 52.25) = 3.82, p = 0.03

Mean RT (neutral)	948.86 (25.19)	956.45 (24.04)	953.71 (21.66)	0.55	0.85	0.75	F (1.93, 54.09) = 0.15, p = 0.85
Emotion recognition test (ERT)							
Tot % correct	71.01 (1.37)	74.19 (1.03)	74.84 (1.32)	0.01	0.48	<0.001	F (1.75, 49.22) = 9.90, p = <0.001
Tot latency	1011.50 (44.13)	976.05 (47.91)	930.58 (40.97)	0.40	0.15	0.08	F (1.74, 48.71) = 2.15, p = 0.13
Happiness % correct	90.92 (1.64)	90.00 (1.82)	91.26 (1.34)	0.55	0.39	0.79	F (1.94, 54.33) = 0.42, p = 0.65
Happiness latency	726.16 (43.26)	732.21 (45.98)	680.12 (42.39)	0.88	0.18	0.26	F (1.98, 55.45) = 1.02, p = 0.36
Sadness % correct	76.09 (2.11)	78.62 (1.79)	79.65 (2.40)	0.14	0.48	0.08	F (1.78, 50.07) = 2.31, p = 0.11
Sadness latency	1064.43 (53.11)	957.13 (50.14)	932.41 (50.56)	0.03	0.58	0.02	F (1.87, 52.54) = 4.19, p = 0.02
Anger % correct	64.94 (1.54)	67.12 (1.96)	67.58 (1.89)	0.14	0.82	0.14	F (1.72, 48.39) = 1.30, p = 0.27
Anger latency	1121.31 (68.90)	1039.89 (57.12)	975.70 (54.96)	0.21	0.11	0.04	F (1.53, 43.03) = 3.14, p = 0.06
Disgust % correct	68.69 (1.97)	74.64 (1.87)	74.99 (1.60)	0.04	0.86	0.01	F (1.73, 46.76) = 4.53, p = 0.02
Disgust latency	1125.91 (56.6)	1019.41 (60.29)	1003.65 (49.46)	0.14	0.73	0.04	F (1.61, 45.31) = 2.62, p = 0.09
Fear % correct	47.01 (3.66)	57.12 (2.87)	57.24 (4.21)	0.00	0.97	0.00	F (1.97, 55.39) = 9.47, p = <0.001
Fear latency	1098.88 (62.36)	1096.51 (57.85)	1092.25 (57.53)	0.97	0.90	0.91	F (1.92, 53.77) = 0.28, p = 0.74
Surprise % correct	81.54 (1.38)	78.92 (2.04)	79.64 (1.60)	0.27	0.73	0.33	F (1.90, 51.41) = 0.84, p = 0.43
Surprise latency	914.41 (43.160	970.46 (47.000	897.11 (43.54)	0.30	0.09	0.77	F (1.78, 49.83) = 1.12, p = 0.32

EEG

There was a significant difference between placebo and probiotic for F3 zero crossings (second derivative) ($p = 0.015$), however, there was no significant difference in this index between baseline and placebo ($p = 0.693$) or between baseline and probiotic ($p = 0.058$). There were no significant differences between placebo and probiotic in any of the other measures. See **(Table 4.6)** for pairwise comparisons.

	Baseline	Placebo	Probiotic	Baseline V Placebo (p value)	Placebo V Probiotic (p value)	Baseline V Probiotic (p value)	Treatment Effect (Repeated Measures ANOVA)
F3 Normalised Power (delta)	0.074 (0.005)	0.083 (.006)	0.093 (0.006)	0.202	0.194	0.023	F (2, 48) = 3.29, p = 0.046
Fz Normalised Power (theta 2)	0.037 (0.005)	0.028 (0.004)	0.031 (0.004)	0.017	0.278	0.069	F (1,.45, 34.69) = 4.57, p = 0.027
Fz Normalised Power (alpha 1)	0.041 (0.005)	0.031 (0.005)	0.033 (0.005)	0.043	0.56	0.041	F (2, 48) = 3.52, p = 0.037
Pz Normalised Power (alpha 2)	0.084 (0.016)	0.052 (0.014)	0.05 (0.012)	0.03	0.84	0.02	F (2, 48) = 4.67, p = 0.014
Pz Normalised Power (Beta)	0.078 (0.012)	0.047 (0.004)	0.051 (0.006)	0.034	0.43	0.065	F (1.23, 29.46) = 4.14, p = 0.044
Fz Shannon Entropy	4 (0.05)	4.13 (0.07)	4.07 (0.06)	0.019	0.22	0.064	F (2.48) = 4.07, p = 0.023
Fz Root mean square amplitude	16.45 (1.22)	19.57 (1.53)	18.13 (1.19)	0.005	0.157	0.053	F (2, 48) = 5.42, p = 0.008
Pz Peak Frequency of spectrum	5.45 (0.75)	3.4 (0.53)	3.36 (0.48)	0.004	0.903	0.01	F (1.35, 32.29) = 7.86, p = 0.005
Fz Activity	318.33 (60.4)	444.57 (75.56)	376.58 (53.93)	0.01	0.122	0.141	F (2, 48) = 4.55, p = 0.016
Pz Complexity	3.93 (0.26)	4.75 (0.36)	4.7 (0.31)	0.05	0.88	0.04	F (2, 48) = 3.28, p = 0.046
Pz Non-linear energy	85.29 (29.24)	46.98 (16.72)	48.66 (18.92)	0.046	0.717	*0.05*	F (1.01, 26.33) = 4.22, p = 0.047
Pz Spectral entropy	5.63 (0.16)|	5.14 (0.14)	5.24 (0.14)	0.019	0.476	0.02	F (2, 48) = 4.92, p = 0.01
Pz Wavelet Energy	22.58 (1.91)	19.5 (1.36)	20.29 (1.55)	0.016	0.491	0.055	F (2, 48) = 3.87, p = 0.03
Pz Fisher information	0.15 (0.01)	0.21 (0.02)	0.2 (0.01)	0.006	0.420	0.005	F (2, 48) = 7.34, p = 0.002

Cz Zero crossings (first derivative)	1056.68 (26.04)	1112.53 (21.81)	1089.64 (19.88)	0.006	0.291	0.212	F (2, 48) = 3.26, p = 0.47
Pz Zero crossings (first derivative)	1015.28 (32.26)	1099.23 (26.07)	1074.44 (21.48)	< 0.001	0.305	0.047	F (2,48) = 6.4, p = 0.003
F3 Zero crossings (second derivative)	1385.68 (7.22)	1388.55 (6.18)	1368.56 (7.56)	0.693	0.015	0.058	F (2, 48) = 3.82, p = 0.03
Fz total power	303423.9 (59749)	430219.4 (74428.2)	359577.3 (51091.7)	0.008	0.1	0.14	F (2, 48) = 4.85, p = 0.012

Table 4.6: Pairwise Comparisons of EEG indices. Mean values (standard errors of the mean in parentheses)

4.5 Discussion

Preclinical data strongly supports the view that *L. rhamnosus* (JB-1) has the capacity to alter central GABA transmission by acting through the vagus nerve (Bravo *et al.*, 2011) and in so doing impact significantly on stress responses and behaviour. In this translational study conducted in healthy volunteers we failed to replicate the preclinical findings, which were conducted in an anxious mouse strain. In contrast to the preclinical data, this cross-over study found that *L. rhamnosus* treatment was not superior to placebo in improving cognitive performance and did not attenuate reported stress in healthy male subjects. Furthermore, probiotic treatment did not have a clear anti-inflammatory effect and did not attenuate the subjective stress response or HPA axis response during an acute stress procedure. This study highlights the challenges in translating the findings from candidate psychobiotics in stress-susceptible animals, to healthy human populations.

The candidate psychobiotic used in this study displayed a strong behavioural signal across multiple aspect of behaviour in well-validated screening assays in an anxious mouse strain (Bravo *et al.*, 2011). However, over the eight week period of this trial, self-reported mood, anxiety, stress and sleep were constant and not significantly altered from baseline during the placebo or probiotic phases **(Figure 1A-H).** The data from other studies is mixed. For example, our results are consistent with a study by (Benton *et al.*, 2007), albeit in an older age group, that showed no overall effect of *Lactobacillus casei Shirota* on mood and only a small improvement when post-hoc analysis of the lowest tertile mood scores were considered. After a 6 week, randomized, double-blind, placebo-controlled trial in petrochemical workers, there was a significant improvement in the general health questionnaire score in the probiotic yogurt group (*L. acidophilus LA5 and B. lactis BB12*) and in the probiotic capsule group (*Actobacillus casei, L. acidophilus, L. rhamnosus, L. bulgaricus, B. breve, B. longum, S. thermophiles*), as well as a significant improvement in the depression anxiety and stress scale score in the probiotic yogurt and the multispecies probiotic capsule group. The improvement in scores in these scales were not seen in the conventional yogurt group (containing the starter cultures of *S.thermophilus and L. bulgaricus*). The probiotic did not alter HPA axis function or the kynurenine/tryptophan ratio (Mohammadi *et al.*, 2015c). The same group did not observe a significant effect between the groups in oxidative stress markers (Mohammadi *et al.*, 2015b).

A more recent study, using a multi-species probiotic (*B. bifidum W23, B. lactis W52, L. acidophilus W37, L. brevis W63, L. casei W56, L. salivarius W24, and Lactococcus lactis (W19 and W58*), did not find significant changes in mood or anxiety as measured by the Beck Depression Inventory or Beck Anxiety Inventory, but reported a reduction on subscales of the Leiden index of depression for rumination and aggressive thoughts (Steenbergen *et al.*, 2015a). Another study in healthy controls, using *L. helveticus R0052* and *B. Longum R0175* found no change in stress, as measured by the perceived stress scale, but did report a reduction in anxiety scores using the Hospital Anxiety and Depression Scale and a reduction in the global severity index, somatisation, depression and anger–hostility scores in the Hopkins Symptoms Checklist (HSCL-90) (Messaoudi *et al.*, 2011). Interestingly, we have recently shown that a *B. Longum* which also had anti-stress and precognitive effects in BALB/c mice (Savignac *et al.*, 2014, Savignac *et al.*, 2015a), also was able to modulate behaviour and stress responses in healthy volunteers.

From a physiological perspective, *L. rhamnosus* (JB-1) also exhibited the capacity to reduce acute stress responses in mice (Bravo *et al.*, 2011). Our participants exhibited an increased cortisol output in response to the acute stressor **(Figure 2I)**. However, probiotic treatment did not attenuate cortisol output and there were no differences in subjective stress reports **(Figure A-H).** Although not utilizing an acute stress procedure, Messaoudi and colleagues found a significant difference in urinary cortisol levels between the *L. helveticus R0052* and *B. longum R0175* group and placebo groups (Messaoudi *et al.*, 2011). In a study administering a prebiotic (galactooligosaccharide) to healthy controls for three weeks a significant decrease in the salivary cortisol awakening response compared to placebo was found (Schmidt *et al.*, 2015).

Our results suggest that *L. rhamnosus* (JB-1) treatment doesn't affect either basal or stimulated immune responses. In contract to our findings, both preclinical and clinical studies have previously shown that *L. rhamnosus* has anti-inflammatory effects (Forsythe *et al.*, 2012, Mortaz *et al.*, 2015, Pessi *et al.*, 2000). Thus, two key pillars of brain-gut axis signalling were not modified following psychobiotic treatment. In terms of cognition, the parallel mode of the PAL test (which presents different shapes at each visit) was used in order to avoid practice effects, and to assess conditional learning of pattern-location associations. PAL test performance is dependent upon the hippocampus (de Rover *et al.*, 2011, Eichenbaum and Bunsey, 1995), which has a high proportion of

glucocorticoid receptors (McEwen, 1999). A deficit in visuospatial memory performance, evident in PAL test performance, has been demonstrated in stress-related brain-gut axis disorders with a cognitive component such as IBS (Kennedy *et al.*, 2015, Kennedy *et al.*, 2014b). In this study, the probiotic was not superior to placebo across multiple cognitive domains including memory, attention, executive function and emotion recognition. Similarly, there were no significant differences of relevance in EEG measures between the probiotic and the placebo.

We employed a rigorous cross-over trial with a repeated measures design to control for potential effects of individual differences. Given that our study consisted of young healthy males, with low baseline mood, stress and anxiety scores and no deficits in HPA, inflammatory or cognitive function, demonstrating a clear probiotic effect over placebo in this population may be challenging. This inability to demonstrate superiority of treatment over placebo is not unusual, either in the assessment of psychotropics in general or in microbiota-directed interventions. For example, a novel and initially promising spore based microbiome therapy (SER-109) in *Clostridium difficile* infection (Khanna *et al.*, 2016), was shown not to be statistically superior to placebo in a larger phase II trial (Seres, 2016). At each study visit, participants were asked whether they experienced any side effects from consumption of the capsules. Side effects were negligible, however, formal assessment of gastrointestinal function, was not carried out and is thus a limitation of the study.

There is an important difference in vulnerability between the anxious mouse strain used in the preclinical study and the healthy human volunteers that make up the clinical sample. It is worth noting that probiotics may be of limited benefit in healthy populations. Comparably, antidepressants also have a limited beneficial effect in healthy controls (Serretti *et al.*, 2010). Moreover, antidepressants have a delayed onset of action (Taylor *et al.*, 2006) and we acknowledge that more than four weeks of psychobiotic treatment may be required in future studies in populations with stress-related psychiatric disorders.A recent systematic review indicated that the impact of probiotic supplementation on gut microbiota structure, including an assessment across features such as α-diversity, richness and evenness, in healthy controls was minimal (Kristensen *et al.*, 2016). However, it is important to consider that probiotics may impact the function of colonizing microbes or promote homeostasis of the gut microbiota, rather than change

its composition (Sanders, 2016). A more defined role for probiotic intervention may be in populations with some degree of pathology, for example IBS (Didari *et al.*, 2015). Recently, several studies have demonstrated altered gut microbiota composition in depression (Jiang *et al.*, 2015, Kelly *et al.*, 2016b, Naseribafrouei *et al.*, 2014) and suggest that this altered gut microbiota composition may play a causal role in the development of certain features of depression (Kelly *et al.*, 2016b, Zheng *et al.*, 2016b), though the precise mechanisms have yet to be elucidated. To date, only one small study has investigated a probiotic intervention in depressed patients (Akkasheh *et al.*, 2016). In this study, eight weeks of a multispecies probiotic containing *L. acidophilus, L. casei and B. bifidum*, reportedly reduced depressive symptoms in moderately depressed patients compared to placebo. Although, microbiota analysis was not completed in this study and it was not clear what other forms of treatment patients were undergoing.

Despite the momentum provided by preclinical microbiome studies, there is a growing appreciation of the challenges in moving this work from bench to bedside (Arrieta *et al.*, 2016, Dinan and Cryan, 2016). This includes the fact that the rodent gastrointestinal tract and microbiota composition differs from the human equivalent (Nguyen *et al.*, 2015). It is worth noting that the effects of *L. rhamnosus* were dependent on the vagus nerve (Bravo *et al.*, 2011). The precise mediators between the gut microbiota and the vagus nerve have not been defined and could not therefore be assessed in this study. Moreover, it is important to note that the preclinical analysis of *L. rhamnosus* was carried out in BALB/c mice which are innately anxious and have different brain and gut responses to stress (Browne *et al.*, 2011, Julio-Pieper *et al.*, 2012, O'Mahony *et al.*, 2010, Savignac *et al.*, 2011).

Moreover, Bercik and colleagues have shown alterations in microbiota composition in this strain compared with strains with normal stress responses. Further, when these mice were transplanted with microbiota from a normo-anxious mouse their behaviour normalised suggesting a strong connection between host microbiota and behaviour (Collins *et al.*, 2013). Recently, *B. longum* **1714,** an alternative candidate psychobiotic selected following a similar preclinical screening battery in BALB/c mice, has been reported to reduce stress and improve memory (Allen *et al.*, 2016), although a detailed mechanistic understanding of its effects in this regard is currently lacking. These diverging outcomes highlight the issue of different putative psychobiotics likely

exhibiting different mechanisms of action. Ultimately, this study, together with the SER-109 study illustrate the need to better understand the mechanisms, for effective translation. Whether the JB-1 strain has potential in the treatment of stress-related psychiatric disorders, either as a single agent, or in combination with other potential psychobiotics, remains an open question and further investigations are warranted.

Conclusions

This eight week randomized cross-over trial did not show that *L. rhamnosus* (JB-1) was superior to placebo in modifying stress-related measures, HPA responses, inflammation or cognitive performance in healthy male participants. These results suggest that some caution is required regarding expectations of targeting the gut microbiome in healthy populations and that there may be challenges in translating candidate psychobiotics with promising preclinical signals in anxious mouse strains into healthy human subjects. Future interventional studies investigating the effect of this probiotic in populations with stress-related disorders are required.

Chapter 5: General Discussion

5.1 Overview and summary

A growing body of literature suggests a role for the gut microbiota in stress-related disorders but the clinical implications of these findings lags behind this promising research. In this thesis, we assessed this hypothesis using a translational approach. We have demonstrated that depression is associated with an altered gut microbiota. Further, this altered gut microbiota contributes to the complex underlying pathophysiology of depression. These findings can be incorporated within a neurobiological model of depression, and together with the existing literature, these results support the possibility of therapeutic targeting of the gut microbiota in stress-related disorders such as depression.

In Chapter 2, we demonstrated that depression is associated with altered gut microbiota composition, including decreased richness and diversity. In addition, we affirmed that depression is associated with increased levels of inflammatory markers, altered tryptophan metabolism along the kynurenine pathway and HPA axis dysfunction – all factors which have been linked to gut microbiota alterations in preclinical studies. In Chapter 3, we showed that transferring the gut microbiota from depressed patients to microbiota-depleted rats can induce behavioural and physiological features characteristic of depression in the recipient animals, including anhedonia and anxiety-like behaviours, as well as alterations in tryptophan metabolism. In Chapter 4, we conducted an interventional study in a healthy male cohort to attempt to translate a candidate psychobiotic identified from our preclinical screening platform. Although we provide evidence that the gut microbiota is altered in depression and that this alteration could have a role in prominent features of depression, therapeutic targeting of the gut microbiota in healthy males using *L. Rhamnosus* (JB-1) was not superior to placebo in modifying self-reported stress, HPA axis response to an acute stressor, inflammation, cognition or neurophysiological measures.

5.2 The Gut Microbiota as a Neurobiological factor in Depression

As we have highlighted throughout, depression is a heterogeneous disorder and the pathophysiology is complex. The factors contributing to the onset of this condition will vary widely from person to person but the interaction of genetic predisposition with environmental factors, such as significant psychosocial stress, particularly in early life, and biological systems such as the neuroendocrine and neuroimmune pathways are

cardinal. To this complex model, we now propose that the gut microbiota be added. In this thesis, we have demonstrated that a depression-associated gut microbiota can induce common features of depression such as anhedonia and anxiety and as well as altered tryptophan metabolism. By incorporating the gut microbiota into existing concepts of depression a more comprehensive model has been developed **(Figure 5.1).**

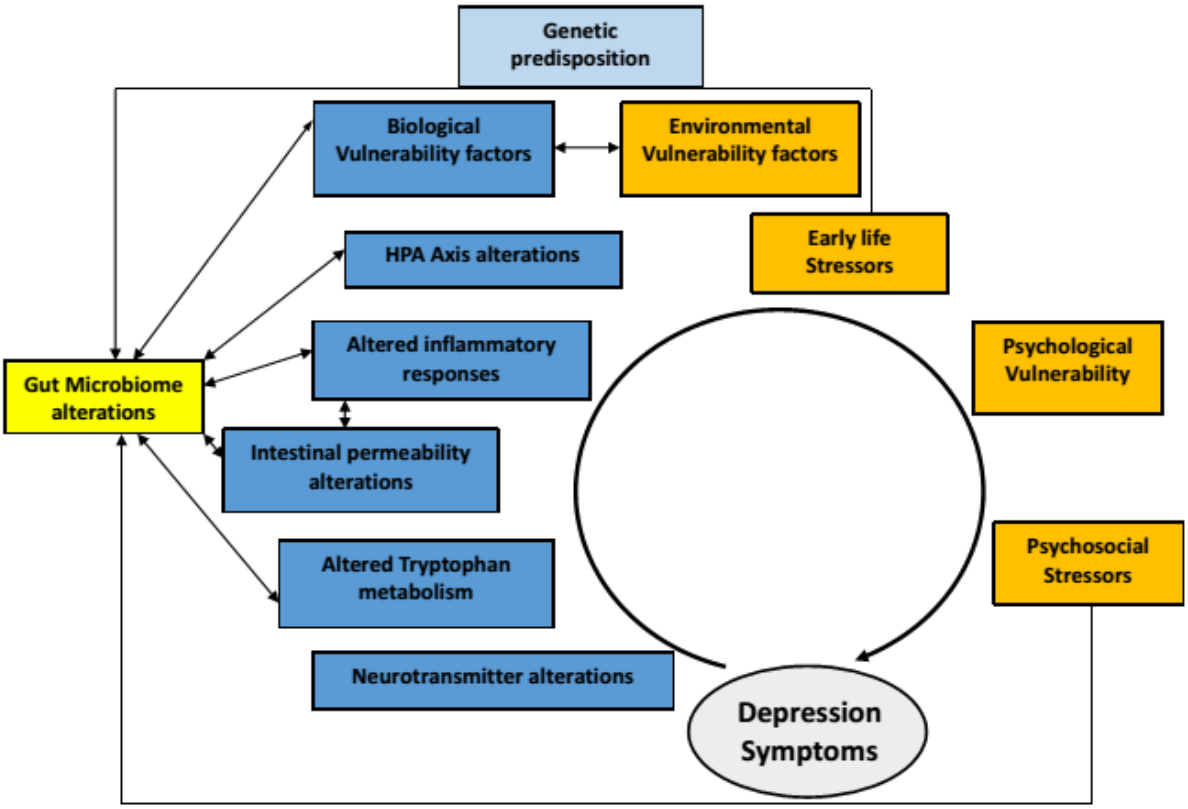


Figure 5.1: Neurobiological model of depression. Genetic and environmental factors interact to predispose the individual to the risk of depression. Due to these gene-environment interactions, alterations in the gut microbiome, acting via the brain-gut-axis, function as an additional biological vulnerability factor. Psychosocial stressors can precipitate depression symptom expression and can result in gut microbiome alterations. At this stage, gut microbiome alterations both increase the biological vulnerability to subsequent stressors and may act as a perpetuating factor for depressive symptom expression.

5.3 The Microbiome as a Stratification tool

What exactly constitutes a "healthy microbiome" has not yet been clearly defined (Backhed *et al.*, 2012), and there are considerable interpersonal differences in healthy

individuals (Falony *et al.*, 2016, Zhernakova *et al.*, 2016). Consequently, there are multiple possible configurations for a healthy gut microbiome and it is also likely that some stable configurations are associated with disease (Relman, 2015). It is important also to appreciate that the functional output of multiple microbiome configurations may in fact be equivalent, given that concepts of redundancy and pleiotropy can also be applied to specific microbial members of the overall consortium. Indeed, our metabolomics analysis indicated that even though the gut microbiome was less diverse in depression, it was not associated with differential microbial metabolite production.

Despite the considerable and complex challenges of defining a healthy gut microbiome, and the relevant features that might be implicated in stress-related disorders, we anticipate that successful translation of our work to date on pathological microbiomes could lead, for example, to stratification based on gut microbiome composition profiles, which in turn could identify sub-groups of patients that may be more likely to respond to a microbiome-based therapeutic approach. A major, as yet unfulfilled objective in psychiatry is the identification of biomarkers. Due to the complexity of the brain, it is perhaps no surprise that psychiatry is the only medical speciality that has no robust biomarker to assist in diagnosis or treatment, and unfortunately the dogma of trial and error prevails. Considerable effort has been invested into rectifying this situation. For example, studies utilizing a range of techniques, including, but not limited to neuroimaging (McGrath *et al.*, 2013), microRNAs (Gururajan *et al.*, 2016b) and inflammatory profiles (Cattaneo *et al.*, 2013, Raison *et al.*, 2013, Uher *et al.*, 2014) have reported to be of benefit. However, none of these approaches are near the stage of benefitting decision making in routine clinical practice.

It is likely that a constellation of biomarkers will be necessary. Thus, the addition of gut microbiota profiling may deliver further diagnostic accuracy and potentially extend personalized medicine. For example, Irritable bowel syndrome (IBS) subtypes have been stratified according to their gut microbiota profiles, specifically those with an increased *Firmicutes: Bacteroidetes* ratio (Jeffery *et al.*, 2012a). Furthermore, depression was the most robust clinical discriminator between a high *Firmicutes: Bacteroidetes* ratio in IBS patients relative to IBS patients with a healthy-like microbiota signature (Jeffery *et al.*,

2012a). In addition, the order *Actinomycetales* and the family *Actinomycetaceae* were inversely associated with clinically significant depression (Jeffery *et al.*, 2012a).

Although, microbiome-based biomarkers in depression have not yet been developed, the current study identified some interesting candidate microbiota signatures which could be considered in this regard. In the depressed group, the most pronounced difference was observed in the reduction of the relative abundance of *prevotellaceae* at the family level and in the *prevotella* at the genus level, whereas the *thermoanaerobacteriaceae* family were increased. At the genus level, the relative proportions of *eggerthella*, *holdemania*, *gelria*, *turicibacter*, *paraprevotella*, and *anaerofilum* were increased in the depressed group, whereas *dialister* was decreased. We also observed more general markers which might have utility, including reductions in richness and diversity. However, given our limited sample size, we did not note any marked correlations between specific alterations and symptoms. Future large scale studies that focus on the possible links between gut microbiota signatures and components of depression are necessary to address this issue. We speculate that in conjunction with a range of other biomarkers (Gururajan *et al.*, 2016a) gut microbiota signatures will be of increasing importance in psychiatry.

It is interesting to note that the emergence of the gut microbiome as a key player in brain and behaviour parallels another major development: The Research Domain Criteria (RDoC) (Glannon, 2015, Morris and Cuthbert, 2012). This research initiative, although the subject of much debate (Frances, 2014, Kraemer, 2015, Weinberger et al., 2015, Yee et al., 2015) presents an exciting opportunity to advance psychiatric research (Casey et al., 2014, Cuthbert and Insel, 2013, Insel, 2014). Rather than using the traditional restricted categorical diagnostic approaches to psychiatric diagnosis, the RDoC matrix of functional dimensions, grouped into broad domains such as cognition and reward-related systems, examined across units of analysis ranging from genetic, molecular, and circuit activity to psychology and behaviour. Furthermore, by removing the constraints of classical psychiatric disease diagnosis, the RDoC leads to a better alignment of pre-clinical and clinical studies to build a common framework of comparable neurobiological abnormalities, for example, based on microbiome alterations, to help form subgroups of patients on the basis of similar pathophysiology (Kaiser and Feng, 2015). For example,

in our study, anhedonia and anxiety emerged in our preclinical model and this may be a useful starting point for stratification across other psychiatric diagnoses.

5.4 The Microbiome as a therapeutic target in Depression

Novel psychiatric drug development is in a state of stagnation (Nutt and Goodwin, 2011). In the last 40 years, very few therapeutics with novel mechanisms have progressed to phase III clinical trials or regulatory approval. In this context, targeting the gut microbiome is an appealing option and potentially represents a major advance. Our results with a potential psychobiotic, which failed to demonstrate an impact in healthy human subjects despite a strong preclinical profile, suggest that the development of microbiome-based therapeutics will also be challenging. It is not immediately obvious why our current psychobiotic exhibited a limited impact while a previous and recent study in our laboratory using *B. longum* **1714**, was translatable (Allen *et al.*, 2016). It is important to note that the preclinical analysis in both studies was carried out in BALB/c mice which are innately anxious and have different brain and gut responses to stress (Browne *et al.*, 2011, Julio-Pieper *et al.*, 2012, O'Mahony *et al.*, 2010, Savignac *et al.*, 2011). These diverging outcomes highlight the issue of different putative psychobiotics likely exhibiting different mechanisms of action.

The overall probability of success of bringing any new drug, through preclinical stages and clinical trial stages I through III to market is approximately 8% (DiMasi *et al.*, 2003). The glycine reuptake inhibitor Bitopertin, serves as a recent example. After an initially promising proof-of-concept study, for the treatment of negative symptoms of schizophrenia (Umbricht *et al.*, 2014), Roche announced that it was not going to proceed with further development of the drug. Indeed, major pharmaceutical companies have shifted drug discovery efforts away from psychiatric toward non-psychiatric disorders with identified biological targets (Cressey, 2010, Miller, 2010, Munos, 2013). A multitude of factors may account for this, including the high levels of heterogeneity and co-morbidity in psychiatry, the absence of molecular targets, the increasing cost and average duration of treatment discovery (Morgan et al., 2011), together with the increasing placebo response rate and failure rates in clinical trials.

Given the stasis in psychiatric drug development, expanding potential therapeutic targets is an essential endeavour. The gut microbiome, acting via the brain-gut-axis, is an easily

accessible and druggable target. Our results show that there is reduced gut microbiota richness and diversity in depressed patients and this was sufficient to produce anhedonia and anxiety-like behaviour in a rodent model. This concept of an entire ecosystem as a potential pathogen is a somewhat unfamiliar concept in clinical psychiatry, but the concept of diversity and stability as key indices in healthy ecosystems is generally accepted (Guarner *et al.*, 2006, Hidaka, 2012, Klerman and Weissman, 1989, Lozupone *et al.*, 2012b, Rook and Lowry, 2008, Rook *et al.*, 2013, Rook *et al.*, 2014, Turnbaugh *et al.*, 2009a, Weissman, 1992). Clearly, gut microbiota alterations are just one of many potential vulnerability factors that may lead to symptom expression in depression (see Figure 5.1).

The formidable challenges of developing an effective drug also applies to microbiome-based therapies. This has recently been highlighted in a study using a spore based microbiome therapy (SER-109) in *Clostridium difficile* infection. After, an initially promising Phase Ib trial of a spore based microbiome therapy (SER-109) for *C. difficile* infection (Khanna *et al.*, 2016), was shown not to be statistically superior to placebo in a larger phase II trial (Seres, 2016). This interim failure, which cost at least 120 million dollars, emphasizes the significant challenges in developing new effective therapies. Considering this failure to achieve the primary efficacy endpoint was in a gastro-intestinal infection, for which FMT is of proven clinical benefit (Kelly et al., 2015, van Nood et al., 2013), it emphasizes the significant challenges for the development of a microbiome based therapy in psychiatric disorders.

To date, only one small study has investigated a probiotic intervention in depressed patients (Akkasheh *et al.*, 2016). In this study, eight weeks of a multispecies probiotic containing *L. acidophilus, L. casei and B. bifidum*, reportedly reduced depressive symptoms in moderately depressed patients compared to placebo. Although, microbiota analysis was not completed in this study and it was not clear what other forms of treatment patients were undergoing, it does provide a platform for larger and more detailed clinical probiotic trials. This suggests that a revision of current preclinical screening platforms to include an assessment of psychobiotics under pathological conditions may be a necessary refinement.

5.5 A new animal model of depression

As is the case in our study, animal models will never recapitulate all the disease symptoms of a psychiatric diagnosis. But many of the questions we have raised will require evaluation in reliable preclinical models. However, most currently used behavioural models do not include the gut microbiota as a factor (relying instead on stressors, for example). The humanized FMT model is an integral component to demonstrate cause and effect in gut microbiota studies involving psychiatric populations. While GF animals are an excellent proof-of principal tool, we and others have shown that these animals have some profound CNS abnormalities in adulthood as a consequence of GF status during critical neurodevelopmental windows, many of which are relevant for the depressive phenotype. The main advantage of antibiotic-induced microbiota depletion during adulthood is that it avoids these potential confounding influences (Arrieta *et al.*, 2016, Luczynski *et al.*, 2016).

The profile of depression-like behaviours and physiological alterations noted following FMT from depressed patients suggest that this model may be a useful paradigm in behavioural pharmacology to investigate microbiota-associated depression. Of course, as with any potential novel model, further rigorous validation is imperative, especially given the well described issues of reproducibility (Baker, 2016, Omary *et al.*, 2016). While, humanized non germ-free mice using antibiotic treatment and human fecal transfer has been performed previously (Hintze *et al.*, 2014), it is also important to acknowledge that some antibiotics act directly on the brain (Nau *et al.*, 2010) to impact behaviour, anatomy, and physiology (Desbonnet *et al.*, 2015, Frohlich *et al.*, 2016, Mohle *et al.*, 2016).

A key question that must be addressed is, why were only some features of depression transferred? We can speculate that different donor profiles may account for this, but to answer this question, we need to investigate whether different donor symptom profiles can be transferred via FMT. For example, would it be possible to further disentangle the contribution of the gut microbiota to the pathophysiology of depression, by attempting to transfer sub-categories of depressed subjects with different levels of severity or different co-morbidities. As an extension, could other domains from other psychiatric disorders, for example anxiety disorders be transferred via FMT? Another question, to be addressed, is the precise temporal dynamics of the emergence and possible persistence of the behavioural alterations post FMT.

Further validation of the model developed in this thesis would provide the opportunity to further dissect the mechanistic properties of potential psychobiotics and expedite the screening process for translation into clinical populations. The current pre-clinical screening process does not include pathological models of depression, and thus the development of this new model could facilitate a superior platform to evaluate potential psychobiotics and delineate further, what the optimal combination of synergistic strains would be for the treatment of stress-related disorders. Furthermore, prebiotics, short chained fatty acids, antibiotics, and indeed currently available antidepressants could also be tested and compared.

5.6 Limitations of the reported studies

Although, the studies reported in this thesis have generated some novel and exciting insights into how the gut microbiota acting via the brain-gut axis contributes to the underlying neurobiological mechanisms of depression, there are a number of limitations which must be accounted for when interpreting our findings. In Chapter 2, the study was cross-sectional in design, thus precluding longitudinal assessment of changes in the gut microbiota as they related to changes in depressive symptoms. Our depressed group consisted of more males than females, whereas epidemiological studies show that females have higher rates of depression (Kessler, 2003, Weich *et al.*, 2001, WHO, 2008). However, it is interesting to point out that this sex difference may partially be attributed to different manifestations of depressive symptoms in males (Martin *et al.*, 2013). Regardless, the unbalanced sex profile in our study was due to the fact that more males than females were willing to provide fecal samples. Although our depressed group and healthy control group were matched on sex, age and body mass index, there were significant differences in alcohol and cigarette consumption. In terms of gut microbiota collection and DNA extraction, a mixture of fresh and frozen samples were used. However, this has been shown to have minimal impact on the integrity of the gut microbiota (Fouhy *et al.*, 2015).

In recent years, there has been a focus on the complex interaction between the gut microbiota and drug metabolism (Clayton *et al.*, 2009, Saad *et al.*, 2012, Swanson, 2015). For example, the gut microbiota can modulate the effects of oncology drugs (Viaud *et al.*, 2013), cardiac drugs (Haiser *et al.*, 2013, Saha *et al.*, 1983), proton pump inhibitors

(Imhann et al., 2015) and statins (Kaddurah-Daouk et al., 2011). In a recent well powered cross-sectional microbiome study, medication had the largest explanatory power on microbiome composition, accounting for 10% of community variation (Falony 2016). Of the medications reported in this study the anti-depressant venlafaxine and the benzodiazepine clonazepam were the psychiatric medications included in the analysis, though a thorough investigation of the effects of anti-depressants on the gut microbiota has not yet been conducted. It is well established that recruiting medication free depressed patients is a significant challenge, and in our study, the majority of the depressed patients, who were at least moderately depressed, were prescribed anti-depressant medication. We acknowledge the possibility of an antidepressant-related and serotonin-driven contribution to the alteration in the gut microbiota. However, antidepressants would be expected to reduce the kynurenine/tryptophan ratio rather than increase it via, for example, activity on TDO (Badawy and Morgan, 1991, Badawy *et al.*, 1991) making it unlikely that residual medication in the fecal transplantation from the depressed patients would increase depressive and anxiety like behaviours in the rats that received the depressed FMT.

The full impact of the effect of psychotropic medication on the gut microbiota has yet to be established, but several pre-clinical and clinical studies have started to examine the bidirectional relationship between the gut microbiota and antipsychotic medication. Antipsychotics result in metabolic dysregulation and weight gain (Bak *et al.*, 2014). In a preclinical study chronic olanzapine treatment altered gut microbiota composition and induced significant body weight gain in the female rats, while both males and females had olanzapine-induced increases in adiposity (Davey *et al.*, 2012). Pre-treatment with an antibiotic cocktail attenuated this weight gain (Davey *et al.*, 2013). Another preclinical study using GF mice, demonstrated that the gut microbiota are necessary and sufficient for weight gain caused by oral olanzapine, which shifted the microbiota profile towards an "obesogenic" bacterial profile (Morgan et al., 2014). Similarly risperidone, alters gut microbiota in mice and a FMT from risperidone-treated mice to naive recipients resulted in a 16% reduction in total resting metabolic rate (Bahra *et al.*, 2015). The same group translated the findings into an adolescent clinical cohort to show that chronic risperidone treatment was associated with an increase in body mass index and a significantly lower ratio of *Bacteroidetes:Firmicutes* compared to antipsychotic-naive psychiatric controls (Bahr *et al.*, 2015).

In Chapter 3, given that rodent and human microbiota are different and the inherent translational challenges in moving from rodent to man, it is perhaps not surprising that overlap of specific taxa were not reflected in the rat microbiota following the FMT. In addition, the microbiota composition was determined using fecal samples in the human study and from cecal samples in the rodent study. Considering, not all behavioural readouts of relevance to depression were recapitulated in our preclinical study, this suggests that those domains which were impacted relate to the missing taxa which are a feature of the rodent microbiota following the transfer. In addition, as mentioned above, our depressed patients were on anti-depressant medication and it would be important to determine the effects of a FMT from medication free depressed patients in future studies.

In Chapter 4, male participants were selected to avoid the need to control for menstrual cycle, which can impact upon cortisol output and other readouts. Future studies will need to assess the impact of probiotic interventions in female participants and may need to consider the incorporation of a washout period into the study design. Given that our study population consisted of young healthy males, with low baseline mood, stress and anxiety scores and no deficits in HPA, inflammatory or cognitive function, this finding does not necessarily preclude the exploration of this probiotic or a combination, in stress-related disorders.

5.7 What Future Studies are needed?

The investigation of the role of the gut microbiota in clinical psychiatric populations is in its infancy and the few clinical studies conducted thus far are cross-sectional. The failure to find a robust and consistent gut microbiota signature in depression thus far is not surprising given the small sample sizes and lack of a standardized approach (Jiang *et al.*, 2015, Naseribafrouei *et al.*, 2014, Zheng *et al.*, 2016a). This is not confined to depression studies and there are also considerable variation in the results reported in IBS. Two recent large scale cross-sectional microbiome studies, with a combined sample size of 3948, have highlighted some of the major challenges in the field of microbiome research (Falony *et al.*, 2016, Zhernakova *et al.*, 2016). These studies re-iterate the problems in defining a “normal” microbiome and the importance of large sample sizes and the need to consider confounding variables, such as stool consistency, medication use, and diet.

Interestingly, and contrary to some studies, early-life events such as birth mode were not reflected in adult microbiota composition. With a strikingly low cumulative, nonredundant effect size of 7.63%, the study emphasizes the influence of additional, currently unknown covariates as well as intrinsic microbial ecological processes such as founder effects, species interactions, and dynamics. Most studies of the gut microbiome in specific disorders have sample numbers well below the impressive size of these studies and have not even taken into account many of the known confounding variables. It is nonetheless striking that we and others have been able to pick up a narrowing of microbiota diversity under various pathologies in much smaller populations, suggesting that some of these factors may be subservient under certain disease-associated conditions.

With these issues in mind, identification and validation of specific microbiome-based biomarkers in psychiatric disorders will require large scale longitudinal studies that assess the trajectory of the gut microbiota and its relationship to neuropsychiatric symptom development across the lifespan. There are already some examples from epidemiological research, focused on the impact of various insults to the microbiome, for example, following different modes of birth (Curran et al., 2016, Curran et al., 2015, Curran et al., 2014, O'Neill et al., 2015) and antibiotic use (Lurie et al., 2015). And large scale studies, such as The American Gut (Goedert et al., 2014) and associated British Gut projects (http://www.britishgut.org/) are ongoing in healthy volunteers and may assist in providing an appreciation of what is meant by a healthy microbiome and a template for future studies in psychiatry.

In order to establish solid clinical relevance in psychiatric populations, microbiome based interventional studies, will also need to assess the functional consequences of microbiota shifts, as well as the impact on central markers of brain activity, before, during and after the intervention. Clinical trials of probiotic interventions in depressed patients at different levels of severity, and in comparison to other treatment modalities including antidepressants would be an interesting prospect. Consideration should also be given to investigation of pharmacokinetic, toxicological and dose-responses, to precisely determine optimal concentration and frequency of probiotic dosing (Marteau and Shanahan, 2003a).

5.8 Perspectives and conclusions

The fusion of neuroscience and the microbiome is a new frontier in psychiatric research. This thesis, together with existing data demonstrates that the contemporary neurobiological models of depression should be extended to incorporate the gut microbiome. This data provides a platform to further delineate the precise gut microbiota signatures and functional outputs of relevance to depression in large scale longitudinal studies. Exploration of the role of the microbiome in other psychiatric disorders is now warranted. We propose that this process should involve breaking down psychiatric diagnoses into biological dimensions using an evolving RDoC framework which includes the microbiome as an additional dimensional construct. Merging the microbiome project and the brain connectome project (Toga et al., 2012) using the RDoC framework, although a significant multi-disciplinary endeavour, provides an important opportunity to advance the understanding of the pathophysiology of these complex brain disorders.

It is an unanswered question whether sub-groups of depressed patients with specific gut microbiome profiles would respond better to microbiome based therapies than other treatments. And although, a promising approach, we acknowledge that targeting the gut microbiome in depression needs to be put into current clinical context. This will depend on many factors, but primarily on level of severity. Probiotics will be of negligible utility in the treatment of severe depression, however, in depression of mild to moderate severity, they may prove useful augmenting agents to other treatment modalities. Perhaps the strongest indication of probiotic intervention may be as part of a pre-emptive strategy, either in those in remission or with other vulnerability factors.

Of course, in clinical practice, probiotic supplementation, would form only one component in the overall multimodal treatment strategy for depressed patients, which includes, but is not limited to, recommendations regarding healthy diet and exercise. A key question is whether probiotic supplementation is superior to dietary alteration. The immediacy of probiotic supplementation, in patient populations with potential motivational issues offers one potential advantage over dietary alteration, and thus this could be the first step for a further and more sustained transition to a healthier diet. Our results certainly highlight the role of the gut microbiome in depression and indicate that

further studies that extend across neurobiological and symptom domains in psychiatric populations, with implications for patient management strategies are now warranted.

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Appendix

Subject information sheet

Study title: The gut microbiome in major depression

Principal Investigators:

Professor Ted Dinan

Professor John Cryan

Professor Catherine Stanton

Professor Paul Ross

Site of Investigation:

Cork University Hospital, Mercy University Hospital and University College Cork.

Site of Laboratory:

Alimentary Pharmabiotic Centre, University College Cork

Why is this study being run?

Depression is the most common mental disorder. It will affect up to 15% of the population during their lifetime. It can have a profound negative impact on the sufferer's quality of life and it can incur significant health care costs.

We do not have a complete understanding of the mechanism underlying depression, however it is well established that exposure to stress, particularly at an early age, is an important factor for the development of depression later in life. Stress can result in changes in the bacterial composition of the gut (microbiota). There is now increasing evidence that this communication pathway works in both directions and it has been shown that bacteria within the gut may influence brain function and behaviour. This study aims to investigate whether the gut microbiota is altered in people with depression.

Study Proceedure

Depending on your location the visits will take place at Cork University Hospital, Mercy University Hospital, University College Cork, or outpatients departments.

Thirty male and female patients diagnosed with major depressive disorder between the ages of 18 and 65 years will be enrolled.

You will be advised of the purpose of the study and the procedures which will be undertaken. You will be given a copy of the subject information sheet, which will explain what is required from you. If interested, you will then be requested to read and sign the informed consent form and will receive a signed copy.

Your suitability to participate in the study will be checked. You will be asked questions about your mood both now and in the past and will be asked about alcohol and drug use. You will also be asked questions about your current medical health, previous medical problems. You will be asked whether anybody in your family suffers from medical or mental health problems. You will be asked about your medications and allergies.

Your height and weight will be measured and your body mass index (BMI) will be calculated. A urine sample will be taken for a HCG pregnancy test if you are a woman of childbearing age.

A blood sample (nine millilitres) will be collected to measure inflammatory markers and a nine millilitre sample for haematology and biochemistry analysis performed as per standard of care.

Three early morning saliva samples will be collected. You will be provided with containers and instructed how to do this.

A faecal (stool) sample will be collected (which has been collected at home, within 12 hours of your visit to the clinic). You will be provided with a container and an information sheet. You will be instructed how to do this.

What happens if I start the study and change my mind later?

You do not have to take part in the study, participation is entirely voluntary. Refusal to participate, or discontinuing participation at any time, will involve no penalty, loss of benefits or denial of treatment or services by the Cork teaching hospital or the participating doctor.

Who is funding the trial?

There are no cost implications for the Health Service Executive (HSE) or to you. The management of patients and investigative tests will comply with current standards of care. Cost of research tests will be incurred by the Alimentary Pharmabiotic Centre, University College Cork.

Is the information confidential?

All information which is collected about you during the course of the research programme will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. To protect your privacy, your sample and any medical information that is transferred to our collaborators, will be labelled (or "coded") only with a study subject number, not your name. Only your doctor at the hospital and his or her staff will keep the link between your subject number and your name. In the event of any publication regarding this study, your identity will not be disclosed.

Blood samples may be sent to third party service providers for specialised analyses or to collaborators in other Universities. In all instances, anonymity will be preserved and the samples will not be used for commercial purposes.

What will happen to the results of the research study?

It is intended that the data will be combined with data obtained using material from several different patients. We hope to publish the results so that as many of our findings as possible will be made available to the medical and scientific community. You will not be personally identified in any publication. Because of the exploratory nature of the work, none of the results will be provided to you or to the physicians who are treating you or may treat you in the future. The timing of any publication will depend mostly on the speed with which we collect the data and cannot be predicted with certainty.

What happens if there is anything I do not understand?

If there is anything you are not sure about, the Doctor will be happy to explain in more detail to yourself or your relatives, guardians (or legal representative if required). The study will be fully explained to you before you decide if you want to take part.

Who has reviewed the study?

This study has been checked and approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

Professor Ted Dinan

Professor of Psychiatry, Cork University Hospital (CUH), and Alimentary Pharmabiotic Centre, University College Cork.

Dr John Kelly, Lecturer in Psychiatry, University College Cork

Telephone number: 021 4901224

Informed Consent Form by subject for participation in a human intervention study

Protocol Number: APC045

Patient Name: _____

Title of protocol:

The gut microbiome in major depression and response to treatment

Principal Investigator(s):

Professor Ted Dinan (021-4901224)

Participation in this study is voluntary and you may withdraw at any time for any reason.

The research project and procedure associated with it have been fully explained to me. All experimental procedures have been identified and no guarantees have been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to heath care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. I understand that the investigators have such insurance as is required by law in the event of injury resulting form this research.

I, the undersigned, hereby consent to participation as a subject in the above described project conducted at Cork University Hospital, Mercy University Hospital and University College Cork.

I have received a copy of this consent form for my records.

I understand that if I have any questions concerning this research, I can contact the Doctor listed below.

If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Informed Consent Form by subject for participation in a human intervention study

Name (Block letters):_____

Subject's Signature:_____ Date:_____

dd.mm.yy

Name (Block letters):_____

Investigator's Signature:_____ Date;_____

dd.mm.yy

SUBJECT INFORMATION SHEET

Study Title: The effect of probiotics on stress and cognition.

Protocol No.: APC057

Principal Investigator: Professor Ted Dinan

Co-Investigators: Professor John Cryan, Professor Paul Ross,

Professor Catherine Stanton, Prof Geraldine Boylan

Dr Gerard Clarke, Dr Paul Kennedy

Dr Andrew Allen, Dr Fahmi Ismail, Dr John Kelly

Site of Investigation: Cork University Hospital,

Wilton, Cork.

Brookfield Health Sciences Complex, Human Nutrition Studies Unit and Alimentary Pharmabiotic Centre,

University College Cork, Cork.

Alimentary Pharmabiotic Centre, UCC

Why is this study being run?

You are invited to participate in a research study to determine how the consumption of probiotics influences cognitive function and stress in healthy individuals. This study will also assess the role that the immune system plays in the effect of probiotics on cognitive function and stress, as well as the role of recent and early life traumatic experiences and sleep patterns.

Study procedures

This study will involve up to 20 – 30 healthy subjects, aged between 18 and 40 years. In total, participation will involve five main study visits, including one screening visit prior to the first main study visit. If you agree to participate in the study you will first

be asked to visit the Alimentary Pharmabiotic Centre UCC for a short screening visit lasting around one hour. You will be asked to visit the study site for collection of blood and will be asked to provide saliva and stool samples at home. You will be provided with probiotic or placebo capsules but you will not be aware of which capsule you are receiving. You will be required to take the capsule once a day every day for four weeks. At the end of the four-week period you will return for another study visit, this time looking at cognitive performance with EEG measures. You will then complete another period of consuming the probiotic/placebo capsule, which will be followed by another cognitive performance/EEG test.

Screening Visit Outline

You will be advised of the purpose of the study and the procedures which will be undertaken. You will be given a copy of the Subject Information Sheet, which will explain what is required from you. If interested, you will then be requested to read and sign the Informed Consent form, and receive a signed copy.

You will be asked some general questions concerning your health and personal details, including your medical history, your family medical history and current medical status. Details of your current and/or past medications will also be collected. Your height and weight will be taken to determine your body mass index (BMI). You will be asked to complete questionnaires regarding recent and early life traumatic experiences, general stress levels and how you respond to stress, your general health and activity levels, sleeping habits, diet and current levels of depression and anxiety. You will complete a brief verbal IQ test. In addition, you will be given containers to collect saliva and stool samples to bring along to the later visits, and specific instructions on how to collect these samples.

Pre-supplementation visit outline

You will be asked to bring a stool sample to this visit. You will fill in a number of questionnaires assessing stress, anxiety and depression, as well as sleep disturbances, and gastrointestinal symptoms. Five venous blood samples (10mls each) will be obtained and assessed for levels of tryptophan (an amino acid) and immune system measurements in the blood. During the collection of blood samples, you may experience a slight scratch, which may be uncomfortable for a moment but quickly passes. Probiotic or placebo capsules will be provided, with instructions for when to take them.

Baseline cognitive performance

You will visit the research centre for assessment of cognitive performance. You will be asked to refrain from strenuous exercise and alcohol for 24 hours prior to the experimental session and from caffeine for two hours prior to testing. Sensors will be

attached to the scalp to measure brain activity (EEG). During this visit you will complete a handedness scale and a number of cognitive tests using a touch screen computer apparatus.

Supplementation phase

During this period you will be required to take the capsule once a day, every day for four weeks. You are requested not to make any major changes to your diet during this phase, **and to avoid taking any probiotics or antibiotics.**

Pre-experimental Day Outline

On the morning of this day you will be required to take a number of saliva samples after awakening. You will be asked to provide these saliva samples, as well as stool samples at this visit. The stool samples are taken to ensure that probiotics have entered the digestive system in the probiotic condition. A blood sample will also be obtained during this visit, to assess effects of the probiotic supplementation on tryptophan and immune factors.

Experimental Study Day Outline: Cognitive performance

At the end of the supplementation phase, you will visit the research centre for assessment of cognitive performance, similar to the baseline cognitive performance visit.

Second supplementation phase

This will follow the same procedure as the first supplementation phase; you will be consuming the capsule you did not consume during the first consumption phase.

Cognitive performance test

At the end of the second supplementation phase you will complete one more assessment of cognitive performance and EEG as before.

What happens if I start the study and change my mind later?

You do not have to take part in the study, participation is entirely voluntary. Refusal to participate, or discontinuing participation at any time, will involve no penalty, loss of benefits or denial of treatment or services by the Cork Teaching Hospital or the participating doctor.

Will I experience any unpleasant side effects?

During the collection of your blood sample, you may experience a slight scratch, which may be uncomfortable for a moment but quickly passes.

Funding of trial

There are no cost implications for the Health Board or to you. The management of patients and investigative tests will comply with current standards of care. Cost of research tests will be incurred by the Alimentary Pharmabiotic Centre, University College Cork. Upon completion of the study, you will receive €200 on completion of the study to cover your costs and expenses.

Confidentiality

All the information gathered from this study will be stored on a computer and paper files and will be treated confidentially. You will be identified only by a subject number. In the event of any publication regarding this study, your identity will not be disclosed.

What happens if there is anything I do not understand?

The study will be fully explained to you before you decide if you want to take part. If there is anything you are not sure about, the investigators will be happy to explain in more detail. If you have any problems or questions after the study has started you may call:

Dr John Kelly

GF Unit

Cork University Hospital

Wilton Cork

Telephone Number: +353 (0)21 4901224

Professor Ted Dinan

GF Unit

Cork University Hospital

Wilton Cork

Telephone Number: +353 (0)21 4901224

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL
Section A

Protocol Number:_____ Patient Name:_____
_

Title of Protocol: **The effects of probiotics on stress and cognition**

Doctor(s) Directing Research: Professor Ted Dinan Phone: +353(0)21 4901224
Dr John Kelly Phone: +353(0)21 4901224

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

Section B

I. NATURE AND DURATION OF PROCEDURE(S): Studies suggest that probiotics are associated with reduced stress and improved cognitive function, but few have actually investigated physiological factors associated with cognitive performance and stress. We hypothesise that probiotic consumption will lead to effects on physiological measurements of the stress response system and changes in cognitive functioning. By carrying out a clinical assessment of factors such as early life trauma and sleep quality we can further characterize the role these factors may play in the stress response and their relationship to cognitive functioning following probiotic administration. Understanding the interactions between probiotic consumption, the physiological response to stress and the subsequent effects on cognitive functioning may potentially aid in developing new products which can aid in the reduction of stress and the improvement of cognitive function.

II. POTENTIAL RISKS AND BENEFITS:

1. Collection of blood samples (in amounts not exceeding 50 millilitres - 4 tablespoons) by venipuncture might cause hematoma, a bruise at the site of vein puncture, inflammation of the vein and possible infection. Care will be taken to avoid these complications.
2. While there is no immediate direct benefit to you from this study, more research like this may ultimately lead to new treatments for stress.

III. POSSIBLE ALTERNATIVES:

You may choose not to participate, or participate voluntarily.

Confidentiality and Anonymisation

Your medical history will be used in the research. To preserve confidentiality, full anonymisation applies to all participants. Your personal data will be stored, processed and analysed in a form that does not allow individuals to be identified. Identifiable data (name, address) should only be accessible to staff who have a formal duty of confidence to the participant.

Section C AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to

withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Subject's Signature: _____ Date: _____

dd mm yy

NAME (BLOCK LETTERS): _____ Time:

_____

Investigator's Signature: _____ Date: _____

dd mm yy

NAME (BLOCK LETTERS) _____