Investigating Neurobiological Mechanisms
Linking Depression and Inflammation

by

Kyoko Hasebe
BPsych (Honours)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

May, 2017
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Kyoko Hasebe was involved in protocol development, experimental studies, analysis, statistical analysis and manuscript preparation.

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Acknowledgement

This thesis could not have been accomplished without the help and support from many people in the Gray laboratory and Metabolic Research Unit at the School of Medicine, Deakin University.

First, I would like to sincerely thank my supervisor, Dr. Laura Gray for giving me the opportunity to undertake my PhD in her laboratory. I would also like to thank her for her help, continual guidance, patience, and constant encouragement throughout my PhD. She has provided me with invaluable training and skill sets that I will require for my future endeavours.

Second, I wish to sincerely thank to Professor Ken Walder, Dr. Olivia Dean and Dr. Leni Rivera who co-supervised my project. I sincerely appreciate the help, guidance and encouragement that they have given me throughout my PhD. Special thanks should also be given for their advice on the methods to evaluate a wide range of dataset, interpretation of the data, and statistical advice.

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Finally, I would like to thank my family and friends. I would like to say a big thank you to my parents and my partner for their constant support and encouragement throughout my studies.
Publications of work contributing to this thesis

The following publication has arisen from the experimental work that I have conducted towards this thesis:

**Chapter 2**


I also contributed to the following paper:

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Work carried out in collaboration and contributions of colleagues:

Chapter 2

“Adjunctive N-acetylcysteine in depression: exploration of IL-6, C-reactive protein and brain derived neurotrophic factor”. This study utilised biological samples collected by the main clinical trial (Berk et al. 2014). I was not involved in the main study conducted by Professor Michael Berk and Dr. Olivia Dean at IMPACT SRC, Deakin University, whereas I was responsible for protocol development, experimental studies, analysis, statistical analysis and manuscript preparation for this inflammatory marker analysis.

Chapter 3 and 4

Regarding 16S metagenomic sequencing, the sequencing process of extracted DNA from faecal samples to raw data was performed at Macrogen Inc (Seoul, South Korea). In addition, bioinformatics which transformed the raw data into a dataset were performed by Associate Professor Tamsyn Crowley and Dr. Theo Allnut, The School of Medicine, Deakin University. I conducted DNA extraction and purification from faecal samples prior to sending samples to Macrogen Inc.
I planned and conducted all experiments including behavioural testing and collecting samples and data. Also, I performed all the biochemical assays, qPCR assays and data analysis that are included in this thesis, where indicated above.
Abstract

Mood disorders including major depressive disorder (MDD) are amongst the most prevalent psychiatric disorders. In addition to the high prevalence and recurrence rates, the low remission rates and limited efficacy of antidepressant therapy imply that currently our understanding of the mechanisms underpinning MDD is still far from complete. Whilst accumulating evidence now suggests that inflammation is involved in the pathophysiology of MDD, current antidepressants are not primarily designed to regulate the inflammatory state in MDD. Therefore, advancing our understanding of the aetiological mechanisms underlying MDD is an urgent and crucial matter that would allow us to improve the efficacy of current medications and develop new therapeutic agents.

This project focused on investigating the underlying pathophysiological mechanisms of MDD with respect to diet, gut microbiome and inflammation. Our current knowledge suggests that poor dietary patterns could alter the gut microbiome and inflammatory signalling systems, and subsequently predispose to MDD. However, we do not yet understand the neurobiological mechanisms underlying how the diet, gut microbiome and inflammation interact, and how this may induce depressive mood. In addition, two novel therapeutic agents, N-acetylcysteine (NAC) and minocycline (MINO) were examined in terms of their capacity to modulate high fat diet-induced changes in behaviour, gut microbiome and inflammation. NAC and MINO have been used in clinical practice and therefore have established safety profiles. Most importantly, these two agents readily cross the blood brain barrier and exert anti-inflammatory and anti-oxidant effects by which NAC and minocycline could potentially modulate aberrant inflammatory status and oxidative biology in MDD. However, there is a paucity of studies examining the therapeutic efficacy of these agents on inflammation in MDD.
In the clinical component of this project, we evaluated therapeutic effects of adjunctive NAC treatment on inflammatory markers, IL-6 and C-reactive protein (CRP), and a neurogenesis marker, brain derived neurotrophic factor (BDNF), were evaluated in a clinical MDD population. While adjunctive NAC treatment significantly improved depressive symptoms, this was not associated with alterations in the levels of IL-6, CRP and BDNF, indicative of a disconnect between these markers and depressive symptoms. This is suggestive of alternate mechanisms of action of NAC.

In the preclinical component of this study, we demonstrated that high fat diet (HFD) consumption can drive depressive-like behaviour/mood, and that this was associated with diet-induced changes in the gut microbiome. Interestingly, this diet-induced depressive-like behaviour was not associated with elevations of pro-inflammatory mediators, instead peripheral and central inflammatory markers were within normal ranges. On the other hand, NAC and MINO did not reverse diet-induced depressive-like behaviour nor affect the inflammatory state. Whereas NAC induced subtle changes in the gut microbiome, MINO treatment resulted in significant changes in the gut microbiome and subsequently attenuated microbial signalling. Our findings suggest that MINO and NAC might not be optimal choices to regulate the mechanisms underpinning HFD-induced depressive mood.

Throughout this PhD project, importance of diet on health and disease states of the body and the brain in the context of MDD were identified and provided empirical evidence to support the epidemiological premise that poor dietary pattern can precede MDD. Dietary factors directly change key microbial populations in the gut, which may result in transforming the
healthy gut microbial population into a disease inducing organism, which is implicated in the pathophysiology of MDD.
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<td>AMOVA</td>
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<td>alpha-amino-3-hydroxy-5-methyl-4-isoxaxoleproponic acid</td>
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<td>HAM-D</td>
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<td>reactive oxygen species</td>
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<td>superoxide dismutase</td>
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<td>TST</td>
<td>tail suspension test</td>
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1.1 Background
Mood disorders including major depressive disorder (MDD) are amongst the most prevalent psychiatric disorders, afflicting approximately one in five individuals within their lifetime (Kessler et al. 2007). Symptoms of MDD include depressed mood, loss of appetite, lack of pleasure, lack of motivation, cognitive deficits and increased likelihood of suicidal ideation, and somatic features such as chronic fatigue, pain and disturbed sleeping patterns (DSM-5 2013). While MDD has at least one core feature, which is depressed mood, MDD patients usually exhibit various combinations of other features. The heterogeneity of the clinical features of MDD points to the underlying aetiological heterogeneity, both of which pose challenges to the identification of the pathophysiological mechanisms of this disease. In addition, MDD is a recurrent disorder, with more than half of patients experiencing more than one episode throughout their lifetime (Burcusa and Iacono 2007; Warden et al. 2007). The high prevalence and recurrence rates mean that MDD is ranked as one of the leading causes of disability worldwide (WHO 2004). Furthermore, the low remission rates and limited efficacy of antidepressant therapy imply that currently our understanding of the mechanisms underpinning MDD is still far from complete. Therefore, advancing our understanding of the aetiological mechanisms underlying MDD is an urgent and crucial matter that would allow us to improve the efficacy of current medications and develop new therapeutic agents.

1.2 Aberrant immune system and MDD
Accumulating evidence shows elevated levels of pro-inflammatory cytokines in MDD patients, which suggests that activation of the immune system might be associated with the pathophysiology of MDD. Both innate and acquired immune responses cause inflammation.
Inflammation is a local response to tissue injury, infection or irritants. In the case of a pathogen, inflammation minimises its spread, and kills and promotes removal of the pathogen through phagocytosis, initiated by macrophages and neutrophils attracted to the site (Kuby 1997). Upon phagocytosis, macrophages in the periphery and microglia in the brain produce a series of pro-inflammatory cytokines, interferon γ (IFN-γ), interleukin 1β (IL-1β), IL-2, IL-6 and tumour necrosis factor α (TNF α) (Watkins, Maier, and Goehler 1995).

Associations between elevations of inflammatory mediators and an increase in depression/anxiety have been documented in numerous studies. Elevated plasma concentrations of cytokines (TNFα, soluble TNF receptors, IL-6 and IL-1β) induced by the endotoxin, Salmonella abortus equi were associated with increased levels of anxiety and depressed mood in healthy volunteers (Reichenberg et al. 2001). Treatment with pro-inflammatory cytokines and IFN α induce behavioural and mood changes such as depressed mood, anxiety, cognitive dysfunction, short-term memory deficit and disrupted sleep patterns in cancer or hepatitis patients (Raison et al. 2010; Wichers et al. 2005). The behavioural and mood changes induced by the treatment were concomitant to the elevation of plasma pro-inflammatory cytokines. Hence, these data indicate an association between pro-inflammatory cytokines and depressive mood.

In addition, patients with MDD have been reported to exhibit increased biomarkers of inflammation, including IL-1β, IL-6 and TNFα in the periphery and the brain (Maes 1999; Maes, Smith, and Scharpe 1995; Hannonstad, DellaGioia, and Bloch 2011; Berk et al. 2013). Furthermore, a meta-analysis showed that some antidepressant treatments, such as selective serotonin reuptake inhibitors (SSRI), reduce levels of IL-6 and TNF α (Hannestad, DellaGioia, and Bloch 2011). Although it is still unclear where these inflammatory mediators originated
from in MDD patients, there is a striking fact that the prevalence of comorbid MDD in patients with coronary heart disease is three times higher than in the general population (Dantzer et al. 2008; Carney and Freedland 2017). This indicates that depressed mood may be attributable to elevations of pro-inflammatory mediators in some patients with systematic disorders. For instance, coronary heart diseases and increased levels of an acute phase protein, C-reactive protein (CRP) and a pro-inflammatory cytokine, IL-6 (Carney and Freedland 2017). In line with this, evidence suggests that inflammation and MDD might have a bidirectional relationship (Renoir, Hasebe, and Gray 2013), in which inflammation might precede symptoms of MDD, or that MDD might lead to a pro-inflammatory state. However, elevated levels of pro-inflammatory mediators does not occur universally in MDD patients (Stewart 2016). For instance, the relationship between inflammation and MDD might not be applicable for certain races or ethnic minorities. Although there might be exceptions of cases of MDD with an absence of elevated inflammatory mediators, a large body of evidence supports an association between inflammation and MDD. However, what remains unclear is the underlying biological pathways underpinning this relationship.

1.2.1 Immune signals from the periphery to the brain
The brain has been believed to be an ‘immune privileged’ organ, as the blood brain barrier restricts the passage of cytokines and circulating immune cells (Capuron and Miller 2011; Dantzer et al. 2008). However, recent evidence suggests that the brain has an extensive lymphatic drainage system connecting to lymph nodes, indicating that immune surveillance of the brain is supported by clearance of immune cells (Aspelund et al. 2015; Dissing-Olesen, Hong, and Stevens 2015; Muller 2014). In addition, substantial evidence now suggests that immune signals from the periphery are able to reach the brain though three pathways;
humoral, neural and cellular pathways (Capuron and Miller 2011). Through the humoral pathway, pro-inflammatory cytokines produced by the periphery reach the brain through “leaky” regions of the blood brain barrier. Once reaching the brain parenchyma, pro-inflammatory cytokines trigger activation of microglia which are responsible for the release of more pro-inflammatory cytokines, as well as second messengers such as reactive oxygen species (ROS) and nitric oxide (NO) (Capuron and Miller 2011; Dantzer et al. 2008). Through the neural pathway, pro-inflammatory cytokines stimulate afferent nerve fibres in the vagus nerve (Capuron and Miller 2011). In the cellular pathway, a pro-inflammatory cytokine, TNFα, originating from the periphery, stimulates immune cells in the brain to produce monocyte chemoattractant protein-1, which is responsible for the recruitment of monocytes into the brain (Capuron and Miller 2011; D'Mello, Le, and Swain 2009). Therefore, immune signals are able to enter the brain and activate the immune network in the brain.

Pro-inflammatory cytokines function in two ways in the brain. Each pro-inflammatory cytokine induces its own synthesis, and some cytokines such as IL-1β, IL-6 and TNFα induce the synthesis of other pro-inflammatory cytokines in a cascade manner (Capuron and Miller 2011). Microglia and astrocytes, the central immune cells, produce pro-inflammatory cytokines, express receptors for pro-inflammatory cytokines and amplify signals from pro-inflammatory cytokines (Capuron and Miller 2011; Dantzer et al. 2008). Hence, glial cells mediate the potent effects of pro-inflammatory cytokines in the brain.

1.2.2 Immune cells in the brain

Microglia are the resident macrophages in the CNS and account for approximately 5-10% of the human adult brain cell population (Turrin and Rivest 2006). Microglia reside throughout
the brain parenchyma, however, the highest density is found in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Lawson et al. 1990). These brain regions overlap the neuronal circuits involving mood regulation, motivation and cognition (Capuron and Miller 2011).

While microglia are the principal mediators of the activation of the central immune system by inflammatory stimuli and produce pro-inflammatory cytokines, chemokines and reactive oxidative species, these immune cells also play a critical role in the recovery process in the CNS (Czeh, Gressens, and Kaindl 2011; Turrin and Rivest 2006). In response to immunological or neuronal signals, microglia regulate the cellular environment in the brain by removing cell debris (phagocytosis), signalling apoptosis, neurogenesis and proliferation of astrocytes/oligodendrocytes (Czeh, Gressens, and Kaindl 2011; Rajkowska and Miguel-Hidalgo 2007; Turrin and Rivest 2006).

Astrocytes are another major glial cell in the brain that perform a wide range of functions including involving neuron-glial signalling, maintaining synaptic homeostasis, regulating synaptic pruning and importantly, modulating glutamate transport which is implicated in the pathophysiology of MDD (Kimelberg and Nedergaard 2010; Verkhratsky, Nedergaard, and Hertz 2015). In addition, astrocytes are critically involved supporting endothelial cells which form the brain blood barrier (Abbott, Ronnback, and Hansson 2006). Astrocytes express receptors for cytokines, chemokines and acute phase proteins, and respond to immune signals from microglia or peripheral macrophages. Upon activation associated with pathological conditions, astrocytes as well as microglia increase surface markers of inflammatory activation such as major histocompatibility complex (MHC) class II (Haroon, Miller, and Sanacora 2017) and produce pro-inflammatory cytokines such as IL-1β, TNF α and IL-6 (Avila-Munoz and Arias 2014; Muller 2014). These neurotoxic effects induced by glial cells

1.2.3 Oxidative stress induced by inflammation

Glial activation upon immune signalling from the periphery initiates the production of ROS such as extracellular superoxide and nitric oxide (NO). Furthermore, nitric oxide synthase isoforms (iNOS) are up-regulated by pro-inflammatory cytokines and the endotoxin, lipopolysaccharide (LPS). The synthesis of ROS and NO by glial cells is a crucial part of the mechanism of cell proliferation and neuronal repair, and contributes to maintenance of a healthy cellular system in the CNS (Block, Zecca, and Hong 2007). However, an excess of superoxide causes an imbalance between ROS and antioxidant defences in microglia and can result in substantial oxidative damage (Novo and Parola 2008). In the context of MDD, glial cells in the CNS are stimulated by pro-inflammatory cytokines, which may mediate simultaneous production of NO and ROS. Accumulating concentrations of NO and ROS cause impairment of mitochondrial ATP production and subsequently increase oxidative damage to the CNS structure (Muller 2014; Tilleux and Hermans 2007). In addition, a recent study suggested that this prolonged glial activation state potentially disrupt the glutamatergic system which is tightly regulated by glial cells and in turn, predispose to depressive mood (Haroon, Miller, and Sanacora 2017).
1.3 Glutamatergic system

The amino acid glutamate is the major excitatory neurotransmitter in the brain (Orrego and Villanueva 1993). Glutamate mediates the majority of excitatory neurotransmission in the CNS while gamma-aminobutyric acid (GABA), another amino acid neurotransmitter, mediates the majority of inhibitory neurotransmission (Sanacora, Treccani, and Popoli 2012). Glutamate neurons are widely distributed in the brain. Following early findings that a subclinical dose of ketamine, a glutamate N-methyl-D-aspartate (NMDA) receptor antagonist improved depressive symptoms in treatment resistant MDD patients (Berman et al. 2000), accumulating evidence points to the antidepressant effects of modulating the glutamatergic system (Zarate et al. 2006; Preskorn et al. 2008; Ghasemi et al. 2014).

1.3.1 Preclinical and clinical evidence of aberrant glutamate system in MDD

Several studies have reported biological changes in the amino acid neurotransmitter system in MDD patients. Elevated concentrations of plasma glutamate in MDD patients were reported in some studies (Kucukibrahimoglu et al. 2009; Mitani et al. 2006). The concentration of plasma glutamate was positively correlated with the Hamilton Depression Rating Scale (HAM-D) score (Mitani et al. 2006). A decline of the ratio of glutamate to glutamine in cerebrospinal fluid (CSF) in MDD patients was correlated with a reduction of depressive symptoms (Hashimoto et al. 2016). Furthermore, antidepressant treatment, fluoxetine- or S-citalopram modulated levels of plasma glutamate, glutamine and GABA levels in MDD patients (Kucukibrahimoglu et al. 2009). The association between levels of glutamate in the periphery and dysfunction of the glutamatergic system in the CNS is still poorly understood, however several lines of evidence supports a mechanistic link between dysfunction of the glutamatergic system in the CNS and MDD.
One of the major glial cells in the brain, astrocytes, play a key role in regulating postsynaptic exchange in glutamatergic neurotransmission (Haroon, Miller, and Sanacora 2017). For instance, pharmacologic ablation of astrocytes in the prefrontal cortex induced depressive-like behaviour in the forced swim test (FST) and anhedonia in the sucrose preference test (SPT) in rats (Banasr and Duman 2008). An animal model of depression, the learned helplessness model, also exhibited significant suppression of both transcript levels and protein levels of the glial glutamate transporter GLT1 in the hippocampus and cerebral cortex compared to littermates with low levels of helplessness and non-helpless animals whereas transcript levels of GLAST, another glial glutamate transporter which is highly expressed in astrocytes, in the areas were not affected (Zink et al. 2010). In addition, inhibition of GLT1 in the prefrontal cortex induced a significant reduction of sucrose intake, indicative of anhedonia (John et al. 2012). A significant downregulation of GLAST protein levels in the hippocampus was also reported in a rat model of depression, the Flinders sensitive line (Gomez-Galan et al. 2013).

In humans, a significant downregulation of excitatory amino acid transporters, SLC1A2 (human nomenclature for GLT 1) and SLC1A3 (human nomenclature for GLAST), that are involved in glutamate signalling pathways in the locus coeruleus in the forebrain (Bernard et al. 2011) and in the hippocampus (Medina et al. 2013; Medina et al. 2016) of post-mortem brains of MDD patients was compared to controls via a microarray technique and in situ hybridization. These data indicate that dysfunction of the glutamatergic system could underpin the pathophysiology of MDD; and there is a critical and intriguing involvement of glial cells in the regulation of glutamatergic neurotransmission.
1.3.2 Inflammation and the glutamatergic system

As discussed earlier, chronic inflammation, as evidenced by subclinical elevations of circulating pro-inflammatory cytokines, is commonly associated with MDD. Chronic inflammation also perturbs glutamatergic neurotransmission. IFN α, a pro-inflammatory cytokine administered therapeutically in patients with Hepatitis C, induced an increase in glutamatergic neurotransmission in the basal ganglia and anterior cingulate cortex (Haroon et al. 2014). The same authors also reported a link between a pro-inflammatory mediator, C-reactive protein (CRP), glutamate levels in the left basal ganglia and depressive symptoms. An elevation of plasma CRP levels in MDD patients was significantly correlated with increased levels of glutamate in the left basal ganglia. The increased glutamate levels were also positively associated with symptoms of MDD, anhedonia and psychomotor slowing (Haroon et al. 2016). In addition, another study showed that elevated CRP levels in MDD patients were associated with the glial marker myoinositol, which is found primarily in astrocytes and functions as a marker of astrocytic activity (Ashwal et al. 2004). These studies imply the link between chronic inflammation and aberrant glutamatergic signalling in MDD may be modulated via glial cells.

1.3.3 Glial cells and the glutamatergic system

As discussed earlier, glial cells play a crucial role in an integrated network responding to pro-inflammatory factors from the periphery, by which pro-inflammatory cytokines appear to be responsible for pathophysiological mechanisms in the CNS. Upon activation, microglia express both glutamate transporters (GLT1, GLAST and System xCT) and glutamate receptors on their surface (Verkhratskii and Butt 2012; Haroon, Miller, and Sanacora 2017; Noda et al. 2000).
Glutamate in the extracellular matrix binds to AMPA and kainite subtypes of ionotropic receptors on activated microglia and promotes microglia to produce and release pro-inflammatory cytokines such as TNF α (Haroon, Miller, and Sanacora 2017; Noda et al. 2000; Verkhratskiĭ and Butt 2012). Microglia also produce ROS upon stimulation of glutamate receptors (Mead et al. 2012). Thus, the evidence suggests that pro-inflammatory and neurotoxic molecules from microglia in a prolonged inflammatory state in the CNS could have detrimental effects on the ability of astrocytes to buffer glutamate from the extracellular space, and subsequently mediate aberrant glutamate neurotransmission.

Hence, we are beginning to develop a picture of how aberrant central immune activation may play a role in the pathogenesis of MDD. However, it remains unclear as to what factors may be contributing to the pro-inflammatory drive in MDD, although altered immune regulation arising from environmental factors such as diet are strong candidates.

1.4 Diet as a risk factor for MDD

Accumulating evidence suggests that for many patients, diet is one of the key environmental factors that contributes to the risk of developing MDD. A substantial number of epidemiological studies have found that unhealthy diets, characterised by high levels of fat and/or sugar, are associated with an increased risk of developing MDD (Jacka et al. 2014; Rahe et al. 2015; Yu, Parker, and Dummer 2014). Furthermore, healthy diet patterns reduce the incidence of MDD (Dipnall et al. 2015; Opie et al. 2015; Ruusunen et al. 2014; Jacka et al. 2017).
Although the development of MDD can alter dietary patterns, longitudinal studies confirm that diet quality often precedes MDD onset, suggesting that diet can be a causal factor (Akbaraly et al. 2009; Rienks, Dobson, and Mishra 2013; Sanchez-Villegas et al. 2009; Sanchez-Villegas et al. 2012). Furthermore, dietary patterns modulate inflammatory status. For example, meta-analyses showed healthy dietary patterns such as the Mediterranean diet significantly decrease plasma levels of CRP (Neale, Batterham, and Tapsell 2016; Schwingshackl and Hoffmann 2014) and IL-6 (Schwingshackl and Hoffmann 2014).

In contrast, Western style diets containing processed food and sweets were associated with an increase in plasma levels of CRP and IL-6 (Lopez-Garcia et al. 2004; Fung et al. 2001). Therefore, poor diet quality could induce elevations of pro-inflammatory mediators which subsequently may predispose to depressed mood. In fact, numerous pre-clinical studies have reported that a high fat and/or Western style diet are associated with cognitive impairment (Boitard et al. 2014; Andre et al. 2014; Jeon et al. 2012) and depressive-like behaviour (Sharma, Fernandes, and Fulton 2013; Sharma and Fulton 2013; Kesby et al. 2015). Other studies, however, demonstrated less conclusive effects of a high fat diet (Andre et al. 2014; Pyndt Jorgensen et al. 2014; Krishna et al. 2016; Takase et al. 2016), which is possibly due to the large variability in the proportion and type of fats used. These studies indicate the capacity of diet to shape behaviour and mood, however, the pathways underpinning this association are poorly understood.

1.5 The gut microbiome

The gut microbiome consists of trillions of microorganisms. It is an ecosystem predominantly dominated by bacteria (Shreiner, Kao, and Young 2015). In recent years, increasing evidence
supports a functional role of the gut microbiome in the regulation of health and disease, including neuropsychiatric disorders. The gut microbiome plays a central role in metabolic function, protection against pathogens, and is critically involved in shaping the healthy immune system (Shreiner, Kao, and Young 2015).

1.5.1 Diet and gut microbiome

Several studies have shown that a poor diet is capable of altering the gut microbial community (Brown et al. 2012). For instance, a Western style diet that contains high fat and high sugar resulted in an increase in Firmicutes and a decrease in Bacteroidetes (Turnbaugh et al. 2009). Other studies found that high-fat diets rich in safflower oil, an omega-6 polyunsaturated fatty acid (PUFA), reduce the abundance of Bacteroidetes while enriching the populations of Firmicutes, Actinobacteria and Proteobacteria in rodents (Turnbaugh et al. 2008; de La Serre et al. 2010).

One study assessed the relative contribution of host genetics and diet in shaping the gut microbiota and metabolic syndrome phenotypes in mice. In high fat diet fed mice, dietary change explained 57% of the total structural changes in gut microbiota, whereas genetic mutation accounted for no more than 12% (Zhang et al. 2010). This strongly indicates that diet plays an important role in regulating the gut microbial community. Collectively, the evidence highlights the potentially critical role that the gut microbiota might play in shaping behaviour and mood. What remains unclear is the mechanism for how the changes in gut microbiota induce depressive mood. However, several lines of evidence indicate a possibility that the gut microbiota could indirectly mediate inflammatory status.
1.5.2 Gut microbiome mediates inflammation

Bacterial lipopolysaccharides (LPS) are the major outer surface membrane components present in almost all gram-negative bacteria and are a major inducer of the inflammatory response (Alexander and Rietschel 2001). Given the large number of microorganisms in the intestine, the gut microbiome serves as a significant reservoir of LPS.

Experimental administration of LPS is also frequently used in animal models of inflammation-mediated MDD. LPS induced inflammation causes sickness behaviour that resembles depressive-like behaviour such as behavioural despair and anhedonic behaviour, which are analogous to MDD symptoms. Upon detection of LPS, LPS binding protein (LBP), CD14 and Toll-like receptor 4 (TLR4) trigger a cascade of immune responses and subsequently release pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF α (Manco, Putignani, and Bottazzo 2010; Moreira et al. 2012; Alexander and Rietschel 2001; Ishii et al. 1993; Tobias et al. 1992; Gutsmann et al. 2001). LBP is a serum/plasma glycoprotein, synthesised by hepatocytes in the liver and intestinal epithelial cells (Gutsmann et al. 2001). Once LBP is in the circulation, LBP forms a complex with LPS that enables binding to CD14 receptors. CD14, a glycosylphosphatidyl-inositol (GPI)-anchored monocyte differentiation antigen, is present in soluble form (sCD14), and is also expressed on the membranes of monocytes and macrophages (mCD14). Both forms of CD14 are associated with TLR4, which transduces a signal from the CD14-bound LPS to the cell nucleus, triggering production of a cascade of inflammatory cytokines (Ishii et al. 1993; Tobias et al. 1992; Gonzalez-Quintela et al. 2013).

Because of the central role of LBP on LPS mediated inflammation, LBP has been used as a surrogate marker of low grade inflammation induced by LPS from the gut (Gonzalez-Quintela...

Several studies showed dietary components can increase levels of LPS in the circulation and elicit inflammatory responses. Under normal conditions, LPS levels fluctuate during the fed and fasted states (Cani et al. 2007). High carbohydrate and fat meals in healthy adults resulted in elevations of markers of oxidative stress, inflammation and levels of LPS (Ghanim et al. 2010). Consumption of a high fat diet also promoted an elevation of LPS and CD14, and subsequently the inflammatory cytokine, IL-6, in healthy adults (Laugerette et al. 2011). High fat diet consumption induces a well-documented increase in intestinal permeability and subsequent elevation of LPS levels (Cani et al. 2007; Cani and Delzenne 2010; Cani et al. 2009). For instance, a four week period of chronic consumption of high fat diet resulted in two to three times higher plasma LPS levels in mice and increased proportion of LPS producing gram-negative bacteria in the intestine (Cani et al. 2007). It has been hypothesised that the elevation of LPS levels was due to an increase in permeability of the intestinal barrier (tight junctions) and resultant bacterial translocation to the systemic circulation (Garate et al. 2011; Kelly et al. 2015; Maes, Kubera, et al. 2012; Slyepchenko et al. 2017). These circulating gram negative bacteria are a source of LPS. This proposed mechanism, “leaky gut” is also implicated in the pathophysiology of MDD (Kelly et al. 2015; Maes, Kubera, et al. 2012; Slyepchenko et al. 2017). Therefore, diet mediated changes in the gut microbiome have the capacity to up-regulate or down-regulate inflammatory signalling, via LPS, in the host.
1.5.3 Glial cells and gut microbiome

As discussed earlier, microglia are critically involved in regulation of health and disease in the CNS. Given that microglia are finely attuned to the inflammatory milieu, the possibility arises that alterations in the gut microbiome may induce immune signalling that influences glial function, and therefore neuronal activity and mood. An intriguing recent study showed gut microbiota are essential for the regulation of microglial homeostasis and activation. Microglia in mice without gut microbiota, germ-free mice, showed defective morphology, reduced cell numbers and diminished immune reactivity in response to LPS compared to conventionally raised mice. Treatment with antibiotics also altered microglial morphology in conventionally raised mice, which was similar to the cells in germ free mice. Interestingly, recolonisation of gut microbiota in germ free mice and oral administration of mixture of short chain fatty acids (SCFAs), which are generated by gut microbiota through fermentation of dietary fibre, restored microglial morphology (Erny et al. 2015). These findings indicate the importance of gut microbiota on development and maturation of microglia. Intriguingly, the authors found receptors for SCFAs were not expressed in the brain.

In line with this, another study also reported that the absence of microbiota alleviated microglial activation and subsequently limited neuroinflammation in the brain and associated motor deficits in a mouse model of Parkinson’s disease (Sampson et al. 2016). Furthermore, transplantation of gut microbiota from Parkinson’s disease patients to mice predisposed to Parkinson’s disease with exaggerated motor impairments compared to the same transgenic mice who received transplantation of gut microbiota from healthy volunteers. In addition, the function of astrocytes is indirectly regulated by the gut microbiota. A recent study reported that dietary tryptophan metabolites from Lactobacillus reuteri exert suppressive effects on
neuroinflammation via the ligand-activated transcription factor aryl hydrocarbon receptor (AHR) in astrocytes using an animal model of multiple sclerosis (Rothhammer et al. 2016).

We are beginning to understand how glial cell functions are regulated by the gut microbiome. Emerging evidence highlights two important implications; gut microbiota are mechanistically involved in glial function and CNS immune reactivity; and alteration of gut microbiota could induce/escalate neuroinflammation in the CNS. What remains unclear are; how gut microbiota signals microglia; and what types of alterations of gut microbiota are associated with disease states.

1.5.4 Gut microbiome and MDD

Accumulating evidence suggests that the gut microbiota is indirectly associated with negative mood (Kelly et al. 2016; Kelly et al. 2015; Maes, Berk, et al. 2012; Maes, Kubera, et al. 2012; Slyepchenko et al. 2017). Interestingly, treatment with prebiotics that promote growth of beneficial bacteria regulated stress-induced depressive-like behaviour and GABA receptor expression in the mouse brain (Bravo et al. 2011). In line with this, an analysis of the mouse brain by magnetic resonance spectroscopy showed an increase in GABA, acetyl aspartate and glutamate in the brain after four weeks of prebiotic treatment (Janik et al. 2016).

Alterations in the gut microbial community are also documented in MDD. A reduction of Bifidobacterium and Lactobacillus were documented in the gut microbiota of patients with MDD (Aizawa et al. 2016), whilst other studies have observed changes in the proportion of Prevotella (Phylum: Bacteroidetes) and Klebsiella (Phylum: Proteobacteria) which correlated with scores on the Hamilton depression rating scale (Lin et al. 2017). Intriguingly, transplantation of the fecal microbiome from patients with MDD into germ-free mice resulted
in an increase in depressive-like behaviours relative to mice transplanted with microbiota from healthy control individuals (Zheng et al. 2016). These studies suggest the gut microbial community may play a causal role in the pathogenesis of depressive behaviour.

Furthermore, transplantation of the cecum and colonic microbiome from mice fed high fat diet induced changes in anxiety-like behaviours in recipient mice (Bruce-Keller et al. 2015). Taken together, these evidence supports complex influence of diet induced changes in the gut microbial community on behaviour and implies an importance of the gut brain axis for the clinical setting and our conceptualisation of MDD.

To summarise, in the context of MDD, unhealthy diet consumption leads to altered gut microbiome communities in the intestine. The subclinical inflammation driven by gut dysbiosis may create a persistent pro-inflammatory state in the CNS. The immune cells in the brain amplify the immune signalling from the periphery, causing dysfunction of glutamate neurotransmission which might predispose to depressive mood. A schematic diagram was depicted in Diagram 1.1.
Diagram 1.1 Interaction between diets, gut microbiome, immune system and behaviour interact in the context of MDD.

1.6 Novel therapeutics

1.6.1 Current antidepressant efficacy

Current pharmacological intervention to treat MDD appears to have moderate efficacy although the therapeutic effects of antidepressants are far from universal. This is evidenced by the fact that only one third of patients achieve complete remission of depressive symptoms and gain functional recovery (Pae et al. 2008). Furthermore, clinical data shows that more than half of patients do not respond to one of the currently available classes of antidepressants, the selective serotonin reuptake inhibitors (SSRI) (Warden et al. 2007). A large cohort study examining 2,800 MDD patients reported the striking fact that only 50 –
60 % of MDD patients respond to the first antidepressant treatment and less than one third of the subjects achieved remission (Trivedi et al. 2006).

Most currently available antidepressants were developed in line with the monoamine hypothesis of MDD, which posits that the pathophysiology of MDD is due to reduced availability of monoamines, such as serotonin and noradrenaline (Sanacora, Treccani, and Popoli 2012). Basic mechanisms of action of current antidepressants are, therefore, aimed at enhancing or restoring availability of monoamines at synaptic levels (Costa-Campos et al. 2013). However, the limited therapeutic efficacy of current antidepressants based on the monoamine hypothesis highlights the fact that there is an urgent need to develop new therapeutics, focusing on different targets or pathways in MDD.

In accordance with current theories of MDD, therefore, agents that could regulate the inflammatory status, levels of ROS and the gut microbiome might be candidates as novel therapeutics for MDD.

1.6.2 N-acetylcysteine

N-acetylcysteine (NAC) is an antioxidant which provides cysteine for synthesis of the most ubiquitous antioxidant in the brain, glutathione (GSH) (Dean, Giorlando, and Berk 2011). NAC has been trialled in psychiatric disorders including obsessive-compulsive disorder, trichotillomania, addiction, schizophrenia and bipolar disorders. Significantly, NAC has shown therapeutic efficacy by modulating negative mood states in these disorders and has been successfully trialled as an adjunctive therapy for bipolar depression (Berk et al. 2011; Lavoie et al. 2008; Magalhaes et al. 2011). Furthermore, adjunctive NAC treatment showed efficacy for depressive symptoms in a recently conducted clinical trial (Berk et al. 2014). Although NAC
has potential efficacy in these disorders, what is unclear is which pathways are responsible for these therapeutic effects, for example whether NAC might exert therapeutic effects through targeting glutamate, oxidative stress, modulating pro-inflammatory responses or altering the gut microbiome.

1.6.2.1 Antidepressant-like effect of NAC

While NAC treatment showed anti-depressant like effects on depressive-like behaviour in rodents (Ferreira et al. 2008; Linck et al. 2012; Wright et al. 2016; Arent et al. 2012; Smaga et al. 2012), there is a paucity of clinical studies investigating therapeutic effects of NAC in MDD. Twenty-four weeks of adjunctive NAC treatment (1g twice daily) in patients with major depressive disorder showed positive effects on depressive symptoms in a recent clinical trial (Berk et al. 2014). Intriguingly, the improvement of depressive symptoms, as measured by the Montgomery-Asberg Depression Rating Scale (MADRS) were observed after 16 weeks, which was 4 weeks after discontinuation of the NAC treatment. On the other hand, NAC also improved major depressive episodes in bipolar disorder (Berk et al. 2008). In this study, depressive symptoms of bipolar disorder were treated by adjunctive NAC treatment (1g twice daily) for 24 weeks. NAC treatment resulted in significant improvement of MADRS scores at the end point (24 weeks of time). In line with this, a recent meta-analysis investigating therapeutic efficacy of NAC on depressive symptoms that examined 574 participants, of whom 291 were randomized to receive NAC and 283 to placebo, concluded that NAC treatment significantly improved depressive symptoms measured by MADRS and that Hamilton Depression Rating Scale (HDRS) (Fernandes et al. 2016). What remains unclear is
the underlying mechanisms of action of NAC that conferred these symptomatic improvements.

1.6.2.2 Potential mechanisms of action of NAC

As mentioned above, NAC is a potent antioxidant which provides cysteine for synthesis of GSH (Dean, Giorlando, and Berk 2011), and this could potentially facilitate the microglial antioxidant defence mechanism. Microglia contain high levels of various anti-oxidants, such as glutathione (GSH), ascorbic acid, and α-tocopherol (Dringen 2005). For example, one study identified the regulation of the synthesis of GSH varied by different pro-inflammatory cytokines (Chatterjee et al. 2000). These antioxidants are believed to be up-regulated in microglia in response to pro-inflammatory cytokines (Dringen 2005). Therefore, NAC could potentially regulate inflammatory status and oxidative biology, and therefore may restore disrupted neurotransmission. In fact, NAC treatment (500mg/kg) alleviated depressive-like behaviour in mice by regulating the glial glutamate transporter (GLT1) and system xCT (Wright et al. 2016). System xCT is expressed on glial cells in the brain and transports cysteine into glial cells (McBean and Flynn 2001). The expression of system xCT is increased upon microglial activation (Mesci et al. 2015). In addition, NAC has direct effects on glutamate neurotransmission. A preclinical study showed that the antidepressant effects of NAC are blocked if an AMPA glutamate antagonist is co-administered, suggesting a key role of NAC on glutamate (Linck et al. 2012).
1.6.2.3 Effects of NAC on the gut microbiota

The effects of NAC on the gut microbiome have not been fully characterised. A preclinical study showed that NAC modulated the redox state and some selected bacteria in the gut in response to early weaning stress in piglets (Xu et al. 2014). A significant increase in *Lactobacillus* and *Bifidobacterium* were observed in the NAC treated animals. Furthermore, the increase in *Lactobacillus* and *Bifidobacterium* were positively correlated with total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), and negatively correlated with hydroxyl radical (IHR), and levels of malondialdehyde (MDA), H$_2$O$_2$, and NO. These results are in line with the potential antioxidant effect of NAC, and also suggest that NAC can mediate changes in the gut microbiota.

Taken together, these studies suggest the potential utility of NAC for MDD. NAC may exert antidepressant-like effects on depressive mood and behaviour through targeting glutamate, oxidative signalling, and subsequently modulating inflammation, or possibly by acting on the gut microbiome.

1.6.3 Minocycline

Minocycline (MINO), a second-generation tetracycline derivative, has potent anti-inflammatory and neuroprotective effects, which are independent of its antibiotic efficacy (Dean et al. 2012; Pae et al. 2008). Most importantly, MINO readily crosses the blood brain barrier and is known to inhibit microglial activation (Pae et al. 2008). Furthermore, MINO has been used extensively as an antibiotic in practice, and hence its safety profile has been established (Dean et al. 2012). The therapeutic actions of MINO may be exerted through inhibition of microglial activation.
In preclinical studies, MINO ameliorated the depressive-like behaviour observed in various animal models of MDD (Henry et al. 2008; Majidi, Kosari-Nasab, and Salari 2016; Liu et al. 2015). However, there is a paucity of clinical studies evaluating the antidepressant effects of MINO in MDD subjects. A clinical open labelled trial investigated the effects of adjunctive MINO treatment on MDD patients, and reported a significant improvement in depressive symptoms (Miyaoka et al. 2012). This preliminary study was open labelled with a small sample population which could be potentially confounded by several factors. However, considering the promising effects of MINO on MDD, more evidence through randomised, placebo-controlled clinical trials to evaluate therapeutic effects of MINO on MDD is urgently required.

A few studies have also reported that MINO treatment exerted antidepressant-like effects on the negative symptoms of patients with schizophrenia (Ghanizadeh et al. 2014; Khodaie-Ardakani et al. 2014). Although there are ongoing clinical studies examining the effects of MINO in MDD subjects (Dean et al. 2014; Husain et al. 2015), little is known about how MINO modulates depressive symptoms.

1.6.3.1 Anti-microbial effects of MINO

MINO is a second generation tetracycline derivative that has been used for over 30 years as an antibiotic to treat moderate to severe inflammatory acne (Kircik 2010). Tetracycline is a bacteriostatic antibiotic that slows growth and reproduction of bacteria, and is categorised as a broad-spectrum antibiotic as it is active against a wide range of aerobic and anaerobic gram-negative and gram-positive bacteria (Garrido-Mesa, Zarzuelo, and Galvez 2013). MINO is effective against both gram-positive and gram-negative bacteria (Garrido-Mesa, Zarzuelo, and Galvez 2013), and its’ mode of action can be either bactericidal (kills bacteria) or
bacteriostatic (slows growth or reproduction of bacteria), and the degree of its effectiveness may differ depending on bacterial strains (Eliopoulos et al. 1994; Gelber et al. 1995; Masuda 1984; Vanhoof et al. 1980).

Surprisingly, the anti-microbial effects of MINO on the gut flora have been documented in very few studies. Chronic MINO treatment (intraperitoneal injection, 5mg/kg, 21 days) in mice resulted in significantly lower abundance of genera *Allobaculum*, *Turicibacter* and *Clostridium* (members of Firmicutes), *Bifidobacterium* (a member of Actinobacteria) and the family S24-7 (a member of Bacteroidetes) and significantly higher relative abundance of the family *Lachnospiraceae* and *Ruminococcaceae incertae sedis* (members of Firmicutes), which was concomitant of a reversal of depressive-like behaviour (Wong et al. 2016). Yang et al. also examined effects of MINO on an animal model of hypertension and the gut microbiome. Chronic MINO treatment (oral gavage, 50mg/kg, four weeks) in rats significantly increased expansion of abundance of *Akkermansia* (a member of Verrucomicrobia), *Bacteroides* (a member of Bacteroidetes), *Enterorhabdus* (a member of Actinobacteria) and *Marvinbryantia* (a member of Firmicutes) compared with controls (Yang et al. 2015). These data suggested that MINO selectively exerts antibiotic effects on specific bacteria; and the anti-microbial effects of MINO on the gut microbiome may differ depending on the host.

In humans, exposure to a single treatment of MINO significantly altered profiles of gut microbiota and the change persisted for a month in healthy adults, whilst the salivary microbiota composition was resilient to the antibiotic (Zaura et al. 2015). Taken together, MINO exerts long lasting anti-microbial effects selectively on the gut microbial communities and mediates significant changes in gut microbial profiles. However, how the significant
alteration of the gut microbiome elicited by MINO interacts with physiological changes and subsequently shapes behavioural changes in the host remains unclear.

1.6.3.2 Anti-inflammatory effects of MINO

A growing body of evidence suggests that up-regulation of pro-inflammatory cytokines is one of the hallmarks of MDD. MINO exerts anti-inflammatory effects as an inhibitor of microglial activation (Dean et al. 2012). Previous studies have demonstrated effects of MINO on pro-inflammatory cytokines and mood. MINO attenuated LPS induced increases in the pro-inflammatory cytokines IL-1β and IL-6 in the cortex and hippocampus in mice (Henry et al. 2008). In the same study, pre-treatment with MINO prevented the development of depressive mood in mice (Henry et al. 2008). In another study, mice were injected with MINO for three consecutive days and on the third day, the mice were challenged by systematic inflammation (LPS). MINO treated mice did not develop anhedonia, a key facet of depressed mood, as measured by the sucrose preference test (O'Connor et al. 2009). These findings suggest that MINO has effects on immune cells in the CNS through inhibition of the synthesis of pro-inflammatory cytokines, and that these effects may flow through to offset mood state.

1.6.3.3 Anti-oxidative effects of MINO

The inhibitory effects of MINO on microglia are associated with anti-oxidant activity in the CNS. Microglia produce free radicals and ROS in response to immunological stimuli, neuronal signals and homeostatic changes in the CNS. Very few studies have examined the relationship between microglial ROS and the anti-oxidant properties of MINO. MINO treatment reduced endotoxin induced NO production in cultured microglia cells (Tikka et al. 2001). MINO also
inhibited hypoxia-induced production of NO in cultured rat microglia (Suk 2004). Furthermore, MINO exerts anti-oxidant properties through direct scavenging of free radicals (Kraus et al. 2005). Given that chronic inflammation may mediate prolonged ROS production in microglia in MDD, minocycline’s capacity to inhibit microglial activation and scavenge extracellular free radicals in the surrounding environment may be central to its antidepressant effects.

Taken together, the anti-inflammatory and the anti-oxidant properties of minocycline, originating from inhibition of central immune cell activation, appear to regulate neuronal function. Furthermore, MINO exerts strong anti-microbial effects on the gut microbiota. The interaction between these three effects of MINO may contribute to an improvement of negative mood. Although further investigations to elucidate the underlying therapeutic mechanisms of MINO to determine how these effects interact in the host are necessary, MINO is a promising candidate as a new antidepressant.

1.7 Aims of this study

Current pharmacological intervention to treat MDD has limited efficacy. Whilst accumulating evidence now suggests that inflammation is involved in the pathophysiology of MDD and some selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) were reported to show anti-inflammatory properties (Hannestad, DellaGioia, and Bloch 2011), these antidepressants are designed to regulate levels of monoamines, not primarily designed to regulate the inflammatory state in MDD. On the other hand, current literature identifies that NAC and MINO exert therapeutic effects on
depressive-like behaviour and depressive/negative mood via pathways including regulating inflammatory state and therefore, have significant potential as new therapeutics for MDD.

In addition, NAC and MINO have been used in clinical practice and therefore have established safety profiles. Current antidepressants exert various side effects include nausea, insomnia, somnolence, fatigue, sexual dysfunction, and weight gain. Because of these side effects, patients can discontinue taking these medications, which contributes to an increased risk of depressive relapse and recurrence (Bostwick 2010; Shultz and Malone 2013). Although MINO and NAC also have side effects, such as pigmentation in skin (Oya, Kishi, and Iwata 2014; Solmi et al. 2017) and abdominal discomfort (Berk et al. 2014) respectively, the degree of adverse effects were milder than antidepressants and well tolerated among patients (Berk et al. 2014; Dean et al. 2017; Oya, Kishi, and Iwata 2014; Solmi et al. 2017).

Most importantly, these two agents readily cross the blood brain barrier and exert anti-inflammatory and anti-oxidant effects, by which NAC and MINO could potentially modulate aberrant inflammatory status and oxidative biology in MDD. However, there is a paucity of studies examining the therapeutic efficacy of these agents on inflammation in MDD.

To evaluate this, we analysed the associations between adjunctive NAC or MINO treatment, inflammatory mediators and depressive mood using serum samples collected during the NAC depression clinical trial and the MINO depression clinical trial run by Dr. Olivia Dean and Prof. Michael Berk at Innovations in Mental and Physical Health And Clinical Treatments (IMPACT) SRC, Deakin University. In the original double-blind clinical trial (Berk et al. 2014), adjunctive NAC treatment for MDD exerted positive effects on depressive symptoms. Given its anti-inflammatory properties, it was hypothesised that the therapeutic effects of NAC were associated with altered inflammatory state in the clinical population. In addition, adjunctive
MINO treatment resulted in a significant improvement of functional measurement in an open labelled and placebo controlled clinical trial to evaluate antidepressant effects in MDD (Dean et al. 2017).

Considering the anti-microbial and anti-inflammatory properties of MINO, we hypothesised that altered gut microbiome and inflammatory state induced by MINO treatment would be associated with the improvement of functional measures in MDD. In addition to inflammatory and microbial markers, levels of brain derived neurotrophic factor (BDNF) were also hypothesised to increase in MDD patients as a result of adjunctive NAC and MINO treatments. It is currently unclear whether the therapeutic effects of MINO and NAC are associated with levels of BDNF, whereas an increase in levels of BDNF associated with an improvement of depressive symptoms after antidepressant treatment has been documented in several studies (Brunoni, Lopes, and Fregni 2008; Matrisciano et al. 2009; Sen, Duman, and Sanacora 2008; Stelzhammer et al. 2014; Wolkowitz et al. 2011; Yoshimura et al. 2009).

This project secondly aimed to explore and profile depressive mood, inflammation and gut microbiome using a high fat diet to induce behavioural changes in an animal model. Our current knowledge suggests that poor dietary patterns could alter the gut microbiome and inflammatory signalling systems, and subsequently predispose to MDD. However, we do not yet understand the neurobiological mechanisms underlying how the diet, gut microbiome and inflammation interact, and how this may induce depressive mood. This animal model allows us to investigate the mechanisms by which diet, the microbiome, and the immune/inflammatory system interact to affect mood. Therefore, we hypothesised that high fat diet will induce depressive-like behaviour in mice as a result of increased inflammatory
tone in the periphery and the brain, and changes in the gut microbial community. To evaluate the underlying mechanisms in depressive mood, it is crucial to use an animal model. It allows us to control confounding factors such as diet, genetic background and living environment. Also, analyses of post mortem samples are necessary to examine underlying mechanisms regarding immune/inflammatory profiles and oxidative biology in the periphery and the brain.

In addition, as clinical and preclinical evidence suggests that NAC and MINO have the potential to regulate the immune/inflammatory response in MDD, it is therefore of interest to examine whether NAC or MINO could modulate diet-induced behavioural changes, and by what mechanism. We thus used NAC and MINO in the animal model of high fat diet induced MDD. Thus, the third aim of this project was to evaluate anti-depressant efficacy of NAC and MINO on high fat diet induced depressive behaviours. It is hypothesised that NAC will reverse depressive-like behaviour in mice by regulating inflammatory tone and oxidative biology in the periphery and the brain. MINO was hypothesised to reverse depressive-like behaviour in mice by regulating inflammatory mediators and the gut microbiome.

Together these preclinical and clinical studies shed light on the efficacy and mechanisms of action of NAC and MINO, and generate an exploration of their potential utility in MDD. In addition, findings from these preclinical and clinical studies will substantially contribute to fill the gaps in our understanding of the underlying pathophysiological mechanisms of MDD.
Chapter 2
Adjunctive N-acetylcysteine in MDD: Exploration IL-6, C-reactive protein and brain neurotrophic factor
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2.1 Introduction

Depression is a widely prevalent psychiatric disorder that imposes significant psychological and physical burdens on individuals and their families. While there are several factors contributing to depression, the association between depression and inflammation has been well documented. Depression is a common co-morbidity in patients with chronic inflammatory diseases (Celano and Huffman 2011; Iaquinta and McCrone 2015; Kozdag et al. 2015; Rusu and Dumitrascu 2015). Moreover, people with Major Depressive Disorder (MDD) also exhibit elevated levels of inflammatory mediators even in the absence of inflammatory comorbidities (Hiles et al. 2012; Yoshimura et al. 2009). Mechanistically, elevated inflammatory mediators affect oxidative biology, bioenergetics, neuronal structure and survival. Glial cells are activated by inflammatory mediators and trigger immune activation in the central nervous system (Czeh, Gressens, and Kaindl 2011). Prolonged immune activation can produce elevated levels of reactive oxygen species and neurotoxins which subsequently damage neuronal structure and survival (Block, Zecca, and Hong 2007; Czeh, Gressens, and Kaindl 2011). Furthermore, magnetic resonance imaging studies show structural changes in the hippocampus and white matter in brains of individuals diagnosed with depression, potentially demonstrating the consequences of continued immuno-inflammatory response, and these changes appear to be associated with recurrence (Samann et al. 2013; Wisse et al. 2015). These data imply that an agent that modulates inflammatory mediators could be a promising therapeutic agent for depression. One such agent is N-acetylcysteine.

N-acetylcysteine (NAC) is an antioxidant which provides cysteine for synthesis of the most ubiquitous antioxidant in the brain, glutathione (GSH)(Dean, Giorlando, and Berk 2011). NAC also has intrinsic anti-inflammatory effects, as illustrated by reduced plasma levels of pro-inflammatory cytokines in haemodialysis patients following NAC treatment (Saddadi et al.
NAC in addition has direct effects on glutamate neurotransmission. Preclinical studies suggest that the antidepressant effects of NAC are blocked if an AMPA glutamate antagonist is co-administered, suggesting a key role of glutamate (Linck et al. 2012). NAC also reverses many models of mitochondrial dysfunction, which is noteworthy given the increased evidence of mitochondrial dysfunction in mood disorders.

NAC has been trialled in psychiatric disorders including obsessive compulsive disorder, trichotillomania, skin picking, addictions, schizophrenia and bipolar disorders. Significantly, NAC has shown therapeutic efficacy by modulating negative mood states in these disorders and has been successfully trialled as an adjunctive therapy for bipolar depression (Berk et al. 2011; Lavoie et al. 2008; Magalhaes et al. 2011). Furthermore, adjunctive NAC treatment showed efficacy for depressive symptoms in a recently conducted clinical trial (Berk et al. 2014). These findings suggest that negative mood states in these psychiatric disorders may share an underlying mechanism. Although NAC has potential efficacy in these disorders, what is unclear is which pathways are responsible for these therapeutic effects, for example whether NAC might exert therapeutic effects through targeting glutamate, mitochondrial energy generation, neurogenesis, apoptosis, oxidative stress and/or modulating pro-inflammatory responses.

2.2 Aim

To further investigate the therapeutic effects of NAC on depressive mood, we conducted exploratory analyses of association between NAC, inflammatory mediators and depressive mood using serum samples collected during the NAC depression clinical trial (Berk et al. 2014). In the current study, we evaluated serum levels of Interleukin-6 (IL6), C-reactive protein (CRP) and brain derived neurotrophic factor (BDNF). We selected IL6 and CRP as inflammatory
markers due to their observed correlations with clinical depression (Haapakoski et al. 2015; Hiles et al. 2012). BDNF was chosen since lower levels of BNDF were found in depressive patients and increased BDNF was associated with improvement of depression scores (Matrisciano et al. 2009; Stelzhammer et al. 2014; Wolkowitz et al. 2011; Yoshimura et al. 2009; Brunoni, Lopes, and Fregni 2008; Sen, Duman, and Sanacora 2008). We thus evaluated; whether inflammatory and neurotrophic markers correlated with depression status; whether these markers had predictive value for a response to NAC treatment; and how NAC may modulate these markers and depressive symptoms. In this exploratory study, we hypothesised that decreases in the serum levels of IL6 and CRP and an increase in levels of BDNF would be associated with improvement of Montgomery Asberg Depression Rating Scale (MADRS) score, and adjunctive NAC treatment ameliorates serum levels of IL6 and CRP and enhances levels of BDNF that subsequently is linked to improvement of MADRS scores.

2.3 Methods
2.3.1 Participants

Participants were recruited between 2007 and 2011 in three locations in Australia; Geelong, Melbourne and Sydney. All participants provided informed written consent including use of biological samples in future studies and the study was conducted according to Good Clinical Practice guidelines. The trial was registered on the Australian and New Zealand Clinical Trials Registry (ACTRN12607000134426) and was approved by the relevant Human Research Ethics Committees. The main trial paper has been published and includes all demographic data (Berk et al. 2014). The demographic information relevant to the current paper is outlined below.

Inclusion criteria required that: Participants fulfilled the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) diagnostic criteria for MDD with a single episode or recurrent episodes; had a score of \( \geq 18 \) on the MADRS score at the time of entry into the study; were at
least 18 years of age; had the capacity to consent to the study; and to follow its instructions and procedures. If undergoing treatment, participants were required to have two weeks of stable treatment (based on their primary treatment; medication or psychotherapy) prior to entry into the study. Exclusion criteria were; a concurrent diagnosis of bipolar I or II disorder or bipolar disorder not otherwise specified; a primary clinical diagnosis of a personality disorder; failure in three or more adequate trials of antidepressant therapy or electroconvulsive therapy (ECT) for the current major depressive episode; presence of a known or suspected clinically unstable systemic medical disorder, including recent gastrointestinal ulcers; pregnant or breast feeding status; current users of greater than 500mg/d of NAC, 200ug/d of selenium, or 500IU/d of vitamin E; and/or history of anaphylactic reaction to NAC or any component of the preparation (Berk et al. 2014). Diagnosis was confirmed using a structured interview, the Mini-International Neuropsychiatric Interview - 5 (MINI-plus).

Participants were randomly allocated to treatment or placebo groups in a double-blind manner. The treatment group received NAC, 2 x 500mg capsules twice daily (total of 2 g/day) in addition to existing treatment for their major depressive episode. NAC was supplied by Zambon (Milan, Italy), and encapsulated by DFC-Pharmamed Pty Ltd (Sydney, Australia) in accordance with Good Manufacturing Practice guidelines. The clinical trial endpoints were at end of treatment (week 12) and post-treatment discontinuation (week 16 – washout). The biological samples were collected at baseline and the end of treatment (week 12)(Berk et al. 2014). In this study, the primary outcomes were effects of adjunctive NAC intervention on levels of inflammatory markers (CRP and IL6) and BDNF. Baseline biological status was used as a predictor variable for clinical outcomes at both end of treatment (week 12) and washout (week 16).
2.3.2 Biological measures and assay methodology (CRP, IL6 and BDNF)

Blood samples for the investigation of inflammatory markers and BDNF were drawn at baseline and treatment endpoint (week 12) in the study. Standard vacutainer blood collection tubes (BD) were used. The current study utilized serum which was drawn into tubes with no additive. Tubes were immediately centrifuged at 1006 g, and the supernatant collected as the serum sample. All samples were stored at -80 degrees Celsius until tested. Serum CRP concentrations were determined using an enzyme-linked immunosorbent assay (human CRP Quantikine ELISA, R&D Systems). Intra- and inter-assay coefficients of variation for the CRP assay were 5.5 and 6.5% respectively. Serum concentrations of IL 6 were measured using a high sensitivity ELISA (R&D systems). Intra- and Inter-assay coefficient were 7.3 and 7.7% respectively. Serum BDNF concentrations were measured using a human BDNF Quantikine ELISA (R&D systems). Intra- and inter-assay coefficient were 4.1 and 9.3% respectively. Each sample was assayed in duplicate. All assays were carried out in accordance with the manufacturer’s instructions.

2.3.3 Statistical analyses

All analyses are based on a modified intention to treat (ITT) model. Parametric modelling was not appropriate due to skewness of CRP, IL6 and BDNF data and the presence of outliers. In order to perform ITT analysis multiple imputation was performed for all missing biological data at treatment endpoint (week 12). Quantile regression models aimed at estimating the conditional median with least absolute errors (LAE) were performed with imputed endpoint serum data to compare the difference of biological correlates at baseline and endpoint between the control and NAC groups (Chamberlain and Chamberlain 1994; Koenker 2005).
For comparing MADRS score (baseline, week12 and week16) and levels of CRP, IL6 and BDNF (baseline and endpoint) between the NAC treatment and control groups, repeated measures split plot in time analysis of variance models were estimated using a generalized estimation equation (GEE) approach with exchangeable working correlation matrix. Datasets of CRP, IL6 and BDNF were transformed and ranked due to outliers and the skewed nature of the data. Intervention by follow-up interaction (IFI) impacts and their 95% confidence intervals (CIs) were estimated from GEEs and reported to evaluate model adjusted mean difference between NAC and placebo groups.

For responders analyses, responders were defined as a reduction of more than or equal to 50% of MADRS score from baseline to treatment endpoint (week 12). For remitters analyses, remitters were defined as less than or equal to MADRS score 7 at treatment endpoint and washout (week 12 and week 16). Median (quantile) regression models were used to explore the relationship between levels of CRP, IL6 and BDNF at baseline in MADRS responders and remitters at the NAC treatment endpoint and washout. Statistical analyses were carried out using STATA (StataCorp, Texas, USA). We set a significance level at 0.05.
2.4 Results

Baseline characteristics of the sample are shown in Table 2.1. The treatment and placebo groups did not differ on demographic variables, clinical characteristics, body mass index (BMI) or collection sites. Of the total number of participants through the clinical trial (n=252) we obtained baseline serum CRP and BDNF for 63 participants in the placebo group and 58 in the NAC group. For IL6, 62 participants in the placebo group had analyses and 56 participants in the NAC group. At treatment endpoint (week 12) serum CRP and BDNF were measured in 32 participants in the placebo group and 28 participants in the NAC group. For serum IL6, 30 samples were analysed in the placebo group and 26 in the NAC group. Missing samples were due to treatment withdrawal from the primary clinical trial or non-attendance for pathology collection (which was an optional component of the primary clinical trial (Berk et al. 2014)).
Table 2.1. Baseline demographic and clinical characteristics

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<th>Placebo (n=63)</th>
<th>NAC (n=58)</th>
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<td>Mean (s.d.)</td>
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<td>Mean (s.d.)</td>
<td>Mean (s.d.)</td>
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**Medication at baseline, % (n)**

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<tr>
<td>Antidepressant</td>
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<td>45 (71.4)</td>
<td>37 (63.8)</td>
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<td></td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>10 (8.3)</td>
<td>5 (7.9)</td>
<td>5 (8.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antipsychotic</td>
<td>9 (7.4)</td>
<td>4 (6.3)</td>
<td>5 (8.6)</td>
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<tr>
<td>Mood stabilizer</td>
<td>5 (4.1)</td>
<td>4 (6.3)</td>
<td>1 (1.7)</td>
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<td></td>
</tr>
</tbody>
</table>

Abbreviations: MADRS, Montgomery-Asberg Depression Rating Scale; BMI, body mass index. BMI was categorised <18.50 = underweight, 18.50 to < 25.00 = normal weight, 25.00 to <30.00 = overweight, and >= 30.00 = Obese.
2.4.1 NAC treatment and serum levels of CRP, IL6 and BDNF

To evaluate effects of adjunctive NAC treatment on serum levels of CRP, IL6 and BDNF, differences in these markers from baseline to endpoint in each treatment group were assessed. Median regression models indicated that the levels of serum CRP, IL6 and BDNF at baseline did not differ from levels of corresponding markers at endpoint in either the NAC or placebo groups (Table 2.2). The changes in levels of CRP, IL6 and BDNF between baseline and endpoint were compared between the NAC and placebo treatment groups, however, the changes in levels of each biological marker over time did not vary between groups (Table 2.2).
Table 2.2. Comparisons of serum levels of CRP, IL6 and BDNF at baseline to endpoint

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>Baseline</td>
<td>Endpoint</td>
<td>Baseline vs. Endpoint</td>
<td>Baseline</td>
<td>Endpoint</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>SD</td>
<td>Mean</td>
<td>Median</td>
</tr>
</tbody>
</table>
| CRP [mg/l]             | 3.58             | 1.41                    | 5.37                    | 3.20                    | 1.8                     | 4.27 p = 0.790
|                        |                  |                         |                         |                         |                         |
|                        |                  |                         | 0.09 mg/l               |                         |                         |
|                        |                  |                         | [−0.83 to 0.643]        |                         |                         |
| IL6 [pg/ml]            | 3.50             | 1.01                    | 8.49                    | 2.76                    | 1.21                    | 4.82 p = 0.924
|                        |                  |                         |                         |                         |                         |
|                        |                  |                         | 0.03 pg/ml              |                         |                         |
|                        |                  |                         | [−0.63 to 0.697]        |                         |                         |
| BDNF [ng/ml]           | 23.03            | 22.73                   | 7.47                    | 22.15                   | 21.90                   | 6.72 p = 0.468
|                        |                  |                         |                         |                         |                         |
|                        |                  |                         | 1.42 ng/ml              |                         |                         |
|                        |                  |                         | [−2.52 to 1.385]        |                         |                         |
| NAC (N = 58)           |                  |                         |                         |                         |                         |
|                        | Baseline         | Endpoint                | Baseline vs. Endpoint   | Baseline                | Endpoint                |
|                        | Mean             | Median                  | SD                      | Mean                    | Median                  |
| CRP [mg/l]             | 4.10             | 2.50                    | 4.88                    | 3.91                    | 1.75                    | 5.29 p = 0.412
|                        |                  |                         |                         |                         |                         |
|                        |                  |                         | −0.57 mg/l              |                         |                         |
|                        |                  |                         | [−2.24 to 1.068]        |                         |                         |
| IL6 [pg/ml]            | 2.68             | 0.97                    | 8.3                     | 2.77                    | 1.14                    | 6.37 p = 0.524
|                        |                  |                         |                         |                         |                         |
|                        |                  |                         | 0.17 pg/ml              |                         |                         |
|                        |                  |                         | [−0.54 to 0.89]         |                         |                         |
| BDNF [ng/ml]           | 21.74            | 20.78                   | 9.29                    | 24.51                   | 22.51                   | 10.26 p = 0.494
|                        |                  |                         |                         |                         |                         |
|                        |                  |                         | 1.42 ng/ml              |                         |                         |
|                        |                  |                         | [−3.14 to 5.98]         |                         |                         |
| Between group comparison | Model adjusted median | Model adjusted median | Model adjusted median | Model adjusted median | Model adjusted median |
|                        | 0.48             |                         |                         |                         |                         |
|                        |                  |                         | 0.48                    |                         |                         |
|                        |                  |                         | [−1.9999 to 1.03998]    |                         |                         |
|                        | 0.14             |                         |                         |                         |                         |
|                        |                  |                         | 0.769                   |                         |                         |
|                        |                  |                         | [−0.8384 to 1.11845]    |                         |                         |
|                        | 2.19             |                         |                         |                         |                         |
|                        |                  |                         | 0.258                   |                         |                         |
|                        |                  |                         | [−1.6974 to 6.07852]    |                         |                         |

CI, confidence interval
2.4.2 Correlations between serum levels of CRP, IL6 and BDNF and demographic data

Correlations between serum levels of CRP, IL6 and BDNF, and duration of illness, comorbidities and BMI were analysed. However, there were no significant correlations (data not shown).

2.4.3 Potential mediation of clinical efficacy (MADRS) by biological markers (CRP, IL6 and BDNF)

The primary results from this clinical trial (Berk et al. 2014) found that adjunctive NAC treatment had positive effects at washout (week 16) - reflected by changes in MADRS score. This study further evaluated whether levels of CRP, IL6 and BDNF may have had mediator effects on the association between 12 weeks of NAC treatment and MADRS score. In this analysis, two separate time points for MADRS score were included; endpoint (week 12) and washout (week 16), which was four weeks after discontinuation of NAC treatment. Follow-up by intervention interaction impacts for MADRS score and CRP, IL6 and BDNF were assessed separately, including; a) the interaction between NAC intervention and week 12 follow-up and week 16 washout for MADRS score controlling for baseline MADRS score; b) the effects of baseline levels of CRP, IL6 and BDNF on MADRS score outcome from baseline to endpoint (week12 and week16) controlling for MADRS score at baseline; and c) the marginal interaction between baseline levels of CRP, IL6 and BDNF and NAC intervention from baseline to endpoint (week12 and week16) controlling for MADRS score at baseline.

MADRS score was significantly improved in the NAC treatment group at follow-up (week 12) compared to MADRS score at baseline (IFI impact = -4.1997; p = 0.04; 95% CI [-8.214 – -0.185])
and at washout (week 16) (IFI impact = -5.4451; p = 0.002; 95% CI [-8.927 – -1.963], consistent with the findings of the overall trial. The main effects of baseline levels of biological markers on MADRS score outcome from baseline to endpoint (week 12 and 16) were assessed. Lower levels of IL6 at baseline were significantly related to a reduction of MADRS score from baseline to endpoint (week 12 and week 16) (model adjusted mean changes in MADRS score = 0.029; p = 0.042; 95% CI [0.0011 – 0.0588]). However, this effect of levels of IL6 at baseline to predict a reduction of MADRS score was not related to NAC intervention as our analysis indicated that the interaction between NAC intervention and levels of IL6 were not significant. Changes in MADRS score from baseline to endpoint (week 12 and 16) were not associated with either baseline CRP levels (model adjusted mean changes in MADRS score mean = 0.005; p = 0.27; 95%CI [-0.004 – 0.015]) nor baseline BDNF levels (model adjusted mean changes in MADRS score = 0.006; p = 0.67; 95%CI [-0.022 – 0.034]) (Table 2.3-1). We then evaluated the marginal interaction effects between baseline levels of biological markers and NAC intervention from baseline to endpoint (week12 and week16) controlling for MADRS score at baseline. Levels of CRP, IL6 and BDNF did not differ between baseline and endpoint by NAC intervention; levels of CRP (model adjusted mean rank difference between NAC and placebo at endpoint = 0.0003; p = 0.98; 95%CI [-0.019 – 0.019]); levels of IL6 (model adjusted mean rank difference between NAC and placebo at endpoint = 0.013; p = 0.67; 95%CI [-0.046 – 0.072]); and levels of BDNF (model adjusted mean rank between NAC and placebo at endpoint = - 0.017; p = 0.56; 95%CI [-0.075 – 0.040]) (Table 2.3-2).
Table 2.3-1. Effects of baseline serum levels of CRP, IL6 and BDNF on MADRS score from baseline to endpoint (week12 and week16) controlling by MADRS score at baseline

<table>
<thead>
<tr>
<th></th>
<th>Model adjusted mean changes in MADRS</th>
<th>p value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>0.005</td>
<td>0.265</td>
<td>-0.004 - 0.015</td>
</tr>
<tr>
<td>IL6</td>
<td>0.029</td>
<td>0.042</td>
<td>0.001 – 0.059</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.006</td>
<td>0.669</td>
<td>-0.022 – 0.034</td>
</tr>
</tbody>
</table>

* Unstandardized regression beta coefficient on ranked serum levels

Table 2.3-2. Marginal interaction effects of baseline serum levels of CRP, IL6 and BDNF and NAC/placebo treatments from baseline to endpoint (week12 and week16) controlling for MADRS score at baseline

<table>
<thead>
<tr>
<th></th>
<th>Model adjusted mean changes in MADRS</th>
<th>p value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>0.008</td>
<td>0.830</td>
<td>-0.011 - 0.027</td>
</tr>
<tr>
<td>IL6</td>
<td>0.010</td>
<td>0.315</td>
<td>-0.009 – 0.030</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.0001</td>
<td>0.990</td>
<td>-0.018 – 0.018</td>
</tr>
</tbody>
</table>

* Unstandardized regression beta coefficient on ranked serum levels for baseline serum levels and NAC/placebo treatments interaction with NAC group as reference category

Table 2.3-1 and 2.3-2: GEE model was used to explore intervention impact on MADRS score (week12 follow-up and week16 washout), levels of each target (CRP, IL6 and BDNF) at baseline and endpoint. Datasets of CRP, IL6 and BDNF were transformed ranked dataset due to outliers.
2.4.4 Responders and remitters analysis

Median regression models were used to analyse differences in levels of CRP, IL6 and BDNF between responders and non-responders, and between remitters and non-remitters. Responders were defined as participants who had more than or equal to a 50% reduction of MADRS scores from baseline to endpoint. Baseline CRP, IL6 and BDNF levels in responders did not differ from non-responders; CRP: model adjusted median difference at baseline between responders and non-responders = 1.49 mg l\(^{-1}\), \(p=0.27\), 95%CI \([-1.1556 – 4.139]\); IL6: model adjusted median differences at baseline between responders and non-responders = -0.002 pg ml\(^{-1}\), \(p=0.997\), 95%CI \([-1.162 – 1.158]\); and BDNF: model adjusted median differences at baseline between responders and non-responders = -0.92 ng ml\(^{-1}\), \(p= 0.84\), 95%CI \([-10.011 – 8.175]\). For exploratory analysis, levels of CRP, IL6 and BDNF at endpoint in responders and non-responders were also assessed. Endpoint CRP, IL6 and BDNF levels in responders did not differ from non-responders; CRP: model adjusted median differences at endpoint between responders and non-responders = 1.13 mg l\(^{-1}\), \(p=0.28\), 95%CI \([-0.931 – 3.199]\); IL6: model adjusted median differences at endpoint in responders and non-responders = 0.314 pg ml\(^{-1}\), \(p=0.73\), 95%CI \([-1.511 – 2.138]\); and BDNF: model adjusted median differences at endpoint between responders and non-responders =3.435 ng ml\(^{-1}\) \(p=0.48\), 95%CI \([-6.170 – 13.040]\). For remitters analysis, participants in each treatment group were categorised either remitters or non-remitters (final MADRS score <7). Baseline CRP, IL6 and BDNF levels in remitters did not differ from non-remitters; CRP: model adjusted median differences at baseline between remitters and non-remitters = -1.117 mg l\(^{-1}\), \(p=0.56\), 95%CI \([-4.875 – 2.640]\); IL6: model adjusted median differences at baseline between remitters and non-remitters = -0.218 pg ml\(^{-1}\), \(p=0.749\), 95%CI \([-1.567 – 1.131]\); and BDNF: model adjusted median differences at baseline between remitters and non-remitters = 0.898 ng ml\(^{-1}\), \(p= 0.87\), 95%CI \([-10.330 – 12.126]\).
Endpoint CRP, IL6 and BDNF levels in remitters did not differ from non-remitters; CRP: model adjusted median differences at endpoint between remitters and non-remitters = -0.39 mg l\(^{-1}\), \(p=0.82\), 95%CI [-3.854 – 3.070]; IL6: model adjusted median differences at endpoint between remitters and non-remitters = -0.70 pg ml\(^{-1}\), \(p=0.83\), 95%CI [-7.008 – 5.609]; and BDNF: model adjusted median differences at endpoint between remitters and non-remitters = 3.207 ng ml\(^{-1}\), \(p=0.48\), 95%CI [-5.795 – 12.208]. Characteristics of responders and remitters can be found in Table 2.4.
Table 2.4. Responders and Remitters

<table>
<thead>
<tr>
<th>Table 2.4. Responders and Remitters</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>CRP (mg l(^{-1}))</strong></td>
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<tr>
<td>Responders (Baseline)</td>
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<tr>
<td>Responders</td>
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<tr>
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<td>43</td>
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<td>Non-responders</td>
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<td>30</td>
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<td><strong>IL6 (pg ml(^{-1}))</strong></td>
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<tr>
<td><strong>BDNF (ng ml(^{-1}))</strong></td>
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<td>Responders (Baseline)</td>
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2.5 Discussion

This study evaluated the potential mechanisms of action of adjunctive NAC treatment on depressive symptoms. We explored serum levels of CRP, IL6 and BDNF to index the inflammatory and neurotrophic elements of NAC’s actions. Adjunctive NAC treatment improved depressive symptoms which was consistent with the findings from the overall trial and a meta-analysis (Berk et al. 2014). However, our data did not show significant effects of adjunctive NAC on levels of CRP, IL6 or BDNF following twelve weeks of treatment. This study therefore does not provide support for the hypothesis that the operative pathway of NAC in depression is via CRP, IL6 or BDNF. What is unclear is whether this result suggests alternative mechanisms of action via alternate pathways impacting inflammation or neurotrophins, or whether this result is an artefact of assay sensitivity and study power, or whether the effects on depression are via entirely other pathways. In particular, the modest clinical effect sizes may compromise the power of detecting a moderating effect of subtle biomarker changes.

Interestingly, the GEE model indicated that lower baseline IL6 levels were significantly associated with reductions of MADRS scores from baseline to treatment endpoint and washout, but the effect was not related to NAC intervention. These findings imply that lower levels of IL6 at baseline appear to predict a reduction of MADRS scores. A possibility remains that the reduction of MADRS score at follow-up which was associated with baseline levels of IL6 might be attributable to ongoing concomitant medications. Since NAC intervention was adjunctive therapy, our clinical population continued on their ongoing medications. A meta-analysis showed that antidepressants can reduce levels of IL6 (Hannestad, DellaGioia, and Bloch 2011). However, this finding should be interpreted with caution, given that our findings that failed to find differences in levels of IL6 between baseline and endpoint in the placebo
and NAC intervention groups, and that levels of IL6 at baseline did not differ between responders and non-responders.

There also remains the possibility that ongoing medications may work in conjunction with the effects of NAC on these markers. Previous studies have shown that established antidepressant treatments have the capacity to influence inflammatory markers. Yoshimura et al. (2009) reported that selective serotonin reuptake inhibitors (SSRI) and serotonin noradrenaline reuptake inhibitors (SNRIs) were associated with levels of IL6 and BDNF. Eight weeks of SSRIs (paroxetine, sertraline and fluvoxamine) or SNRI (milnacipran) treatment significantly reduced levels of IL6 and increased plasma levels of BDNF in participants who achieved a 50% reduction or more on the Hamilton Rating Scale for depression (HAM-D). Furthermore, higher plasma levels of IL6 were related to responsivity to SSRIs or SNRI treatments. Findings from Yoshimura et al. (2009) indicated that plasma levels of IL6 and BDNF were associated with reduction in depressive symptoms following SSRI or SNRI treatments, and thus the mechanism of action of these drugs may involve both IL6 and BDNF.

Although limited information regarding the cohort characteristics of this study was available, the age (39.6 year compared to average age 49.6 years in our clinical sample) and the sample size were different from our clinical population, raising the possibility that levels of IL6 and BDNF could fluctuate with age. The plasma IL6 concentration of depressed participants at baseline in Yoshimura et al. (2009) was in a range between 2.5-3pg/ml. On the other hand, the mean level of IL6 was 3.11pg/ml and the median level of IL6 was 0.99pg/ml in our clinical population. Considering the highly skewed distribution of IL6 concentration in our study, a comparison of the median of levels IL6 in our cohort to the range of levels of IL6 in Yoshimura et al. (2009) indicates that there is a considerable difference in levels of IL6, which may be age related. In this regard, Saddadi et al. (2014) reported that changes in serum levels of IL6 in
response to NAC treatment in subjects whose age was under 40 years differed from subjects whose age was 40 years or over.

NAC treatment reduced levels of IL6 and CRP in chronic kidney disease patients. Nascimento et al. (2010) showed that eight weeks of NAC treatment (600mg x two times daily) significantly reduced plasma levels of IL6 but not plasma levels of CRP in peritoneal dialysis patients. Three months of NAC treatment, 600mg twice a daily, also reduced serum levels of IL6 and CRP in patients with end-stage renal disease (Saddadi et al. 2014). Although both studies included study populations of relatively small size, demographic characteristics in these study populations and our study were very similar in terms of age and gender. However, the dose of NAC in these studies was slightly higher than our study. Furthermore, levels of IL6 at baseline (pre-treatment) in Nascimento et al. and Saddadi et al. were higher compared with our clinical population. The difference in the capacity of NAC to alter inflammatory markers may therefore reflect baseline IL6 levels, with low levels potentially not amenable to further reduction. There may also be dose effects or differences in the underlying inflammatory burden in these disorders.

For levels of BDNF, our cohort had the mean of 22.42 ng/ml at baseline which is considerably higher compared to a range between 1-1.5ng/ml in Yoshimura et al. (2009). Bus et al. (2012) found that serum levels of BDNF declined with age in females in a community population but levels in males remained stable. The authors also found serum BDNF levels were lower with increased levels of depressive symptoms. This conflicts with the comparisons of our study and Yoshimura et al. as our cohort was older than Yoshimura et al. and the plasma levels of BDNF at baseline in our study appeared to be higher than Yoshimura et al. However, antidepressant use may have contributed to an increase in plasma levels of BDNF (Brunoni, Lopes, and Fregni 2008; Sen, Duman, and Sanacora 2008). Our cohort had a long duration of illness, with a mean
of 14.7 years, and had ongoing standard pharmacotherapy including SSRIs, mood stabilisers, atypical antipsychotics and sedatives, and therefore these medications may contribute to higher levels of BDNF at baseline in this study. There were 51 depressed participants (31 SSRI- or SNRI-responsive; 20 SSRI or SNRI-refractory patients; compared to 30 healthy controls) in Yoshimura et al. (2009) compared to 121 in our cohort. Small sample size also could lead to Type I or Type II errors. Uher et al. (2014) also reported that serum levels of CRP predicted different outcomes following Escitalopram (SSRI) and Nortriptyline (SNRI) treatment. Our clinical population appeared to be older and BMI scores appeared to be higher compared to the Uher et al. (2014) study population. These differences could be confounding factors that contribute to the finding that serum levels of CRP in Uher et al. (2014) were lower than in our population. In our clinical population, mean CRP level was 3.83mg/L and median CRP level was 2.03mg/L, while Uher et al. (2014) reported mean CRP level was 1.30-1.66 mg/L. Apart from differences in demographic characteristics between studies, a notable difference was that our study was using NAC as an adjunctive therapy. Therefore, there is a possibility that the mechanism of action of NAC may interact with current medications, which may affect levels of CRP, IL6 and BDNF. While levels of CRP, IL6 and BDNF might be modulated by current antidepressants, these markers may not indicate the efficacy of NAC treatment.

SSRIs reduce the production of Th2 type cytokines such as IL6 and regulate cellular immune response (Martino et al. 2012), while SNRIs suppress Th1 type cytokines such as TNFα and modulate humoral balance (Martino et al. 2012). On the other hand, the mechanism of action of NAC differs distinctively from simply suppressing production of Th1 and Th2 cytokines. NAC is also a potent antioxidant and the therapeutic capacity of NAC to restore mitochondrial dysfunction has been well documented in preclinical studies (Wright et al. 2015; Sandhir et al. 2012; Guo et al. 2015; Sharma, Kaur, and Singla 2015). Mitochondrial dysfunction could
mediate increases in production of reactive oxidative and nitrosative species, and subsequently contribute to increases in production of pro-inflammatory cytokines, DNA damage and lipid peroxidation (Morris and Berk 2015; Dean, Giorlando, and Berk 2011; Guo et al. 2015).

NAC also has the capacity to modulate glutamate flux in the CNS, a feature with particular relevance to depression considering the accumulating evidence for the antidepressant effects of glutamatergic agents such as ketamine (Krzyzanowska et al. 2016). NAC has effects on glutamate cysteine exchange as well as on the glutamate transporter GLUT1 (Roberts-Wolfe and Kalivas 2015). Lastly, there is preclinical evidence that the glutamate AMPA receptor is involved in the antidepressant effects of NAC (Linck et al. 2012). The glutamate pathway merits closer scrutiny as a major operative pathway.

We also assessed whether levels of CRP, IL6 and BDNF at baseline predicted the response to adjunctive NAC treatment, through responders and remitters analyses. Neither baseline levels of IL6, CRP and BDNF nor endpoint levels of IL6, CRP and BDNF were associated with more than or equal to 50% of reduction of MADRS score or remission at follow-up. Our findings consistently indicated that levels of CRP, IL6 and BDNF were not related to adjunctive NAC treatment outcome nor the severity of depressive symptoms.

The results from multilevel mixed-effects linear regression models were consistent with the findings that adjunctive NAC treatment had a positive effect on the improvement of MADRS score at treatment endpoint and washout (Berk et al. 2014). However, this effect was independent from levels of CRP, IL6 and BDNF. The greater the clinical effect size, the greater the statistical power to detect an operative mechanism - that the trials primary effect was modest compromises this capacity. Our findings from a comprehensive series of analyses
indicated that CRP, IL6 and BDNF may not intersect with the pathways by which NAC exerted therapeutic effects on depressive symptoms in this population. Therefore, solely relying on levels of inflammatory markers to measure effectiveness of NAC may not be reflective of the mechanism of action of NAC. A combination of a wide range of markers including indicators of oxidative stress and glutamatergic function could provide a more comprehensive view of the efficacy of NAC and also the underlying pathophysiology of depression. Another limitation in this study was the number of missing samples at endpoint. We used multiple imputations to overcome this limitation, however, this could affect our results. Imputed data were based on values from other participants, which may not necessarily be fully representative of the missing data.

2.6 Conclusion

In summary, three months of adjunctive NAC treatment improved depressive symptoms in patients with major depression. Lower levels of IL6 at baseline were associated with a greater reduction of MADRS score regardless of NAC intervention. However, the finding needs to be interpreted cautiously as it contradicts other findings in this study that failed to find differences in serum levels of IL6 at baseline between responders and non-responders and levels of IL6 did not differ between baseline and endpoint in both placebo and treatment groups. Overall, our results failed to provide support for an association between serum levels of CRP, IL6 and BDNF, and changes in depressive symptoms in our clinical sample, and suggest that alternate mechanisms including mitochondrial energy generation and glutamate need to be examined as potential mechanisms of action.
Chapter 3

Effects of high fat diet on behaviour and therapeutic effects of N-acetylcysteine
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3.1 Introduction

N-acetylcysteine (NAC) is a potent anti-oxidant which provides cysteine for synthesis of the most ubiquitous anti-oxidant in the brain, glutathione (GSH) (Dean, Giorlando, and Berk 2011) with intrinsic anti-inflammatory effects (Saddadi et al. 2014). In the context of major depressive disorder (MDD), adjunctive NAC treatment resulted in positive effects on depressive symptoms in MDD (Berk et al. 2014), and elicited a significant improvement of depressive episodes in bipolar disorder (Berk et al. 2008). A recent meta-analysis investigating the therapeutic efficacy of NAC on depressive symptoms concluded NAC treatment significantly improved depressive symptoms measured by the Montgomery-Asberg depression Rating Scale (MADRS) and the Hamilton Depression Rating Scale (HAM-D) (Fernandes et al. 2016). These findings indicate that NAC shows considerable promise as an antidepressant. While the mechanisms of current antidepressants mainly focus on regulating the availability of neurotransmitters at synapses (Ferreira et al. 2008), NAC might regulate neuronal function via its potent anti-oxidant efficacy and regulation of the inflammatory state.

What remains unclear is the underlying mechanisms of action of NAC that conferred the improvement in depressive symptoms.

One potential mechanism of action of NAC is through its direct effects on glutamate neurotransmission (Gipson 2016). In preclinical studies, NAC treatment showed antidepressant like effects on depressive-like behaviour; behavioural despair in rats (Ferreira et al. 2008), in an animal model of Huntington’s disease in mice (Wright et al. 2016), and in the tail suspension test in mice (Linck et al. 2012). These results showed reversal of depressive-like behaviour was associated with regulation of the glutamatergic system through modulating the glial glutamate transporter (GLT-1) and AMPA receptor elicited by NAC. Other
studies showed NAC induced a reduction in the levels of oxidative stress-mediated depressive-like behaviour (Smaga et al. 2012) and anhedonic behaviour (Arent et al. 2012). Although these preclinical studies suggest that anti-depressant like effects of NAC potentially involve regulating the glutamate system and oxidative biology, we still do not have a complete picture regarding which pathways are involved in the therapeutic effects of NAC. Such evidence is crucial in clinical settings to optimise the therapeutic effects of NAC. Therefore, this study investigated underlying pathways that may indicate NAC therapeutic effects in MDD using high fat diet feeding to induce depressive-like behaviour in mice.

3.2 Chronic high fat diet feeding to induce depressive-like behaviour

Accumulating evidence clearly suggests that poor diet consumption often precedes the onset of depressive mood (Akbaraly et al. 2009; Rienks, Dobson, and Mishra 2013; Sanchez-Villegas et al. 2009; Sanchez-Villegas et al. 2012). Although the mechanisms underpinning how diet mediates depressive mood are still poorly understood, currently available evidence implies that diet, gut microbiome and inflammation may interact to mediate effects on mood. Epidemiological literature indicates a strong association between the consumption of diets high in fat, such as a western style diet, and an increased risk of MDD (Jacka et al. 2014; Rahe et al. 2015; Yu, Parker, and Dummer 2014).

Poor dietary patterns such as a high fat diet also alter the structure and function of the gut microbial communities via changing the balance of two dominant bacteria, Firmicutes and Bacteroidetes (Brown et al. 2012; Zhang et al. 2010). In addition, several lines of studies reported that high fat diet feeding mediated elevations in the levels of a pro-inflammatory cytokine IL-6 and an endotoxin, lipopolysaccharide (LPS), via potential microbial signalling.
from the gut microbiota (Cani et al. 2007; Cani and Delzenne 2010; Cani et al. 2009; Ghanim et al. 2010; Laugerette et al. 2011).

Therefore, we used chronic high fat diet feeding to induce depressive-like behaviour in mice in this study. Currently, there is a paucity of comprehensive investigations of the impact of high fat diet feeding on pathways linking central and peripheral inflammatory states, gut microbiota and depressive mood, and the findings from literature (summarised in Table 3.1) are far from conclusive. Using the high fat diet feeding paradigm, we investigated effects of high fat diet on depressive mood that encompasses the currently proposed pathophysiology of MDD, including central and peripheral inflammatory states, oxidative biology, blood brain barrier (BBB) integrity, the glutamatergic system and gut microbiota. To elaborate how these factors interact and predispose to depressive mood, it is essential to use an animal model that minimises confounding factors such as diets, genetic backgrounds and living environment, and that enables us to conduct post mortem analysis. Hence, findings from our study shed light on the gap in our current knowledge of MDD.

Furthermore, given that NAC has the potential to regulate the immune and inflammatory responses, oxidative biology and the glutamatergic neurotransmission in the brain, it is therefore of interest to examine whether NAC could modulate diet-induced behaviour, and by what mechanisms. In addition, effects of NAC on the gut microbiota are largely unknown. Hence, the investigations of effects of NAC on high fat diet mediated immune profile, the gut microbial community, markers of oxidative stress and the glutamatergic system significantly contribute to an enhancement of our understanding of the mechanisms of action of NAC in MDD.
### Table 3.1 Summary of preclinical studies examining diet-induced depressive-like behaviour

<table>
<thead>
<tr>
<th>Study</th>
<th>Diet</th>
<th>Duration</th>
<th>Findings related to depressive- and anxiety-related behaviour and inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pistell et al., 2010</td>
<td>Western diet (41% kcal from fat)</td>
<td>21 weeks</td>
<td>↑ GFAP protein levels and — Iba1 protein levels in the cortex; — Levels of IL-6, TNFα and MCP1 in the cortex; — cognitive function measured by T-maze</td>
</tr>
<tr>
<td></td>
<td>High fat diet (60% kcal from fat)</td>
<td>21 weeks</td>
<td>↑ levels of IL-6, TNFα and MCP1 in the mouse cortex; ↑ GFAP and Iba1 protein levels in the cortex; ↓ cognitive function by measured T-maze</td>
</tr>
<tr>
<td>Andre et al., 2014</td>
<td>Western diet (49% kcal from fat)</td>
<td>20 weeks</td>
<td>↓ cognitive function measured by Y maze; ↑ anxiety-like behaviour in elevated plus maze; — depressive-like behaviour in TST; — plasma concentration of IL-6, TNFα, IL-1β and LPS; — levels of IL-6, TNFα, IL-1β and INF gamma in the hippocampus and hypothalamus</td>
</tr>
<tr>
<td>Takase et al., 2016*</td>
<td>High fat diet (45% kcal from fat)</td>
<td>24 weeks</td>
<td>↑ anhedonic behaviour in SPT; — duration of immobility in FST; — anxiety-like behaviour in light-dark box and OFT</td>
</tr>
<tr>
<td>Del Rosario et al., 2012*</td>
<td>High fat diet (45% kcal from fat)</td>
<td>8 weeks</td>
<td>↑ anxiety-like behaviour in OFT; — depressive-like behaviour in FST</td>
</tr>
<tr>
<td>Pyndt Jørgensen et al.,</td>
<td>High fat/no sucrose diet (60% kcal from fat)</td>
<td>13 weeks</td>
<td>— plasma concentrations of IL-1β, IL-6 and TNFα; — levels of IL-1β, IL-6 and TNFα in the prefrontal cortex and hippocampus; — anhedonic behaviour in SPT; — depressive-like behaviour in FST</td>
</tr>
</tbody>
</table>

*Studies which did not include examinations of inflammatory mediators

↑ increased; ↓ decreased; — unchanged; FST, forced swim test; SPT, sucrose preference test; OFT, open field test; TST, tail suspension test; GFAP, Glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; IL-6, Interleukin 6; TNFα, tumor necrosis factor α; IFN gamma, interferon gamma; MCP1, monocyte chemoattractant protein 1; LPS, lipopolysaccharide.
<table>
<thead>
<tr>
<th>Study</th>
<th>Diet Description</th>
<th>Duration</th>
<th>Outcome Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del Rio et al., 2016*</td>
<td>High fat diet (45% kcal from fat)</td>
<td>8 weeks</td>
<td>↑ depressive-like behaviour in FST; ↓ anxiety-like behaviour measured in OFT and elevated plus maze</td>
</tr>
<tr>
<td>Yamada et al., 2011*</td>
<td>High fat diet (60% kcal from fat)</td>
<td>16 weeks</td>
<td>↑ depressive-like behaviour in FST however, ↓ depressive-like behaviour in pre-test in FST; ↑ anhedonic behaviour in SPT</td>
</tr>
<tr>
<td>Sharma &amp; Fulton, 2013*</td>
<td>High fat diet (58% kcal from fat)</td>
<td>12 weeks</td>
<td>↑ anxiety-like behaviour in elevated plus maze and OFT; ↑ depressive-like behaviour in FST</td>
</tr>
<tr>
<td>Krishna et al., 2015*</td>
<td>High fat diet (60% kcal from fat)</td>
<td>12 weeks</td>
<td>↑ locomotor activity in OFT; ↓ depressive-like behaviour in FST</td>
</tr>
<tr>
<td>Krishna et al., 2016</td>
<td>High fat diet (60% kcal from fat)</td>
<td>32 weeks</td>
<td>↑ anxiety-like behaviour in OFT; ↓ depressive-like behaviour in FST; ↑ Levels of IL-6 gene expression in the liver at earlier (at 6 weeks), however the elevation did not found at 32 weeks.</td>
</tr>
</tbody>
</table>

*Studies which did not include examinations of inflammatory mediators

↑ increased; ↓ decreased; — unchanged; FST, forced swim test; SPT, sucrose preference test; OFT, open field test; TST, tail suspension test; GFAP, Glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; IL-6, Interleukin 6; TNFα, tumor necrosis factor α; IFN gamma, interferon gamma; MCP1, monocyte chemoattractant protein 1; LPS, lipopolysaccharide.
3.3 Aims

This study aimed to identify depressive-like behaviours arising from high fat diet consumption. We then assessed high fat diet induced alteration in the intestinal gut microbiome. In parallel with this, inflammatory and microbial signalling pathways in the periphery and the brain were examined to determine whether the changes in the gut microbiota may have an association with depressive-like behaviour. In addition, the capacity of NAC, a novel antidepressant with potent anti-oxidant properties, to reverse behaviour, the gut microbiome and inflammatory signalling was evaluated.
3.4 Materials and methods

This experiment and the experiment described in Chapter 4 shared the same control groups, standard diet and vehicle group (SD/Veh) and high fat diet and vehicle group (HFD/Veh). Four separate cohorts of animals were used, with each cohort including minocycline (MINO) and N-acetylcysteine (NAC) treatment groups. There were no variations by cohort in any of the main experimental outcomes, so the data was pooled across cohorts. As the results of our investigation into the two agents are in the process of being prepared for publication as separate papers, they are presented as separate chapters in this thesis. However, the outcomes of the experiment as a whole and the contrasts between the two agents will be discussed in Chapter 5.

3.4.1 Animals and diet

All experiments were performed in accordance with the guidelines of Deakin University Animal Ethics Committee and the National Health and Medical Research Council. Four-week old male C57BL/6J mice were purchased from Animal Resources Centre (Perth, Western Australia, Australia). Animals were group housed (four per cage) and maintained on a 12h light/dark cycle (7am – 7pm) and temperature (20-22 °C) with access to food and water ad libitum. After two weeks of acclimatisation, animals were assigned into two groups: high fat diet (HFD; n = 76), and standard chow (SD; n=76), and were maintained on each respective diet until the end of the study. Groups were matched at this baseline time point across body weight, fat mass and lean mass measured by EchoMRI. The SD group was fed with standard chow (BARASTOC Rat and Mouse Cubes, Ridley AgriProducts, Victoria, Australia) that contains 5% kcal of fat and 20% kcal of protein. The HFD group was fed with high fat diet (SF04-001,
Speciality feeds, Western Australia, Australia) that contains 45% kcal of fat (45% of available energy in the form of fat) and 20% kcal of protein. Detailed dietary compositions of standard chow and high fat diet are shown in Table 3.2. Body weight was measured once per week throughout of the study. Mice were 24 weeks old by the time of behavioural assessments.

Table 3.2. Nutritional content of standard chow and high fat diets

<table>
<thead>
<tr>
<th></th>
<th>Standard chow diet Barastoc, Mice and Rodent cube</th>
<th>High fat diet Specialty Feeds SF04-001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>20.0%</td>
<td>22.6%</td>
</tr>
<tr>
<td>Total Fat</td>
<td>5.0%</td>
<td>23.5%</td>
</tr>
<tr>
<td>Crude Fibre</td>
<td>5.0%</td>
<td>5.4%</td>
</tr>
<tr>
<td>Digestible Energy</td>
<td>12.6MJ/Kg</td>
<td>19MJ/Kg</td>
</tr>
</tbody>
</table>

3.4.2 Experimental design

The Experimental design used for this study is shown in Figure 3.1.

![Figure 3.1. Schematic diagram of experimental design.](image)
3.4.3 Drugs

N-acetylcysteine (NAC) (Astral scientific, Taren Point, NSW, Australia) was prepared in 0.9% saline at a concentration of 100mg/ml for dosing 500mg/kg of high dose of NAC (HNAC) and at a concentration of 50mg/ml for dosing 250mg/kg of low dose of NAC (LNAC) per day. Dosage volume was determined every day by measuring body weight. Solutions were pH-matched to that of the vehicle (saline; dosage 200ul). LNAC, HNAC and saline were orally gavaged daily for a 4-week period, which corresponded to weeks 14 – 17 after the assigning of HF and SD groups.

3.4.4 Behavioural testing

All behavioural testing was conducted between 10:00am and 14:00pm and was recorded and analysed using Noldus EthoVision XT8 (Noldus Information Technology, The Netherlands).

3.4.4.1 Open field test

The open field test (OFT) was used to measure locomotion and exploratory behaviour in a novel environment. The apparatus comprised a clear plexiglass box (50cm x 50cm, with 36 cm high walls). The light intensity was low in the examination room and there was no difference in the light intensity between the centre and the outer zones. Mice were placed in the corner of the area, and allowed to move freely for 6 min and filmed from above. Time spent in the centre, travel distance and mobility were analysed using a tracking system (Ethovision XT 8.5, Noldus).
3.4.4.2 Porsolt forced swim test

The Porsolt forced swim test (FST) displays predictive validity for testing the efficacy of antidepressants, and may measure ‘behavioural despair’ which is considered a depressive-like symptom (Krishnan and Nestler 2011). Mice were placed individually in a glass cylinder (diameter: 18.5 cm; height: 26.5 cm) filled with 25°C±1°C water to a height of 13.5 cm for 6 min. Mice were filmed from the side, and the duration of immobility and latency to first immobility were scored manually by a blinded experimenter, where immobility (i.e. the Porsolt posture) was defined as immobility in all four limbs.

3.4.4.3 Sucrose preference test

The sucrose preference test (SPT) was used to measure anhedonic behaviour (Krishnan and Nestler 2011). Mice were housed 2 per cage due to compliance with the guidelines of Deakin University Animal Ethics Committee, and were given free access to two water bottles, one with tap water and another with 1% sucrose solution. These bottles were set at 5pm and removed at 9am the following day. At the removal of bottles, consumptions of sucrose water and tap water were measured by a blinded experimenter.

3.4.5 16S metagenomics sequencing

Faecal samples were collected during the final week of behavioural testing. DNA was isolated using QIAamp DNA stool kits (Qiagen Pty Ltd, Victoria, Australia), modified to include a bead-beating step. The extracted DNA was quantified and purity was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Sequencing was performed at Macrogen Inc (Seoul, South Korea). The extracted DNA was prepared for
sequencing on the Illumina MiSeq platform using the Illumina 16S Metagenomic Sequencing Library Preparation protocol. DNA was amplified using 16S Amplicon primers selected from Klindworth et al. (2013). The amplicon primers were targeted at the 16S V3 and V4 region. Forward Primer was 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGGCAG. Reverse Primer was 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTACHVGGGTATCTAATCC. The PCR product was purified of free primers and primer dimer species using AMPure XP beads. The amplicons were indexed using the Illumina Nextera XT Index kit, and purified again using AMPure XP beads. The amplicons were quantified by a fluorometric quantification method that uses dsDNA binding dyes.

3.4.6 Bioinformatics

Unprocessed reads were submitted to the NCBI SRA archive (Bioproject PRJNAXXXX). The Uparse Illumina paired read pipeline was used for 16S amplicon analysis (Edgar 2013) with a 3% cluster radius. Reads were filtered, prior to Uparse, to a minimum length of 300 bp. The RDP 16S database v16 (http://drive5.com/utax/data/rdp_v16.tar.gz) was used to taxonomically classify operational taxonomic units (OTUs) in Uparse with a 90% confidence threshold. OTUs with abundance less than 0.5% of the total number of reads or occurrence of less than two across all samples were removed from the analysis. QIIME 1.8 (Caporaso et al. 2010) was used to calculate diversity metrics and statistically compare alpha diversity (on rarefied data) and OTU abundance between species. OTU abundance was cumulative sum scaling (CSS) normalised prior to statistical tests (Paulson et al. 2013). All commands and associated python scripts in the analysis are provided in the GitHub repository: https://github.com/bioinformatics-deakin/Hasebe_and_Allnutt_et.al_2017. Principal
coordinates analysis (PCoA) was performed on weighted UniFrac distances (Lozupone and Knight 2005) calculated from a maximum likelihood tree (FastTree v2.3.1) (Price, Dehal, and Arkin 2010) of the filtered OTUs on raw counts and CSS normalised counts. Beta diversity was analysed by Analysis of Molecular Variance (AMOVA) (Excoffier, Smouse, and Quattro 1992).

3.4.7 Analyses of inflammatory mediators

Plasma levels of inflammatory mediators in SD/Veh, SD/HNAC, HFD/Veh and HFD/HNAC groups were assessed using commercially available ELISA kits: lipopolysaccharide binding protein, LBP (Boster Bio, mouse LBP ELISA kit), soluble CD14, IL6 and TNFα (R&D systems, Quantikine ELISA kits).

3.4.8 Real time quantitative qPCR analysis

Mice were humanely killed by cervical dislocation, the brain removed, and the hypothalamus and bilateral whole hippocampal formations dissected. Hypothalamic and hippocampal tissues were snap frozen in liquid nitrogen and stored at -80 °C for later use. The brain tissues were homogenised and processed for total RNA extraction using RNeasy mini Kit (Qiagen Pty Ltd, Victoria, USA). RNA was quantified and purity assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Complimentary strand DNA was synthesised using Dynamo cDNA synthesis kit (Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (qPCR) was performed using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for ionized calcium-binding adapter molecule 1 (Iba1) (Mm00479862_g1), Major histocompatibility complex class II molecules (MHC II) (Mm658576_m1), cluster of differentiation molecule 11b (CD11b)
(Mm00434455_m1), *Glial fibrillary acidic protein (gfap)* (Mm01253033_m1), *DNA-damage-inducible transcript 4 protein (Ddit4)* (Mm00512503_g1), *Glutamate aspartate transporter (GLAST)* (Mm00600697_m1), *Excitatory amino acid transporter (GLT1)* (Mm00441457_m1), *Cysteine-glutamate antiporter (xCT)* (Mm00442530_m1), *inducible Nitric oxide synthase (iNOS)* (Mm00440502_m1), *Occludin* (Mm00500912_m1) and 18S rRNA (Mm03928990_g1). Each sample was amplified in triplicate. Relative quantification was calculated by the ΔΔCt method and compared with endogenous levels of 18S rRNA.

3.4.9 Statistical analyses

All behavioural and biochemical data were analysed using Prism Graph Pad (GraphPad Software, Inc. La Jolla, CA, USA). Mean ± standard errors of measurements are displayed. Normality of each dataset was assessed by Kolmogorov-Smirnov test. Two-way ANOVA was conducted to examine main effects of diet and treatment and interaction effects of diet and treatment, followed by Bonferroni post hoc comparisons to determine between and within group effects. For alpha diversity and F/B ratio, Kruskal-Wallis test followed by Dunn’s multiple test was used to determine statistical significance. A weighted distance-based analysis of molecular variance (AMOVA) was used to assess spatial distance of each treatment group observed based on the weighted UniFrac distances in each treatment groups in principal coordinate analysis (PCoA). For gene expression analysis, differences in levels of target genes in HFD/Veh and SD/Veh groups were assessed by unpaired t tests. Statistical significance was set for all analyses at $p < 0.05$. 
3.5 Results
3.5.1 High fat diet induced significant body weight and fat mass gain

HFD fed mice weighed significantly more than SD controls at the end of the study, and this effect was roughly equivalent within both vehicle and NAC treated mice (main effect of diet, $F_{(1,85)} = 183.6, p < 0.0001$; main effect of drug treatment, $F_{(2,85)} = 0.65, p > 0.05$; diet x drug treatment interaction, $F_{(2,85)} = 1.72, p > 0.05$; Figure 3.2).

Figure 3.2 Body weight

Figure 3.2 Body weight after long term high fat diet feeding followed by NAC treatment. Two-way ANOVA indicated a main effect of diet, a significantly higher body weight in HFD groups compared to SD groups ($p < 0.0001$). There was no main effect of treatment and no interaction effect between treatment and diet. Data are mean ± s.e.m. Veh = vehicle group; LNAC = low dose NAC (250mg/kg); HNAC = high dose NAC (500mg/kg) **** $P < 0.0001$ (n=17-22)
HFD induced a similar increase in fat mass (main effect of diet, $F_{(1,85)} = 328.1$, $p < 0.0001$; diet x drug treatment interaction, $F_{(2,85)} = 0.98$, $p > 0.05$; Figure 3.3), which was also unaffected by LNAC and HNAC treatment (main effect of drug treatment, $F_{(2,85)} = 1.60$, $p > 0.05$).

Figure 3.3 Fat mass

![Fat mass graph](image)

**Figure 3.3 Fat mass after long term high fat diet feeding followed by NAC treatment.** Two-way ANOVA indicated a main effect diet, a significant fat mass gain in HFD groups compared to SD groups ($p < 0.0001$). Data are mean ± s.e.m. Veh = vehicle group; LNAC = low dose NAC (250mg/kg); HNAC = high dose NAC (500mg/kg) **** $P <0.0001$ (n=17-22)
NAC treatment and HFD had an interaction effect on an increase in lean mass (diet x drug treatment interaction, $F_{(2,85)} = 3.98$, $p < 0.05$; Figure 3.4), which was not affected by NAC treatment (main effect of drug treatment, $F_{(2,85)} = 2.89$, $p > 0.05$) nor diet (main effect of drug treatment, $F_{(2,85)} = 0.44$, $p > 0.05$). Lean mass in HNAC treated HFD mice was significantly higher compared to HF/Veh mice ($t = 2.81$, $p < 0.05$) and HF/LNAC mice ($t = 3.27$, $p < 0.01$).

Figure 3.4 Lean mass

![Figure 3.4 Lean mass](image)

**Figure 3.4 Lean mass after long term high fat diet feeding followed by NAC treatment.** Diet nor NAC treatment did not affect lean mass, however Two-way ANOVA indicated NAC treatment and HFD had an interaction effect on lean mass ($p < 0.05$). Data are mean ± s.e.m. Veh = vehicle group; LNAC = low dose NAC (250mg/kg); HNAC = high dose NAC (500mg/kg); *$p < 0.05$; **$p < 0.01$ (n=17-22)
3.5.2 High fat diet mediated depressive-like behaviours

HFD fed mice spent significantly more time immobile in the FST (main effect of diet, $F_{(1, 109)} = 20.94, \ p < 0.0001$), compared to SD mice (Figure 3.5). This effect was relatively consistent in both vehicle and NAC treated mice (diet x drug treatment interaction, $F_{(2, 109)} = 0.51, \ p > 0.05$), however post hoc comparisons detected significant differences between diet groups within vehicle treated mice ($t = 3.0, \ p < 0.01$) and within HNAC treated mice ($t = 3.16, \ p < 0.01$). LNAC and HNAC treatment did not alter the time spent immobile (main effect of drug treatment, $F_{(2, 109)} = 1.63, \ p > 0.05$).

**Figure 3.5 Forced swim test: Duration of immobility**

**Figure 3.5 Duration of immobility in the Porsolt forced swim test.** Two-way ANOVA indicated a main effect of diet ($p < 0.001$). HF groups displayed significantly longer duration of immobility during FST compared to SD groups. Post hoc comparison indicated Veh/HFD and HNAC/HFD groups had significantly longer duration of immobility compared with the Veh/SD and HNAC/SD groups, respectively ($p < 0.01$). Data are mean ± s.e.m. Veh = vehicle group; LNAC = low dose NAC (250mg/kg); HNAC = high dose NAC (500mg/kg); ** $p < 0.01$; n = 18-22.
Because increased immobility might conceivably result from increased fat mass (rather than diet-induced neurological/behavioural changes), a correlation analysis between fat mass and the duration of immobility was performed. However, a linear regression model indicated that fat mass was not significantly associated with the time spent immobile in HF fed mice ($F_{(1, 55)} = 0.09$, $p > 0.05$; Figure 3.6). HFD and NAC treatment did not affect the latency to the first period of immobility (main effect of diet, $F_{(2, 106)} = 0.11$, $p > 0.05$; main effect of treatment $F_{(2, 106)} = 0.86$, $p > 0.05$; diet x drug interaction, $F_{(2, 106)} = 2.43$, $p > 0.05$; Figure 3.7).

Figure 3.6 Correlation between fat mass and duration of immobility

Figure 3.6 Correlation between fat mass and duration of immobility. Fat mass was not associated with duration of immobility. Linear regression model indicated that fat mass in HFD mice did not predict duration of immobility $F_{(1,38)} = 0.1079$, $p > 0.05$, $R$ square $= 0.0016$, $n = 17-22$. 
Figure 3.7 Forced swim test: Latency to first period of immobility

Diet and NAC treatment did not affect latency to first period of immobility (Two-way ANOVA), n = 17-22.

Figure 3.7 Latency to first period of immobility in Porsolt forced swim test. Diet and NAC treatment did not affect latency to first period of immobility (Two-way ANOVA), n = 17-22.
3.5.3 High fat diet reduced consumption of sucrose solution

HFD mice consumed significantly less sucrose solution in the SPT compared to SD mice (main effect of diet, $F_{(1,51)} = 17.07, p < 0.0001$; Post hoc pairwise comparisons, within vehicle treated mice, $t = 2.89, p < 0.05$; Figure 3.8). NAC treatment did not affect sucrose consumption (main effect of drug treatment, $F_{(2,51)} = 0.99, p > 0.05$; diet x treatment interaction, $F_{(2,51)} = 0.08, p > 0.05$).

Figure 3.8 Sucrose preference test: sucrose consumption

![Sucrose consumption graph](image)

**Figure 3.8 Sucrose consumption overnight.** HF diet had a main effect on sucrose consumption overnight (Two-way ANOVA, $p<0.001$). Veh/HFD group consumed significantly less sucrose solution (1%) compared to Veh/SD group. Animals were housed in pairs while testing in accordance with animal ethics guidelines. Data are mean ± s.e.m. * $p < 0.05$ (n of pairs =8-11; n = 17-22).
3.5.4 High fat diet and NAC altered locomotor activity

NAC treatment significantly increased the time spent in the centre zone of the OFT (main effect of drug treatment, \( F_{(2,109)} = 3.56, p < 0.05 \); Figure 3.9). In contrast, diet did not affect the time spent in the centre region (main effect of diet, \( F_{(1,109)} = 3.17, p > 0.05 \); diet x drug treatment interaction, \( F_{(2,109)} = 0.02, p > 0.05 \)).

Figure 3.9 Open field test: Time spent in centre

![Figure 3.9 Time spent in the centre in the open field test. NAC treatment significantly affected time spent in centre (Two-way ANOVA, \( p < 0.05 \)), although post-hoc comparisons were not significant. Data are mean ± s.e.m. n=17-22.](image-url)
HFD mice travelled significantly less distance compared to SD controls in the OFT (main effect of diet, $F_{(1,109)} = 17.70, p < 0.0001$; Figure 3.10). This effect was similar within both vehicle and NAC treatment groups (diet x drug treatment interaction, $F_{(2,109)} = 0.07, p > 0.05$). Post hoc comparison detected, within vehicle treated and HNAC treated groups, HFD fed mice travelled significantly less distance compared to SD/Veh mice ($t = 2.80, p < 0.05$) and SD/HNAC mice ($t = 2.46, p < 0.05$). NAC treatment did not affect the distance travelled (main effect of drug treatment, $F_{(2,109)} = 0.19, p > 0.05$), suggesting that the increased time that NAC treated mice spent in the centre region (see above) was not simply due to hyperactivity. Although fat mass can potentially alter patterns of locomotor activity, correlation analysis using a linear regression model indicated that travel distance and fat mass were not associated in HFD mice ($F_{(1,55)} = 1.29, p > 0.05$; Figure 3.11).

**Figure 3.10** Open field test: travel distance

![Graph showing travel distance in the open field test](image)

**Figure 3.10** **Travel distance in the open field test.** HFD significantly affected travel distance (Two-way ANOVA, $p<0.01$). HFD/Veh group travelled significantly less distance compared to SD/Veh group. Data are mean ± s.e.m. * $p<0.05$. $n = 17-22$.  

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Figure 3.11 Correlation between travel distance and fat mass. Travel distance during the open field test was not associated with fat mass in HFD groups. Linear regression model indicated that fat mass in HFD mice did not predict travel distance, $F_{(1,55)} = 1.29$, $p > 0.05$, $R^2 = 0.023$, $p > 0.05$, $n = 17-22$. 
Duration of mobility was defined as the amount of time that the animal was moving during the OFT. HFD mice were significantly less mobile compared to SD controls overall (main effect of diet, $F_{(1,109)} = 39.11, p < 0.0001$; Figure 3.12), and within each drug treatment group (post hoc pairwise comparisons, within vehicle treated mice, $t = 3.73, p < 0.001$; within LNAC treated mice, $t = 3.46, p < 0.01$; within HNAC treated mice, $t = 3.67, p < 0.01$). NAC treatment did not affect duration of mobility (main effect of treatment, $F_{(2,109)} = 0.13, p > 0.05$; diet x drug interaction, $F_{(2,109)} = 0.03, p > 0.05$).

**Figure 3.12 Open field test: Duration spent mobile**

![Duration of time spent mobile in the open field test](image)

**Figure 3.12 Duration of time spent mobile in the open field test.** Diet significantly affected mobility during the OFT (Two-way ANOVA, $p<0.0001$). SD/Veh, SD/LNAC and SD/HNAC groups were more active compared to HFD/Veh, HFD/LNAC and HFD/HNAC groups, respectively in the field. Data are mean ± s.e.m. ** $p<0.01$, *** $p<0.001$ n = 17-22.
Interestingly, HFD mice moved significantly faster compared to SD mice control overall (main effect of diet, $F_{(1,109)} = 28.59, p < 0.0001$; Figure 3.13), and within each drug treatment group (post hoc pairwise comparisons, within vehicle treated mice, $t = 2.51, p < 0.05$; within LNAC treated mice, $t = 3.62, p < 0.01$; within HNAC treated mice, $t = 3.10, p < 0.01$), indicating that HFD mice were faster than SD mice while they were moving, despite the reduced duration of mobility and distance travelled. NAC treatment did not affect speed (main effect of treatment, $F_{(2,109)} = 0.25, p > 0.05$; diet x drug interaction, $F_{(2,109)} = 0.52, p > 0.05$). Interestingly, a linear regression model showed significant but weak association between speed and fat mass within HFD fed mice ($F_{(1,55)} = 6.25, p < 0.05$; Figure 3.14).

Figure 3.13 Open field test: Speed

![Figure 3.13 Moving speed during the open field test. HFD significantly affected moving speed during the open field test (Two-way ANOVA, $p < 0.0001$). HFD groups were faster than SD groups overall. Data are mean ± s.e.m. * $p<0.05$, *** $p<0.001$, n = 17-22.](image)
Figure 3.14 Correlation between speed and fat mass. Moving speed during the open field test was significantly associated with fat mass in HFD groups. Linear regression model indicated that an increase in fat mass in HFD mice predicts an increase in speed, $F_{(1,55)} = 6.25$, $p < 0.05$, $R^2 = 0.042$, $n = 17-22$. 
3.5.5 High fat diet and NAC mediated alteration in gut microbiota

Examination of our 16S sequencing data revealed that the read length was 100 base pairs on average instead of the expected 300 base pairs. This step of the process was outsourced to a commercial company and repeating the process was prohibited by cost. A possible reason for the poor read quality might be due to insufficient duration of amplification by QPCR. This issue limited the depth of the analyses, possibly making the definition of class or genus levels potentially inaccurate. Therefore, the following 16S rRNA analyses in this study were based on bacterial phyla.
3.5.5.1 Alpha diversity (Chao1 richness index)

Alpha diversity, the Chao1 richness index, is one measurement to assess structure and function of the microbial community that accounts for the number of species, rareness and commonness of abundant bacteria in a community (species richness) (Lozupone and Knight 2008). The Chao 1 richness index in each treatment group is depicted in Figure 3.15. To test for statistically significant difference between groups, a Kruskal-Wallis test followed by Dunn’s multiple test was used. HFD/Veh mice exhibited a statistically significant reduction of richness in the gut microbial diversity compared with SD/Veh mice \( (p < 0.001) \). The same trend was found in HFD/HNAC mice compared with SD/HNAC mice \( (p < 0.01) \). There were no differences in the LNAC groups.

Figure 3.15 Chao 1 richness index

![Box plot showing Chao 1 richness index](image)

**Figure 3.15 Chao 1 alpha diversity.** Upper and lower quartiles defined the box with median midline, and the whiskers were assessed using Tukey’s method. Kruskal-Wallis test showed that HFD/Veh mice exhibited a statistically significant reduction in Chao 1 alpha diversity compared with SD/Veh microbiota. Similarly, HFD/HNAC group also showed significantly less diverse gut microbiota compared to SD/HNAC group. ***\( p < 0.001 \); **\( p < 0.01 \); n= 6-17
3.5.5.2 Principal coordinate analysis (PCoA)

Principal coordinate analysis (PCoA) is a method to assess similarities or dissimilarities of the gut microbiome communities of each treatment group (Lozupone and Knight 2008). The PCoA plot (Figure 3.16) showed two distinct groups separated by diet, with HFD groups clustered together and SD groups clustered together. On the other hand, the NAC treatment groups did not have separate, distinct clusters. This indicates that HFD is a stronger determinant of the variance in gut microbiota. Principal coordinate 1 (PC1) explains 45.3% of variation and PC2 explains 15.6% variation of the dissimilarities between groups. A weighted distance-based analysis of molecular variance (AMOVA) was used to assess the statistical significance of the spatial separation observed among each treatment group of the principal coordinate analysis plots. Statistically significant dissimilarities were observed between HFD and SD groups with respect to bacterial diversity, within vehicle treated ($p = 0.001$); within LNAC treated ($p = 0.001$); and within HNAC treated mice ($p = 0.001$). Furthermore, within the SD group, vehicle treated and HNAC treated mice also exhibited statistically different microbial membership ($p = 0.048$) and within the HFD group, the gut microbial membership in vehicle treated mice was significantly different from LNAC treated mice ($p = 0.01$).
Figure 3.16 Principal coordinate analysis (PCOA). PCoA of weighted UniFrac matrix showed HFD groups were separated from SD groups. Percent of dataset variability explained by each principal coordinate is shown in brackets in axis titles. Each data point represents an individual animal.
3.5.5.3 Relative abundance at phylum level

Figure 3.17 depicts the relative abundance of bacterial phyla based on cumulative sum scaling (CSS) normalised operational taxonomic unit (OTU) abundance between each treatment group. At the phylum level, there were minimal changes in the bacterial phyla distribution between the groups. However, NAC treatment appeared to promote an increase in the relative abundance of Verrucomicrobia in HFD mice (15-16%) compared with SD mice (5-9%).

Figure 3.17 Relative abundance at phylum level

Figure 3.17 Relative abundances of bacterial phyla in each treatment group. Numbers in the bar chart represent proportions of the relative abundance of each phylum.
3.5.5.4 Firmicutes to Bacteroidetes (F/B) ratio

Firmicutes and Bacteroidetes are the two dominant phyla of the gut microbiota. Thus, the F/B ratio can be used as an indicator of a shift in the ecological balance of the gut microbiota (Figure 3.18). Kruskal-Wallis test followed by Dunn’s multiple comparison test were used to assess differences in F/B ratio between groups as the data were not normally distributed. The tests indicated that HFD mice in each treatment group exhibited a significant increase in F/B ratio compared with SD mice in the corresponding treatment group (SD/Veh compared to HFD/Veh, \( p < 0.001 \); SD/LNAC compared to HFD/LNAC, \( p < 0.05 \); SD/HNAC compared to HFD/HNAC, \( p < 0.01 \)).

Figure 3.18 Firmicutes to Bacteroidetes ratio

![Figure 3.18 Firmicutes to Bacteroidetes (F/B) ratio. Kruskal-Wallis tests followed by Dunn’s multiple comparison test indicated a consistent trend that HFD significantly increased F/B ratio compared to SD groups in each treatment group. Upper and lower quartiles defined the box with median midline, and the whiskers were assessed using Tukey’s method. ***\( p < 0.001 \); **\( p < 0.01 \); *\( p < 0.05 \); n= 6-17.](image-url)
3.5.6 Effects of high fat diet and NAC on inflammatory mediators in the periphery

Levels of lipopolysaccharide binding protein (LBP) in the periphery in HNAC and Vehicle treated groups were assessed by commercially available ELISA kits (Boster Bio). The LNAC group was excluded from this analysis as the two doses of NAC, LNAC and HNAC, were not differentiated on any of the previous tests. HFD significantly increased plasma levels of LBP (main effect of diet, $F_{(1,66)} = 4.37, p < 0.05$), compared to SD mice (Figure 3.19). HNAC treatment did not affect plasma LBP levels (main effect of treatment, $F_{(1,66)} = 0.71, p > 0.05$; diet x drug interaction, $F_{(1,66)} = 0.19, p > 0.05$). On the other hand, both HFD and HNAC treatment did not affect the levels of soluble CD14 in plasma (main effect of diet, $F_{(1,47)} = 1.23, p > 0.05$; main effect of treatment, $F_{(1,47)} = 0.20, p > 0.05$; diet x drug interaction, $F_{(1,47)} = 0.49, p > 0.05$; Figure 3.20).

Plasma concentrations of IL-6 and TNFα were assessed using ELISA kits. However, the readings of plasma IL-6 and TNFα concentrations were below the detection limits of the kits.
**Figure 3.19 Plasma LBP levels.** HFD had a main effect of an increase in plasma LBP levels ($p < 0.05$). However, HNAC treatment did not affect plasma LBP levels. Post-hoc comparisons were not significant. Data are mean ± s.e.m. Veh = vehicle group; HNAC = high dose NAC treatment (500mg/kg) group; (n=13-21)

**Figure 3.20 Plasma soluble CD14 levels.** Two-way ANOVA indicated that neither HFD nor NAC treatment affected plasma soluble CD14 levels. Data are mean ± s.e.m. Veh = vehicle group; HNAC = High dose NAC treatment (500mg/kg) group; (n=12-14).
3.5.7 Effects of high fat diet and NAC on inflammatory mediators in the CNS

Levels of Iba1, MHC class2, CD11b, GFAP, Ddit4, GLAST, GLT1, xCT, iNOS and Occludin in the hippocampus (Table 3.2) and the hypothalamus (Table 3.3) in HFD and SD groups were assessed using real time QPCR. Levels of MHC class2, CD11b, Ddit4, GLAST, GLT1, xCT, iNOS and Occludin in LNAC and HNAC groups were not assessed as NAC treatment did not differentiate on any of the previous tests. Microglial activation was assessed by Iba1, MHC class2 and CD11b (Guillemin and Brew 2004). Astrocyte hypertrophy was measured by GFAP (Pistell et al. 2010). Ddit4 was the product of which regulates cell growth, proliferation and survival (Erny et al. 2015). GLAST, GLT1 and xCT were selected to assess glutamatergic neurotransmission (Haroon, Miller, and Sanacora 2017; Mesci et al. 2015; Wright et al. 2016). Oxidative biology was assessed by iNOS expression (Block, Zecca, and Hong 2007). Occludin was used as a marker of brain blood barrier (BBB) permeability (Freeman and Granholm 2012). Two-way ANOVA tests indicated that diet and treatment did not have main effects on gene expression levels of Iba 1 and GFAP levels and unpaired t tests indicated there were no differences in levels of the rest of target genes in the hypothalamus or the hippocampus.
Table 3.2 Impact of HFD feeding and NAC treatment on gene expression in the hippocampus

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<tr>
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<th>SD/HNAC</th>
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<th>HFD/LNAC</th>
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Table 3.1 Levels of gene expression in the hippocampus. HFD nor NAC treatment did not alter gene expression levels in the hippocampus. Results are expressed as relative fold change in mRNA expression and compared with the SD/Veh group; means ± S.E.M; n =7-10 for Iba1 and GFAP; n=10 for GLAST, GLT1, xCT, CD11b, MHC class2, Ddit4, iNOS and Occludin.
Table 3.3 Impact of HFD feeding and NAC treatment on gene expression in the hypothalamus

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<th>Genes</th>
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<td>GLAST</td>
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Table 3.2 Levels of gene expressions in the hypothalamus. HFD nor NAC treatment did not alter gene expression levels in the hypothalamus. Results are expressed as relative fold change in mRNA expression and compared with the SD/Veh group; means ± S.E.M; n =7-10 for Iba1 and GFAP; n=10 for GLAST, GLT1, xCT, CD11b, MHC class2, Ddit4, iNOS and Occludin.
3.6 Discussion

This study examined the behavioural changes, inflammatory state and gut microbiome alterations elicited by a HFD. The effects of NAC on these factors were also investigated. HFD induced depression-like behavioural changes, paralleled by significant shifts in the population of the gut microbiome. This was accompanied by elevations of LBP, which might point to the way the immune system responds to microbiome changes and the means by which such signals are linked to changes in the brain. NAC did not alter diet-induced changes in behaviour or the gut microbial population.

3.6.1 Effects of high fat diet on behaviour

HFD mice displayed significantly longer time immobile in the FST, possibly indicative of behavioural despair and displayed reduced sucrose consumption in the SPT, potentially indicative of anhedonia. Both key behavioural findings are indicative of a depression-like behavioural state (Krishnan and Nestler 2011). Here, we will discuss the key behavioural findings in our study in relation to currently available literature which was summarised in Table 3.1.

3.6.1.1 High fat diet-mediated behavioural change: Behavioural despair

Our HFD mice exhibited an increase in the immobility time which is consistent with Sharma et al. (2013) and Yamada et al. (2011). What is in common among these two studies and our study were the strain of mouse (C57BL/6J), the age of animals (7-8-week-old) when HFD feeding was started and the age of animals (20-22-week-old) at behavioural testing, whereas
fat content is higher in Sharma et al. (58% kcal from fat) and in Yamada et al. (60% kcal from fat). The age of the mice at the commencement of the diet is equivalent to adolescence and young adulthood in human, whilst a mouse age of 20-22 weeks approximately corresponds to adulthood (Dutta and Sengupta 2016). This consistency of timing and age among the three studies may indicate that there may be a vulnerable age window by which HFD can mediate a specific depressive-like behaviour. This speculation needs to be interpreted cautiously as there are only a few studies which have used this paradigm. However, a large body of epidemiological evidence is in line with this speculation, pointing to the strong association of poor diet during adolescence and young adulthood with increased risk of developing mood disorders (Jacka et al. 2011; Jacka et al. 2010; Jacka et al. 2013; Weng et al. 2012; Gall et al. 2016).

On the other hand, several studies using similar high fat diets reported HFD consumption induced a reduction of immobility in the FST, which contrasts to our findings. For instance, consumption of HFD (45% kcal from fat, the same fat content as this study) resulted in a significant decrease in the duration of immobility in the FST in the CD1 strain of mice (Del Rosario, McDermott, and Panee 2012) and in C57BL/6J mice (Del Rio et al. 2016). Western diet (49% kcal from fat) consumption also resulted in no difference in the duration of immobility in the tail suspension test (TST) (a test also measuring behavioural despair) compared with SD fed C57BL/6J mice (Andre et al. 2014). Interestingly, Andre et al. reported a significant reduction in the immobility time in the FST in these mice after a LPS challenge. Similarly, there were no differences in the duration of immobility between HFD and SD mice in the FST after twenty-four weeks of HFD (45% kcal from fat) consumption (Takase et al. 2016). Furthermore, an increase in the fat content of the diet is also not necessarily associated
with depressive-like behaviour. Thirteen weeks of HFD (60% kcal from fat) resulted in no difference in the duration of immobility in Balb/C mice (Pyndt Jorgensen et al. 2014). Similar findings are also reported using female mice. Female C57BL/6J mice did not exhibit differences in behaviour in the FST compared to SD mice after 32 weeks of HFD consumption (Krishna et al. 2016) whereas five weeks of HFD (60% kcal from fat) consumption induced a significant decrease in immobility time in the FST in female mice (C57BL/6J) (Krishna et al. 2015).

Several factors can possibly contribute to the discrepancy between our study and others. Firstly, the sample size of our study is considerably larger than others. Our study utilised 17 to 22 animals per group which is a more robust sample size compared to 5 animals per group in Del Rosario, McDermott, and Panee (2013) and 8 animals per group in Takase et al.(2016), Andre et al. and Del Rio et al. Small sample size could limit the ability to detect significant effects and thus could lead the Type I or Type II errors. Secondly, animal age at the time of behavioural testing could also affect the results. Our animals were approximately 22-23-weeks-old at the time of behavioural testing and this age falls between 12-week-old in Del Rosario, McDermott, and Panee (2012), 13-week-old in Del Rio et al. (2016) and 32-week-old in Takase et al. (2016). In fact, a study showed that aged mice behaved differently in an array of behavioural tests compared with adult mice under the influence of long term consumption of a high fat diet (Kesby et al. 2015). Thirdly, the duration of high fat diet feeding may contribute to behavioural differences. Takase et al. (2016) employed 24 weeks of HFD feeding whereas Del Rosario, McDermott, and Panee (2012) and Del Rio et al. (2016) employed 8 weeks of HFD feeding, compared with 17 weeks of HFD feeding in this study. This may suggest that different behaviours could be affected by different time points under the influence of
HFD, instead of by the length of duration of diet feeding. For instance, by examining the effect of high fat diet (60% kcal from fat) on behaviour at different time points (at 1, 3 and 6 weeks), Gainey et al. (2016) found anxiety related behavioural changes in HFD mice only at 3 week time point. In line with this, Krishna et al. (2016) also reported HFD (60% kcal from fat) induced anxiety related behaviour in female mice at 32 weeks but not at 21 weeks. Thus, effects of HFD on behaviour may not be universal. For instance, different strains of mice appear to have different susceptibility to HFD effects on behaviour. Furthermore, the timing of HFD-evoked behaviour may differ depending on the types of behaviour assessed. Alternatively, HFD mediated reduction of the immobility time in the FST reported in other studies could be interpreted as an increase in locomotor activity.

3.6.1.2 High fat diet-mediated behavioural change: Anhedonia

Another notable behavioural change induced by HFD in our study was a reduction of sucrose water consumption in the SPT, potentially indicative of anhedonia. With regard to this specific depressive-like behaviour, our finding is consistent with others. In fact, HFD-induced anhedonic behaviour in the SPT paradigm is found regardless of mouse strain, animal age, dietary fat content on duration of HFD feeding (Sharma, Fernandes, and Fulton 2013; Takase et al. 2016; Yamada et al. 2011; Del Rosario, McDermott, and Panee 2012). Anhedonia is a key facet of MDD. The consistency seen in the literature strongly supports epidemiological evidence that poor diet consumption shapes depressive mood. Furthermore, it provides compelling face validity to the HFD-induced animal model of MDD.

A possibility however remains that the anhedonic behaviour in HFD mice in the SPT may be explained due to altered taste preference. Anhedonia, being unable to feel pleasurable
rewards, is one of the core symptoms of MDD (Krishnan and Nestler 2011). The brain rewarding system is regulated by monoamines, such as dopamine. Highly palatable stimuli such as sucrose solution may have been highly rewarding to SD mice and resulted in the release of dopamine, while for HFD mice, this stimulus is only mildly rewarding. Thus, the dietary intervention, long term HFD feeding, may have changed the reward system in the HFD mice. As altered reward responses are intrinsic to the symptoms of MDD this supports the proposal that the HFD-induced behavioural changes are akin to depression. What is less clear is whether HFD-induced behaviours are representative of patterns observed in MDD not associated with diet.

3.6.1.3 High fat diet-mediated behavioural change: locomotor activity
We observed that HFD induced an increase in moving speed and mobility during the OFT, although HFD significantly decreased travel distance. Importantly, correlation analysis found no conclusive evidence that these behavioural changes were attributable to fat mass gain itself or motor impairment due to obesity, and hence may instead represent the effects of neurological changes. This supports the conclusion that the altered behaviour in the FST is not due to obesity-related impairments in swimming capacity. HFD influenced changes in locomotor activity were also reported in the literature. Some studies reported that HFD increased locomotor activity, including a longer travel distance than controls (Krishna et al. 2015; Sharma and Fulton 2013). Others found there was no differences in locomotor activity between HFD fed mice and controls (Del Rosario, McDermott, and Panee 2012; Takase et al. 2016). In addition, as discussed above, several studies reported HFD elicited a significant reduction of immobility time in FST (Del Rio et al. 2016; Del Rosario, McDermott, and Panee
2012; Krishna et al. 2015). Although some groups claimed these locomotor changes as an antidepressant-like effect of HFD consumption, it is not clear which mechanistic pathways are involved in shaping these behavioural changes in HFD animals.

On the other hand, given that both the FST and OFT settings could be perceived as stressful events for a mouse, it is possible that increased speed and mobility in our OFT results indicated that HFD altered their behavioural reactivity to a stressful environment. For instance, FST is a situation of acute stress, in which a mouse is placed in an inescapable environment (Krishnan and Nestler 2011). Yamada et al. (2011) reported that HFD did not alter behaviour in a pre-test session in the FST, whilst the same HFD mice exhibited a significantly longer duration of immobility during the test session conducted 24hr later. In addition, the FST paradigm which Yamada et al. (2011) employed, in fact, is widely used in rats (Slattery and Cryan 2012). For mice, a single exposure to a test is sufficient to ensure a stable immobility at baseline, which can be reduced by a wide range of antidepressants (Slattery and Cryan 2012). Thus, this behavioural change between pre-test and test sessions could be interpreted as learned helplessness behaviour or an adaptive learned behaviour to save energy.

3.6.2 Effects of high fat diet on the gut microbiome

HFD resulted in changes in the gut microbiota that were characterised by significant reductions of richness of the gut microbial community (alpha diversity), and the number of bacterial species assigned by operational taxonomic units (OTUs) detected in the sample. In addition, HFD significantly altered the diversity of the gut microbiota (beta diversity), which is the extent of similarity between microbial communities by measuring the degree to which
membership or structure is shared between communities (Lozupone and Knight 2008). This change was also accompanied by a significant increase in F/B ratio in HFD mice. Firmicutes and Bacteroidetes are two major bacterial phyla that comprise more than 90% of bacterial phylogenetic types of the mouse gut microbiota as well as humans (Cani and Delzenne 2010; Turnbaugh et al. 2008). Dysbiosis, characterised as an increase in Firmicutes and a decrease in Bacteroidetes has been reported in numerous diet-induced obesity models (Cani and Delzenne 2010; Turnbaugh et al. 2008; Boulangé et al. 2016; Geurts et al. 2011). For instance, eight weeks of Western Diet in mice (41% kcal from fat) significantly increased F/B ratio and decreased alpha diversity (Turnbaugh et al. 2008). Whereas HFD induced dysbiosis has been documented in obesity research using a diet induced animal model, what remains unclear is how these changes, such as changes in the microbial richness and diversity and F/B ratio in the gut microbiota are associated with depressive-like behaviour.

3.6.2.1 Comparisons with clinical studies

There is a paucity of clinical studies regarding association between gut microbiota and MDD, and the findings are inconclusive. Naseribafroei et al. (2014) examined the gut microbiome from 37 MDD patients in comparison with 18 non-depressed outpatients. With regard to microbial richness, the microbiota from MDD patients did not differ from controls. By analysing OTUs, however, they found a significant overrepresentation of Bacteroidetes and underrepresentation of Lachnospiraceae (a member of Firmicutes) in MDD patients compared with healthy controls. Furthermore, correlation analysis indicated the genera Alistipes (a member of Bacteroidetes) and Oscillibacter (a member of Firmicutes) were highly correlated with MDD in the cohort (Naseribafrouei et al. 2014). An increase in the levels of
Alistipes were associated with healthy diet (Drescher, Thiele, and Mensink 2007) whilst a decrease in the levels of Oscillibacter was reported in the gut microbiota after HFD feeding (Qin et al. 2012). However, Naseribafroei et al. (2014) did not provide any dietary information regarding their clinical population, thus, it is unclear whether these two bacterial genera were associated with dietary patterns of the clinical population.

In contrast, Jiang et al. (2015) reported significant differences of bacterial phyla between active MDD patients, treatment-responsive MDD patients and healthy controls. Firmicutes and Actinobacteria were significantly less abundant in actively depressed patients than in healthy controls, whilst Bacteroidetes was significantly more abundant in the MDD patients. On the other hand, Firmicutes, Fusobacteria and Actinobacteria were significantly less abundant in treatment-responsive MDD patients compared with healthy controls, whereas Bacteroidetes and Proteobacteria were significantly less abundant in those responsive MDD patients (Jiang et al. 2015). Although Jiang et al. (2015) highlighted a significant difference in the relative abundance of bacterial phyla between MDD patients and healthy controls, the richness and beta diversity of these three cohorts (active MDD, responsive MDD and healthy controls) did not differ from each other. Both the Naseribafroei et al. (2014) and Jiang et al. (2015) indicated that the richness and microbial diversity between MDD patients and healthy controls. In addition, both studies pointed out significant changes in levels of abundance of Bacteroidetes and Firmicutes in MDD patients, although the direction of the changes were not the same.

Furthermore, Kelly et al. (2016) reported a significant reduction in the richness and changes in microbial diversity in MDD patients compared with healthy controls. This finding was accompanied by a significant increase in Prevotellaceae (a member of Bacteroidetes) and a
decrease in *Thermoanaerobacteraceae* (a member of Firmicutes). Opposite directional changes in levels of Bacteroidetes and Firmicutes in MDD were found in Lin et al. (2017). By examining the gut microbiota in 10 MDD patients, an increase in the levels of Firmicutes and a decrease in the levels of Bacteroidetes in MDD patients were reported in comparison with healthy controls (Lin et al. 2017). Statistically higher abundance of the genus *Prevotella* (a member of Bacteroidetes), *Klebsiella* (a member of Proteobacteria), *Streptococcus* (a member of Firmicutes) and *Clostridium XI* (a member of Firmicutes) were also reported in MDD patients compared with healthy controls. It is unclear whether indices of richness and diversity of the gut microbiota in MDD patients differed from healthy controls in Lin et al. (2017).

While a shift of the balance between Bacteroidetes and Firmicutes appears to be common in MDD, measures of the richness and diversity in the gut microbiota in MDD were insufficiently provided. Given that the gut microbial community is a complex ecosystem and the fact that not all species within a sample are equally related to each other (Lozupone and Knight 2008), highlighting specific bacteria as biomarkers of MDD without acknowledging this assumption could be misleading and may result in wrong conclusions being drawn. Furthermore, microbial diversity could be strongly influenced by several factors, such as health status, age, diet and antibiotic use (Lozupone and Knight 2008) and hence, differences in these factors in the clinical populations in these studies may have influenced the results. In fact, the gut microbiome in MDD patients in Naseribafroei et al. (2014) was compared with the gut microbiome from non-depressed patients who were recruited from an outpatient neurological unit. On the other hand, Jiang et al. (2015), Kelly et al. (2016) and Lin et al. (2017) recruited healthy age and sex matched controls. In addition, the clinical populations in
Naseribafroei et al. (2014), in Jiang et al. (2015), Lin et al. (2017) and Kelly et al. (2016) were recruited from geographically different locations, in Norway, China and Ireland respectively and hence, different dietary patterns may also contribute to the abundance of particular bacteria and the richness and diversity on the gut microbiota. The large discrepancy in age between the three studies is also a confounding factor. Naseribafroei et al. (2014) reported the average age of their MDD population was 49.2 which is close to the average age of 45.8 in Kelly et al. (2016), whereas the average age of MDD population in Jiang et al. (2015) was 25.3 (active MDD patients) and 27.1 (responsive MDD patients). The average age of the Lin et al. (2017) population was 36.2 years old.

Furthermore, a possibility arises that dysbiosis may not be causally associated with all types of MDD, considering that different underlying aetiologies underpin the heterogeneity of this disease. Nevertheless, the dysbiosis elicited by high fat diet consumption and concomitant depressive-like behaviour in our study exemplified a certain type of MDD which was evidenced by the strong association between MDD and poor dietary patterns supported by substantial epidemiological literature.

Taken together, dysbiosis in MDD patients is apparent in these limited studies, characterised as alterations in the abundance of certain bacteria, and the richness and diversity of the microbial community. Whilst the dysbiosis found in our HFD mice partly fits with the MDD literature, interpreting these findings relative to clinical studies is potentially confounded by several environmental factors such as age, diet, health status and medication use. In addition, differences in the composition of gut microbiota between mice and humans, and a complex dietary pattern in humans, should be considered when interpreting these results. The gut microbiota is a complex ecosystem that we are only now beginning to understand. The
discrepancy between the clinical studies could be addressed by using animal models which allow for controlling these factors.

3.6.2.2 Comparisons with preclinical studies

A limited number of preclinical studies have examined effects of HFD on gut microbiota and depressive-like behaviour. A study reported that ten weeks of HFD (60% kcal from fat) feeding resulted in a significant increase in Firmicutes and a significant decrease in Bacteroidetes and subsequently an increase in the F/B ratio in HFD group compared with SD group. However, HFD induced dysbiosis was not associated with anhedonia or behavioural despair (Pyndt Jorgensen et al. 2014). Although the dysbiosis found in this study was consistent with the alterations in gut microbiota in our findings, one possible reason that this study resulted in different behavioural outcomes might be attributed to the different mouse strains; Balb/C mice instead of C57BL/6J. Differences in mouse strains could affect behavioural reactivity in the FST (Lucki, Dalvi, and Mayorga 2001).

On the other hand, several lines of literature using animal models of depression reported similar alterations of the gut microbiota that were associated with anxiety-like and depressive-like behaviour. An exposure to social defeat stress (confronting an intruder mouse for a short period) for 10 days induced anxiety-like behaviour in CD1 mice that was accompanied by a significant reduction in the richness and an alteration of the diversity of the gut microbiota. However, at the phylum level, a shift in the balance between Firmicutes and Bacteroidetes did not occur (Bharwani et al. 2016). A chronic restraint stress model, which involved 4-6 hours of restraint stress for 21 days, showed a non-significant trend towards an increased F/B ratio which was accompanied by depressive-like behaviour.
(prolonged immobility time during the FST) (Wong et al. 2016). These studies showed that chronic stress could induce alterations of the gut microbiota which are similar to the changes elicited by HFD. Given the resultant depressive- and anxiety-like behavioural changes in the HFD and stress models, this raises the question of how the disruption in the gut microbial community elicited by stress was similar (or dissimilar) to the changes induced by HFD. We need to standardise methods across studies to classify and identify the specific changes in the gut microbiota.

Furthermore, faecal microbiota transplantation from MDD patients to Sprague Dawley rats partially replicated behavioural changes and the gut microbial community which resembled the profile of the gut microbiome of the human donor (Kelly et al. 2016). Interestingly, an analysis of the relative abundance at phylum level in the rat microbiota after faecal microbiota transplantation did not match with the relative abundance in donor MDD patients. Whereas a significant decrease in Candidate Division TM7 and Actinobacteria was found in the MDD microbiota recipient rats compared with the healthy control recipient rats, a significant increase in Firmicutes and a decrease in Bacteroidetes were reported in the donor MDD patients. Moreover, these animals only exhibited anhedonic behaviour, a reduction of sucrose preference in the SPT but not behavioural despair in the FST (Kelly et al. 2016). Given that the recipient rats received an antibiotic treatment (a cocktail of ampicillin, metronidazole, vancomycin, ciprofloxacin, and imipenem) in drinking water for 28 consecutive days prior to the faecal microbiota transplantation, there is a possibility that the antibiotic treatment might have altered their taste preference, and hence sucrose preference. These results point to the complexity of the gut microbial community, in which the changes in this eco system may not be uniform, despite similar behavioural changes.
3.6.3 Effects of high fat diet on microbial signalling

We measured plasma LPS binding protein (LBP) and soluble CD14 levels in the periphery as indicators of dysbiosis-induced inflammation. LBP is an acute phase protein that is synthesised in the liver. LBP is bound to CD14 to initiate pro-inflammatory responses upon detection of the endotoxin lipopolysaccharide (LPS) in the circulation (Garate et al. 2011; Jerala 2007; Kitchens and Thompson 2005). CD14 is found in two forms, in a membrane bound form on the cell surface (mCD14) and in a soluble form in the circulation (Kitchens and Thompson 2005). High fat diet consumption induces a well-documented increase in intestinal permeability and subsequent elevation of LPS levels (Cani et al. 2007; Cani and Delzenne 2010; Cani et al. 2009). It has been hypothesised that the elevation in LPS levels is due to an increase in the permeability of the intestinal barrier (tight junctions) and resultant bacterial translocation to the systemic circulation (Garate et al. 2011; Kelly et al. 2015; Maes et al. 2012; Slyepchenko et al. 2017). These circulating gram negative bacteria are a source of LPS. This proposed mechanism, “Leaky gut” is also implicated in the pathophysiology of MDD (Kelly et al. 2015; Maes et al. 2012; Slyepchenko et al. 2017). Hence LBP has been used as a surrogate marker of low grade inflammation induced by LPS from the gut (Gonzalez-Quintela et al. 2013; Kheirandish-Gozal et al. 2014; Ruiz et al. 2007; Lequier et al. 2000; Lepper et al. 2007).

3.6.3.1 Effects of high fat diet on plasma LBP concentration

Our HFD mice exhibited a significant elevation of plasma LBP levels, which indicates that HFD-induced disruption of the gut microbiota mediated LPS signalling, possibly via an increase in permeability in the gut wall and/or bacterial translocation. In line with our findings, elevations of plasma LBP and LPS levels along with depressive-like behaviour in the FST were reported.
in rats submitted to 21 days of chronic mild stress (CMS), an animal model of MDD (Garate et al. 2011). The elevations of plasma LBP and LPS levels in the rats were attenuated by antibiotic treatment, however, the stress-induced depressive-like behaviour was not reversed by the antibiotic. Garate et al. (2011) suggests that concentrations of LBP and LPS levels can fluctuate with possible changes in the gut microbiome, as it is unknown what changes in the gut microbiota were elicited by chronic stress and antibiotic treatment as the authors did not include this information. The findings also indicate that changes in concentrations of LPS and LBP may not necessarily be associated with behavioural changes and therefore, implies that changes in the gut microbiome may not always exert effects on behavioural changes in the host. For instance, Kelly et al. (2016) found that there was no difference in plasma LBP levels between MDD patients and healthy controls, despite the fact that gut microbiota from MDD patients exhibited dysbiosis compared with healthy controls. The authors conducted faecal gut microbiota transplant from subsets of the MDD patients with the most severely depressed symptoms and healthy controls to rats. The faecal transplantation did not cause changes in plasma LBP levels in rats receiving MDD gut microbiota compared with rats receiving healthy control gut microbiota (Kelly et al. 2015).

Taken together, the currently available evidence suggests that concentrations of LBP are associated with some changes in gut microbiota in MDD. As such, our HFD-induced changes in gut microbiota may be associated with a disease state that resulted in depressive-like behaviour. What remains unclear is what underlying pathways are involved in HFD-induced elevation of LBP levels.
3.6.3.2 Effects of high fat diet on plasma soluble CD14 concentration

HFD did not affect plasma soluble CD14 concentration in our study, despite an increase in LBP concentrations and depressive-like behaviour in HFD mice. Our finding is in line with findings from clinical studies. Currently available evidence regarding soluble CD14 in MDD showed that there was no difference in the levels of plasma soluble CD14 in MDD patients and healthy controls (Musil et al. 2011), and that plasma soluble CD14 in MDD patients was not correlated with MDD symptoms nor disease course (Landmann et al. 1997).

A known function of soluble CD14 is to mediate the effects of LPS on cells lacking the membrane form of CD14 (Musil et al. 2011). Elevations of soluble CD14 were found in several autoimmune diseases. For example, increased levels of soluble CD14 were correlated with elevations of C-reactive protein and IL-6 in rheumatoid arthritis (Bas et al. 2004). Serum soluble CD14 levels were significantly higher in multiple sclerosis compared to neurological and healthy controls (Lutterotti et al. 2006). However, the pro-inflammatory state in MDD is modest compared to that typically observed in autoimmune or infectious diseases (Raison and Miller 2011). Therefore, our finding and others raise the possibility that levels of soluble CD14 might be different in subclinical inflammation compared with florid inflammation. For instance, a recent study showed concentration of LBP was associated with a pro-inflammatory state without an increase in soluble CD14 levels in patients with metabolic syndrome, a condition known to be associated with low grade inflammation (Jialal et al. 2015).

Another possibility is that elevated LBP levels in our HFD mice might be bound to membrane CD14 instead of soluble CD14 or involved in other pathways. A study reported that soluble CD14 acts to diminish monocyte response to LPS by transferring cell bound LPS to lipoproteins and thus, reducing the release of pro-inflammatory mediators (Kitchens and Thompson 2005).
This indicates that soluble CD14 and membrane bound CD14 may have different functions via LBP in response to LPS. Hence, elevations of plasma LBP levels might not necessarily be associated with elevations of plasma soluble CD14 levels.

3.6.4 Effects of high fat diet on inflammatory state

The immune system has been proposed to be an important interface connecting the gut microbiota and the brain (Bharwani et al. 2016; Kelly et al. 2015; Maes et al. 2012; Maes et al. 2009). In our study, concentrations of plasma pro-inflammatory cytokines in HFD mice, TNFα and IL-6, were below detection levels and therefore were unable to be measured using commercially available ELISA kits. According to the manufacturer’s manual, the detection ranges of the kits are designed to measure transient elevations of these pro-inflammatory cytokines in response to immune challenge such as LPS and hence, reported sample values of serum/plasma concentrations of these pro-inflammatory cytokines in healthy animals are below the lowest standard. Accordingly, our findings indicate that plasma levels of TNFα and IL-6 in HFD mice were below the lowest standard, indicating that our samples fall in the normal range.

Concentrations of pro-inflammatory mediators reported in MDD were modest compared to concentrations of these mediators found in autoimmune and infectious diseases, and the values found in MDD patients were in fact, regarded as within the “normal” range (Dowlati et al. 2010; Raison and Miller 2011). Hence, our findings concur with the evidence. However, this also points to a limitation of the methodology we used in this study, by which we were unable to identify subtle differences in these pro-inflammatory mediators within normal
ranges. In addition, it implies the need for alternate markers that are indicative of subtle inflammatory changes.

Nevertheless, although a close connection between inflammatory status, the gut microbiota and depressive-like behaviour has been hypothesised in MDD, the current literature suggests that disruption of these factors may not co-occur in some cases, which might reflect the heterogeneity of MDD. For example, Jiang et al. (2015) reported that there were no differences in serum IL-6, TNFα and IL-1β concentrations between MDD patients and healthy controls, whereas significant elevations of serum IL-6, TNFα and CRP levels in MDD patients compared with healthy controls were reported in Kelly et al. (2016). Both studies reported dysbiosis was associated with MDD patients. Furthermore, concentrations of IL-6, TNFα and IL-1β in the periphery and the brain were not affected in Western diet (41-49% kcal from fat) fed mice (Andre et al. 2014; Pistell et al. 2010).

On the other hand, HFD feeding (60% kcal from fat) in mice resulted in significant elevations of IL-6 and TNFα in the circulation which were concomitant with a significant increase in TNFα and IL-1β protein levels in the hippocampus (Jeon et al., 2012) and a significant increase in Iba1 and GFAP mRNA expression levels in the cortex (Pistell et al., 2010). To note, the age of the mice in the Pistell et al. (2010) was 12 months (48 week old) at the beginning of the study and approximately 17-18 months (69 weeks old) at the end of study. An increased number of activated microglia and astrocytes was found in the aging brain (Conde and Streit 2006; Robillard et al. 2016), thus, the elevations of the glial activation markers in this study may not be simply due to effects of HFD.

In addition, we can only speculate whether the florid inflammatory states elicited by HFD (60% kcal of fat) in these studies were associated with depressive-like behaviour, which
neither of the studies examined. Taken together, the current literature suggests that HFD-induced changes in depressive-like behaviour/mood may not necessarily be associated with pro-inflammatory state. Our finding indicated HFD-induced behaviour was associated with normal ranges of inflammatory mediators. Whether there were subtle changes in these pro-inflammatory mediators within the normal range in HFD mice compared with SD fed mice are only speculative due to methodological limitations. Investigations of alternate pathways or signals to identify inflammatory states associated with depressive mood/behaviour are warranted.

3.6.4.1 Effects of high fat diet on oxidative biology

This study also evaluated levels of inducible nitric oxide synthase (iNOS) in the hippocampus and hypothalamus to assess oxidative damage elicited by HFD, as a diet rich in saturated fat is associated with an increase in the levels of oxidative stress in the brain, in addition to inflammation (Uranga et al. 2010). However, we did not find any upregulation of iNOS mRNA expression in these brain areas.

Gainey et al. (2016) reported that HFD (60% kcal from fat) feeding in mice induced upregulation of iNOS expression and suppression of GSH: GSSG ratio in the hippocampus only at the 3rd week of a 6 week period of the study and these signals were absent at the 1st or 6th week. In addition, a 12 week period of HFD feeding (60% kcal from fat) in mice did not result in differing iNOS expression in the hippocampus in HFD mice HFD and SD mice (Liu et al. 2015). These data suggest that upregulation of iNOS, indicative of oxidative stress damages, may be an early, transitory outcome of chronic HFD consumption. Our study examined hippocampal
and hypothalamic iNOS mRNA expression at the end of the experiment, at the 17th week. The timing of the evaluation in our study may not be suitable for identifying transitory changes.

3.6.4.2 Effects of high fat diet on the brain blood barrier

Finally, the brain blood barrier (BBB) function was assessed by measuring gene expression levels of occludin in the hippocampus and hypothalamus. Occludin is a key protein component of the tight junctions of the BBB (Freeman and Granholm 2012). The BBB has a critical function in preventing pro-inflammatory agents and neurotoxins entering from the circulation into the brain. Hence, this study assessed the integrity of the BBB by evaluating levels of occludin mRNA in the hypothalamus and hippocampus. Chronic HFD consumption did not alter gene expression levels of occludin in this study, which indicates at least one of the tight junctions protein of the BBB was not affected by HFD.

In contrast, the current literature reported that disturbance of the tight junctions protein including occludin in mice and rats occurred after 3 months (Takechi et al. 2013) and 12 months (Freeman and Granholm 2012) of HFD feeding, respectively. The discrepancy between our study and the literature may be due to differences in HFD feeding duration or in dietary components (high cholesterol content in the diet). Furthermore, occludin is not the only protein constituent of the BBB where endothelial cells connect together with various proteins such as tight junction proteins (i.e., claudin), scaffolding proteins (i.e., ZO-1 and ZO-2) and astrocytes (Freeman and Granholm, 2012). Therefore, relying on levels of occludin mRNA might be insufficient to evaluate BBB integrity.
3.6.5 Effects of high fat diet on the glutamatergic system

The glutamatergic system is tightly regulated by glutamate transporters and receptors expressed by activated glial cells (Haroon, Miller, and Sanacora 2017). Therefore, our study evaluated levels of glutamate transporters located on astrocytes and microglia, namely GLT-1, GLAST and System xC.

Our results indicated that HFD-induced depressive-like behaviour was not associated with changes in glial glutamate uptake in the hippocampus and the hypothalamus, as evidenced by no changes in the gene expression levels of GLT-1, GLAST and System xC between HFD and SD mice. In contrast, changes in glial glutamate transporters were implicated depressive-like behaviour in several studies. Altered mRNA expression levels of GLT-1 and GLAST were associated with depressive-like behaviour in mice measured by the FST and SPT (Skupio et al. 2015; Wright et al. 2016). The hippocampal GLT-1 levels were downregulated in learned helplessness rats, a common animal model of MDD (Zink et al. 2010). Bentea et al. (2015) showed that System xC was critically involved in depressive-like behaviour as absence of System xC in mice decreased depressive-related behaviour. The discrepancy between our study and others implies that HFD-induced depressive-like behaviour might employ different pathways, possibly including the monoamine system. In fact, HFD (60% kcal from fat) induced depressive- and anxiety-like behaviour coincided with a significant increase in D2 receptor and a significant reduction in D1 receptor expression in the nucleus accumbens (Sharma and Fulton 2013). Therefore, HFD evoked depressive mood might be mediated via changes in other neurobiological mechanisms.
3.6.6 Effects of NAC on behaviour

In our study, neither the high dose (500mg/kg) nor the low dose (250mg/kg) of NAC treatment exerted antidepressant-like effects on behaviour in HFD mice. Considering the dissociation between depressive-like behaviour and changes in inflammatory/oxidative stress markers and glutamatergic system found in our HFD mice, it is not surprising to observe that NAC did not exert therapeutic effects on HFD-induced behaviour as the anti-depressant effects of NAC has been attributed to its potent anti-oxidant efficacy and modulation of the glutamatergic system.

Antidepressant-like effects of NAC were documented in several studies. For example, chronic NAC treatment (50 and 100mg/kg) reversed depressive-like behaviour in rats measured by the FST in an animal mode of depression, which was associated with a significant increase in superoxidase dismutase (SOD) activities in the frontal cortex, hippocampus and dorsal striatum (Smaga et al. 2012). In our study, iNOS mRNA expression was the only marker of oxidative stress measured. NAC is an anti-oxidant which provides cysteine for synthesis of the most ubiquitous anti-oxidant in the brain, glutathione (GSH) (Dean, Giorlando and Berk, 2011). Hence, it is possible that NAC might exert therapeutic effects on other oxidative markers such as NADPH oxidase markers and the ratio between reduced and oxidised glutathione (GSH/GSSG). Another possible therapeutic mechanism of NAC is via regulation of the glutamatergic system. Acute NAC (25mg/kg) and chronic NAC (500mg/kg) treatment improved depressive-like behaviour in mice measured by the TST and in the FST via modulating glutamate AMPA receptor (Linck et al. 2012) and cysteine dependant transporters (Wright et al. 2016), respectively. Our results suggest that HFD induced depressive-like
behaviour may not be due to dysfunction of glutamatergic system and hence, NAC treatment may not result in a reversal of HFD induced depressive behaviour.

3.6.7 Effects of NAC on the gut microbiota

Our 16S rRNA analysis indicated that NAC did not affect the richness of the gut microbiota nor the F/B ratio. However, the beta diversity, a measurement of similarity/dissimilarity between groups, assessed by AMOVA analysis, indicated significant dissimilarities in the microbial communities between the SD/Veh and SD/HNAC groups and between the HFD/Veh and HFD/LNAC groups. These somewhat contradictory results may be due to the methods used in this study. In our study, Chao1 richness was not altered by NAC and this indicates that the number of species in the samples (weighted by abundance), was unaffected by NAC treatment. However, the beta diversity was assessed using weighted UniFrac distance, which accounts for prevalence and phylogenetic relationships between communities of bacteria. According to our relative abundance data (Figure 3.17), it appears there were some increases and decreases in taxa between SD/Veh and SD/HNAC groups and between HFD/Veh and HFD/LNAC groups. These shifts in relative abundance may contribute to the statistical significance in the AMOVA results.

3.6.8 Limitations of this study

To note, our study has several limitations. The findings from our study failed to provide evidence that supports HFD-induced elevations of pro-inflammatory and oxidative stress markers and dysfunction of the glutamatergic neurotransmission, which may correlate with
HFD-mediated depressive-like behaviour. Although it might be the case that underlying pathways that manifested diet-induced depressive mood may not interact with pathways involving inflammation, oxidative stress and glutamatergic systems, it is possible that our findings were confounded by several limitations.

Firstly, concentrations of inflammatory mediators in the circulation were at undetectable levels using the commercially available assays which were designed to detect transient inflammatory elevations, such as those induced by infection. Collating available evidence indicates that HFD-induced changes in inflammatory and oxidative biology states may differ from the florid inflammatory responses observed during infection. This implies that HFD-induced changes in inflammatory states may still fall in “normal” ranges which is under detectable levels using these assays. Therefore, the methodology we employed did not possess appropriate sensitivity to capture the subtle changes elicited by HFD and it hence might not be an optimal choice. Secondly, 16S rRNA processing issues limited us from in-depth analysis. Given that new methodologies and more in-depth analysis is becoming available for gut microbiome analysis, evaluating at the phylum level only provides an incomplete picture of any alterations in the gut microbiota.

Furthermore, NAC treatment did not mediate significant changes in the gut microbiome nor reversal of behavioural change. It is possible that the dosage of NAC used (250mg/kg and 500mg/kg) might not be optimal to reverse HFD-induced behavioural changes. In addition, NAC is known to have low bioavailability (Bavarsad Shahripour, Harrigan, and Alexandrov 2014). We administered NAC through oral gavage to ensure its delivery to the intestine however, the majority of studies cited here directly administered NAC via intraperitoneal injections. The difference in administration routes might affect the outcomes, and hence
therapeutic effects of NAC on the gut microbiota might be confounded by these factors. These limitations should be addressed in further studies.

3.6.9 Impact of high fat diet on the periphery and the CNS

To summarise, we showed that chronic high fat diet consumption mediated depressive-like behaviour, behavioural despair and anhedonia, indicative of core MDD symptoms in humans. Our findings supported the premise that poor diet consumption has a causal role in shaping depressive mood, a link strongly advocated by the epidemiological literature. In addition, high fat diet consumption resulted in significant alterations in the gut microbial community. Therefore, it was of great interest to examine how high fat diet mediated changes in the gut microbiota affect the immune system, and the glutamatergic system and subsequently contribute to depressive mood.

Evaluating current literature implies that high fat diet consumption might have a critical influence on shaping negative mood in adolescence and young adulthood. Furthermore, this could be attributed to high fat diet-elicited disruption of gut microbial communities which significantly differentiated from the healthy state. Alteration of gut microbiota was also documented in MDD populations and preclinical models of MDD. However, a diverse range of analytical methods to assess gut microbiota and inconsistent patterns of presenting findings across the literature posed significant challenges to collate the findings from these studies.

Intriguingly, HFD did not affect the inflammatory state, oxidative biology, glutamate system or BBB integrity despite HFD mice exhibiting depressive-like behaviour. Collating available
evidence and our findings resulted in several possibilities; firstly, HFD feeding may mediate subtle shifts in the inflammatory state however, we were unable to detect such changes due to insufficient sensitivity of our method; secondly, HFD may change immune responsivity to a systemic challenge rather than the basal inflammatory state; and thirdly, HFD-induced depressive-like behaviour might involve alternate mechanisms. Given the heterogeneity of MDD, it is possible that our study replicated a subtype of MDD by using the chronic high fat diet feeding paradigm, and highlighted the significant importance of diet on the health of the body and the brain.

Nevertheless, our study has some limitations. We acknowledge that our methodological issues in 16S sequencing provided insufficient information to comprehensively compare and evaluate the changes in gut microbial communities and hence, restricted our in-depth analyses. On the other hand, our study clearly demonstrated that poor quality diet has a capability to shape depressive mood. Analyses of contributing factors to the diet-induced depression in our study implicated the complexity of underpinning mechanisms of this disease and further investigations are warranted. The biological link between diet and MDD found in this study sheds light on our current knowledge of MDD. The clinical significance of this study highlights potential anti-depressant effects of dietary intervention on MDD.

3.7 Conclusions
Our study demonstrated that high fat diet consumption resulted in depressive-like behaviour that was associated with significant alterations of the gut microbial community, including a reduction of the number of bacteria and changes in diversity. Chronic HFD feeding mediated two key facets of MDD core symptoms, behavioural despair and anhedonia in rodents,
equivalent to depressive mood and a lack of pleasure in humans respectively. Our finding provided support for the concept that poor diet consumption precedes depressive mood. Furthermore, findings from this study raise the possibility that a healthy diet can potentially be used as a novel intervention for MDD. Interestingly, HFD fed animals did not exhibit florid inflammation, excessive oxidative stress, disruption of the BBB integrity or dysfunction of glutamatergic neurotransmission, which indicates a possibility that HFD-mediated depressive mood interacted with other pathways. This speculation was further emphasised by our finding that NAC treatment did not result in a reversal of HFD-induced depressive-like behaviour, despite documentation that the therapeutic effects of NAC were mediated by these factors.

Another significant finding in our study was that HFD feeding significantly altered the gut microbial community from the original state, which occurred in parallel with development of depressive mood. Although further in-depth analysis is required to elaborate HFD-mediated changes in gut microbiota and the underpinning mechanisms to connect gut microbiota and behaviour, our study implies that changes in gut microbiota potentially shape depressive mood. In addition, we documented effects of NAC on gut microbiota which shed light on currently unknown effects of NAC on gut microbiota. Our findings indicated that NAC mediated subtle changes in the gut microbial community compared to HFD-induced changes. What is clear from this study is that a HFD can result in behavioural changes which resemble ‘behavioural despair’ and anhedonia in rodents, which are considered to be core symptoms of MDD in humans; and that these are associated with striking shifts in the gut microbial population. This highlights the critical role the gut microbiome might play in regulating
behaviour and the necessity of considering drug effects on gut microbiota when developing new therapeutics.
Chapter 4

Therapeutic effects of minocycline on high fat diet-induced depressive-like behaviour
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4.1 Introduction

Minocycline (MINO) is a broad-spectrum tetracycline antibiotic which has been investigated for its potential as an antidepressant via modulation of neuroinflammation and suppression of microglial activity (Henry et al. 2008; Zheng, Kaneko, and Sawamoto 2015; Garrido-Mesa, Zarzuelo, and Galvez 2013). MINO ameliorates the depressive-like behaviour observed in various animal models of MDD (Henry et al. 2008; Majidi, Kosari-Nasab, and Salari 2016; Liu et al. 2015). Adjunctive treatment with MINO also alleviated depressive symptoms in MDD (Miyaoka et al. 2012) and negative symptoms of schizophrenia (Ghanizadeh et al. 2014; Khodaie-Ardakani et al. 2014). Furthermore, MINO is anticipated to influence the gut microbiota as it is an antibiotic, which may contribute to MINO’s putative antidepressant effects. For instance, chronic restraint stress-induced depressive-like behaviour in mice was reversed by MINO, and this was associated with changes in gut microbiota (Wong et al. 2016), which suggests that MINO might exert its antidepressant efficacy not only through regulation of inflammation but also via its antimicrobial effects. Currently, the specific effects of MINO on the gut microbiome are largely undocumented. Most importantly, if MINO has a significant influence on the gut microbiome, how these changes are associated with depressive-like behaviour and changes in inflammatory pathways is of great interest in this study.

4.2 Aims

This study investigated the effects of MINO on high fat diet-induced depressive-like behaviour, peripheral and central inflammatory status, and the gut microbiome. We hypothesised that MINO would reverse high fat diet-induced depressive-like behaviour via a combination of regulating inflammatory status and altering the gut microbiome.
4.3 Materials and methods

This experiment shared the same control groups, standard diet and vehicle group (SD/Veh) and high fat diet and vehicle group (HF/Veh) with Chapter 3. Thus, methods and materials used for this study were described in Chapter 3. Four separate cohorts of animals were used, with each cohort including minocycline (MINO) and N-acetylcysteine (NAC) treatment groups. There were no variations by cohort in any of the main experimental outcomes, so the data was pooled across cohorts. As the results of our investigation into the two agents are in the process of being prepared for publication as separate papers, they are presented as separate chapters in the thesis. Hence, statistical analysis was performed separately in Chapter 3 and 4, however the same SD/Veh and HF/Veh groups were used as baseline. The outcomes of the experiment as a whole and comparisons between the two agents will be discussed in Chapter 5.

4.4 Drugs

Minocycline hydrochloride (MINO) (PCCA, Houston, USA) was prepared in 0.9% saline at concentrations of 10mg/ml for dosing at 50mg/kg of MINO per day. Dosage was determined every day by body weight, between 130ul and 210ul. Solutions were pH-matched to that of the vehicle (dosage 200ul). MINO and saline were orally gavaged daily for a 4-week period, which corresponded to weeks 14 – 17 after the assigning of HFD and SD groups.
4.5 Results

4.5.1 MINO did not affect body weight, fat mass or lean mass

MINO treatment did not mediate changes in body weight (Two-way ANOVA; main effect of treatment, $F_{(1,68)} = 0.67, p > 0.05$; Figure 4.1) or fat mass (main effect of treatment, $F_{(1,68)} = 0.44, p > 0.05$; Figure 4.2). Lean mass was also not affected by MINO (main effect of treatment, $F_{(1,68)} = 0.006, p > 0.05$; Figure 4.3). MINO and HFD did not interact to affect body weight (diet x drug treatment interaction, $F_{(1,68)} = 0.1, p > 0.05$), fat mass (diet x drug treatment interaction, $F_{(1,68)} = 2.04, p > 0.05$) or lean mass (diet x drug treatment interaction, $F_{(1,68)} = 2.31, p < 0.05$).

On the other hand, HFD fed mice displayed significantly heavier body weight than SD controls, and this effect was equivalent within both the vehicle and MINO treated groups (Two-way ANOVA; main effect of diet, $F_{(1,68)} = 178.2, p < 0.0001$). HFD induced a similar increase in fat mass (main effect of diet, $F_{(1,68)} = 325.0, p < 0.0001$). In contrast, lean mass was not affected by HFD (main effect of diet, $F_{(1,68)} = 3.08, p > 0.05$).
Figure 4.1 Body weight after HFD feeding followed by MINO treatment. Two-way ANOVA indicated a main effect of diet, a significantly higher body weight in HFD groups compared to SD groups (p < 0.0001). Data are mean ± s.e.m. Veh = vehicle group; MINO = minocycline treatment group; **** p < 0.0001 (n=18)

Figure 4.2 Fat mass after HFD feeding followed by MINO treatment. Two-way ANOVA indicated a main effect of diet, a significantly higher fat mass in HFD groups compared to SD groups (p < 0.0001). Data are mean ± s.e.m. Veh = vehicle group; MINO = minocycline treatment group; **** p < 0.0001 (n=18)
Figure 4.3 Lean mass after HFD feeding followed by MINO treatment. Two-way ANOVA indicated diet and MINO did not affect lean mass. Data are mean ± s.e.m. Veh = vehicle group; MINO = minocycline treatment group; (n=18)
4.5.2 High fat diet and MINO mediated alteration in gut microbiota

Examination of our 16S sequencing data revealed that the read length was 100 base pairs on average instead of the expected 300 base pairs. This issue limited our capacity to conduct further in-depth analysis down to class or genus levels. Thus, the following 16S rRNA analyses in this study were based on bacterial phyla.
4.5.2.1 Alpha diversity (Chao1 richness index)

Alpha diversity is a measurement to assess structure and function of microbial diversity within a community (a sample) which accounts for the number of species, commonness and rareness of abundant bacteria (Lozupone and Knight 2008). A Kruskal-Wallis test followed by Dunn’s multiple comparison test indicated that Chao 1 richness index suggested a significant reduction of richness in the gut microbial diversity in HF/Veh mice compared with SD/Veh mice ($p < 0.05$; Figure 4.4). MINO treatment also contributed to a significant reduction of diversity of the gut microbiota in SD/MINO mice ($p < 0.001$) and HF/MINO mice ($p < 0.01$).

**Figure 4.4 Chao 1 richness index**

**Figure 4.4 Chao 1 richness index (alpha diversity).** Upper and lower quartiles defined the box with median midline, and the whiskers were assessed using Tukey’s method. A Kruskal-Wallis test followed by Dunn’s multiple test showed that HFD/Veh mice exhibited a statistically significant reduction in Chao 1 alpha diversity compared with SD/Veh microbiota. Similarly, HFD/MINO mice also showed significantly less diverse gut microbiota compared to HFD/Veh group. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n= 9-17
4.5.2.2 Principal coordinate analysis (PCoA)

Principal coordinate analysis (PCoA) is a method to assess similarities or dissimilarities of the gut microbiome communities which takes into account relative abundance of bacteria of each sample within each treatment group. The PCoA of the weighted UniFrac Matrix (Figure 4.5) showed the gut microbiota in SD/Veh, SD/MINO, HFD/Veh and HFD/MINO clustered distinct from each other. HFD (PC1) explained 45.3% of the variation and MINO treatment (PC2) explained 15.6% of the variation between groups. A weighted distance-based analysis of molecular variance (AMOVA) was used to assess the statistical significance of the spatial separation observed between treatment groups. Statistically significant dissimilarities were observed between SD/Veh, HFD/Veh, SD/MINO and HFD/MINO groups with respect to bacterial diversity ($p = 0.001$).
Figure 4.5 Principal coordinate analysis (PCoA) of weighted UniFrac matrix. HFD/Veh, HFD/MINO, SD/Veh and SD/MINO groups clustered separated from each other. Percent of dataset variability explained by each principal coordinate is shown in brackets in axis titles. Each data point represents a single sample.
4.5.2.3 Relative abundance at phylum level

Figure 4.6 showed the relative abundance of bacterial phyla in each treatment group. At the phylum level, there were not notable changes in bacterial phyla distribution between groups.

**Figure 4.6 Relative abundance at phylum level**

![Relative abundance at phylum level](image)

**Figure 4.6 Relative abundances of bacterial phyla in each treatment group.** Numbers in the bar chart represent proportions of the relative abundance of each phylum.
4.5.2.4 Firmicutes to Bacteroidetes (F/B) ratio

Firmicutes and Bacteroidetes are two dominant phyla of the gut microbiota. Firmicutes to Bacteroidetes (F/B) ratio (Figure 4.7) is used to assess differences in the main composition of the gut microbiota. A Kruskal-Wallis test followed by Dunn’s multiple comparison test indicated that HFD significantly increased the F/B ratio compared to the gut microbiota in SD/Veh mice ($p < 0.001$). In addition, MINO treatment mediated a significant increase in the F/B ratio in HFD mice compared to SD/MINO mice ($p < 0.05$).

Figure 4.7 Firmicutes to Bacteroidetes ratio

![Box and whiskers plot](image)

**Figure 4.7 Firmicutes to Bacteroidetes (F/B) ratio.** Box and whiskers plot displayed the analysis of the differences of the main composition of the microbiota (Firmicutes to Bacteroidetes). A Kruskal-Wallis test indicated HFD significantly increased F/B ratio compared to SD groups overall. Upper and lower quartiles defined the box with median midline, and the whiskers were assessed using Tukey’s method. ***$p < 0.001$; *$p < 0.05$; n= 9-17.
4.5.3 Effects of MINO on inflammatory mediators in the periphery

Two-way ANOVA indicated that HFD significantly increased plasma levels of LBP (main effect of diet, $F_{(1,67)} = 4.70$, $p < 0.05$), compared to SD fed mice (Figure 4.8). In addition, MINO treatment significantly decreased plasma LBP levels (main effect of treatment, $F_{(1,67)} = 5.73$, $p < 0.05$; diet x drug interaction, $F_{(1,67)} = 0.18$, $p > 0.05$). On the other hand, both HFD and MINO treatment did not affect the levels of soluble CD14 in plasma (Two-way ANOVA; main effect of diet, $F_{(1,46)} = 0.003$, $p > 0.05$; main effect of treatment, $F_{(1,46)} = 0.038$, $p > 0.05$; diet x drug interaction, $F_{(1,46)} = 0.28$, $p > 0.05$; Figure 4.9).

Plasma concentrations of IL-6 and TNFα were assessed using ELISA kits. However, the readings of plasma IL-6 and TNFα concentrations were below or equal to the lowest standard point and thus, we were unable to conduct statistical analysis.

Figure 4.8 Plasma LBP levels

Figure 4.8 Plasma LBP levels. Two-way ANOVA indicated a main effect of diet ($p < 0.05$) and a main effect of MINO treatment ($p < 0.05$). Data are mean ± s.e.m. Veh = vehicle group; MINO = minocycline treatment group; (n=14-22)
Figure 4.9 Plasma soluble CD14 levels

Two-way ANOVA indicated there were no main effects of diet or treatment. Data are mean ± s.e.m. Veh = vehicle group; MINO = minocycline treatment group; (n= 14-22)
4.5.4 Effects of MINO on inflammatory mediators in the CNS

As markers of glial activation, levels of \textit{Iba1} and \textit{MHC class2} in the hippocampus and the hypothalamus were assessed using real time QPCR. In the hypothalamus, two-way ANOVA indicated that MINO treatment significantly reduced levels of \textit{GFAP} expression (main effect of treatment, $F_{(1,31)} = 8.76, p < 0.01$; Table 4.1). However, post hoc comparison only detected a significant downregulation of hypothalamic \textit{GFAP} expression levels in SD/MINO mice compared to SD/Veh mice ($t = 2.59, p < 0.05$). The effect was independent from HFD (main effect of diet, $F_{(1,31)} = 2.89, p > 0.05$; diet x drug interaction, $F_{(1,31)} = 0.62, p > 0.05$). Levels of \textit{Iba1} expression did not alter with either HFD nor MINO in the hypothalamus (Two-way ANOVA; main effect of diet, $F_{(1,32)} = 0.07, p > 0.05$; main effect of drug $F_{(1,32)} = 0.70, p > 0.05$; diet x drug interaction, $F_{(1,32)} = 0.02, p > 0.05$).

In the hippocampus, levels of \textit{Iba1} expression did not alter with either HFD nor MINO (Two-way ANOVA; main effect of diet, $F_{(1,34)} = 0.27, p > 0.05$; main effect of treatment, $F_{(1,34)} = 0.83, p > 0.05$; diet x drug interaction, $F_{(1,34)} = 0.91, p > 0.05$) nor \textit{GFAP} levels (Two-way ANOVA; main effect of diet, $F_{(1,34)} = 0.28$; main effect of drug, $F_{(1,34)} = 0.04, p > 0.05$; diet x drug interaction, $F_{(1,34)} = 0.24, p > 0.05$).
Table 4.1 Impact of HFD feeding and MINO treatment on gene expression in the hippocampus and hypothalamus

<table>
<thead>
<tr>
<th>Genes</th>
<th>Hippocampus</th>
<th>Hypothalamus</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SD/Veh</td>
<td>SD/MINO</td>
</tr>
<tr>
<td><em>Iba1</em></td>
<td>1.000 ± 0.3708</td>
<td>0.5445 ± 0.1353</td>
</tr>
<tr>
<td><em>GFAP</em></td>
<td>1.000 ± 0.2096</td>
<td>1.1680 ± 0.2460</td>
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Table 4.1 Gene expression levels of glial activation markers in the hippocampus and hypothalamus. MINO treatment significantly decreased GFAP gene expression levels overall (Two-way ANOVA, \( p < 0.01 \)). HFD did not alter gene expression levels in the hypothalamus. Results are expressed as relative fold change in mRNA expression compared with the SD/Veh group; means ± S.E.M; \( n = 7-11 \). *\( p < 0.05 \) compared to SD/Veh group.
4.5.5 MINO did not reverse high fat diet-mediated depressive-like behaviours

Two-way ANOVA indicated that HFD mice spent significantly more time immobile in the FST (main effect of diet, $F_{(1,76)} = 12.11, p < 0.001$), compared to SD mice (Figure 4.10). This effect was consistent in both the vehicle and MINO treated mice (diet x drug treatment interaction, $F_{(1,75)} = 0.18, p > 0.05$), however post hoc comparisons only detected a significant difference between diet groups within vehicle treated mice ($t= 2.91, p < 0.01$). MINO treatment did not reverse the time spent immobile (main effect of drug treatment, $F_{(1,76)} = 2.54, p > 0.05$) which was elicited by the HFD.

Figure 4.10 Forced swim test: Duration immobile

**Figure 4.10 Duration of immobility in the Porsolt forced swim test.** Two-way ANOVA indicated a main effect of diet ($p < 0.001$). HFD groups displayed significantly longer duration of immobility during the FST compared to SD groups. Post hoc comparison indicated HFD/Veh group had significantly longer duration of immobility than the SD/Veh group ($p < 0.01$). Data are mean ± s.e.m. Veh = vehicle group; MINO = minocycline treatment group; ** $P <0.01$ (n=18-22)
Interestingly, both HFD and MINO treatment significantly increased the latency to the first period of immobility (main effect of diet, $F_{(1,75)} = 4.74, p < 0.05$; main effect of treatment $F_{(1,75)} = 5.31, p < 0.05$; diet x drug interaction, $F_{(1,75)} = 0.67, p > 0.05$; Figure 4.11).

**Figure 4.11 Forced Swim Test: Latency to first period of immobility**

![Graph showing latency to first period of immobility for Veh and MINO groups.]

**Figure 4.11 Latency to first period in the Porsolt forced swim test.** Diet and MINO treatment had significant impact on latency to first period of immobility, with a main effect of diet ($p < 0.05$) and a main effect of treatment ($p < 0.05$) (Two-way ANOVA). (n=18-22).
Because increased immobility might conceivably result from increased fat mass (rather than diet-induced neurological/behavioural changes), a correlation analysis between fat mass and the duration of immobility was performed. However, a linear regression model indicated that fat mass was not significantly associated with the time spent immobile in HFD fed mice (F(1, 38) = 0.11, p > 0.05; Figure 4.12).

Figure 4.12 Correlation between fat mass and duration of immobility

![Figure 4.12 Fat mass was not associated with duration of immobility. Linear regression model indicated that fat mass in HFD mice did not predict longer duration of immobility. R square = 0.003; p > 0.05; n = 18-22.](image)
4.5.5.1 MINO did not affect consumption of sucrose solution

MINO treatment did not affect sucrose consumption (Two-way ANOVA; main effect of drug treatment, $F_{(1,36)} = 1.48, p > 0.05$; diet x treatment interaction, $F_{(1,36)} = 0.02, p > 0.05$; Figure 4.13). HFD mice consumed significantly less sucrose solution in the SPT compared to SD mice overall (main effect of diet, $F_{(1,36)} = 13.89, p < 0.001$), and within each drug treatment group (Post hoc pairwise comparisons, within vehicle treated mice, $t=2.89, p < 0.05$; within MINO treated mice, $t=2.41, p<0.05$).

**Figure 4.13 Sucrose preference test: sucrose consumption**

*Figure 4.13 Sucrose consumption overnight.* HFD had a main effect on sucrose consumption overnight (Two-way ANOVA, $p < 0.001$). HFD/Veh group and HFD/MINO group consumed significantly less sucrose solution compared with the SD/Veh and SD/MINO groups, respectively. Data are mean ± s.e.m. * $p < 0.05$ (n=18-22)
4.5.5.2 MINO altered time spent in the centre of the OFT

MINO treatment significantly increased the time spent in the centre zone in the OFT overall (Two-way ANOVA; main effect of drug treatment, $F_{(1,76)} = 10.37, p < 0.01$; Figure 4.14), and this effect was most noticeable within HFD fed mice (Post hoc comparison, within HFD between drug treatments, $t = 1.23, p < 0.01$). In contrast, diet did not affect the time spent in the centre region (main effect of diet, $F_{(1,76)} = 0.08, p > 0.05$; diet x drug treatment interaction, $F_{(1,76)} = 1.81, p > 0.05$).

Figure 4.14 Open field test: Time spent in centre

![Figure 4.14 Time spent in the centre of the open field test.](image)

**Figure 4.14 Time spent in the centre of the open field test.** MINO had a main effect on time spent in the centre (Two-way ANOVA, $p < 0.01$). HFD/MINO group spent significantly longer duration in the centre zone compared to HFD/Veh group. Data are mean ± s.e.m. n=18-22. ** $p < 0.01$
Two-way ANOVA indicated that MINO treatment did not affect the distance travelled (main effect of drug treatment, $F_{(1,76)} = 0.84, p > 0.05$; Figure 4.15), suggesting that the increased time that MINO treated mice spent in the centre region (see above) was not simply due to hyperactivity. HFD mice travelled significantly less distance compared to SD controls (main effect of diet, $F_{(1,76)} = 7.83, p < 0.01$). This effect was similar within both vehicle and MINO treatment groups (diet x drug treatment interaction, $F_{(1,76)} = 0.63, p > 0.05$), despite only reaching statistical significance within vehicle treated mice (post hoc comparison, within vehicle between diets, $t=2.68, p < 0.05$). Although fat mass can potentially alter patterns of locomotor activity, correlation analysis using a linear regression model indicated that travel distance and fat mass in HFD/Veh and HFD/MINO mice were not associated ($F_{(1,38)} = 0.50, p > 0.05$; Figure 4.16).

Figure 4.15 Open field test: travel distance

![Figure 4.15 Travel distance in the open field test.](image)

HFD had a main effect on travel distance (Two-way ANOVA, $p < 0.01$). HFD/Veh group travelled significantly less distance compared to SD/Veh group. Data are men ± s.e.m. n=18-22. * $p < 0.05$
Figure 4.16 Open field test: Correlation between travel distance and fat mass

Figure 4.16 Correlation between travel distance and fat mass. Travel distance during the open field test was not associated with fat mass in HFD groups (Linear regression model). R square = 0.013; n = 12-14
Duration of mobility was defined as the total amount of time that the animal was moving in the field during the OFT. MINO treatment did not alter duration of mobile (Two-way ANOVA; main effect of treatment, $F_{(1,76)} = 2.02, p > 0.05$; diet x drug interaction, $F_{(1,76)} = 0.19, p > 0.05$; Figure 4.17). HFD mice were significantly less mobile compared to SD controls overall (main effect of diet, $F_{(1,76)} = 19.52, p < 0.0001$), and within each drug treatment group (post hoc pairwise comparisons, within vehicle treated mice, $t = 3.62, p < 0.01$; within MINO treated mice, $t = 2.69, p < 0.05$).

**Figure 4.17 Open field test: Duration spent mobile**

![Duration mobile in the open field test](image_url)

**Figure 4.17 Duration mobile in the open field test.** Diet had a main effect on mobility during open field test (Two-way ANOVA, $p < 0.0001$). SD/Veh and SD/MINO groups were more active than HFD/Veh and HFD/MINO groups in the field. n=18-22. Data are mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$
Interestingly, HFD mice moved significantly faster compared to SD mice control overall (Two-way ANOVA; main effect of diet, $F_{(1,76)} = 19.41, p < 0.0001$; Figure 4.18), and within each drug treatment group (post hoc pairwise comparisons, within vehicle treated mice, $t = 3.71, p <0.001$; within MINO treated mice, $t = 2.59, p <0.05$), indicating that HFD mice were faster than SD mice while they were moving, despite the reduced duration of mobility and distance travelled. MINO treatment did not affect speed (main effect of treatment, $F_{(1,76)} = 2.89, p > 0.05$; diet x drug interaction, $F_{(1,76)} = 0.33, p > 0.05$). A linear regression model showed no association between speed and fat mass within HFD fed mice ($F_{(1,38)} = 1.59, p > 0.05$; Figure 4.19).

Figure 4.18 Open field test: Speed

![Graph showing moving speed during the open field test.](image)

**Figure 4.18 Moving speed during the open field test.** High fat diet had a main effect on moving speed during the open field test (Two-way ANOVA, $p < 0.0001$). HFD/Veh and HFD/MINO groups were faster than SD/Veh and SD/MINO groups. n=18-22. Data are mean ± s.e.m. * $p < 0.05$, *** $p < 0.001$
Figure 4.19 Open field test: Correlation between speed and fat mass

Figure 4.19 Correlation between speed and fat mass. Moving speed during the open field test was not associated with fat mass in HFD groups (Linear regression model). R square = 0.042; $p > 0.05$; $n = 12-14$
4.6 Discussion

This study examined the effect of MINO on behavioural changes, inflammatory status and gut microbiome alterations elicited by a HFD. We observed depressive-like behaviours and changes in gut microbiota induced by HFD, as discussed in Chapter 3. MINO appeared to alter the gut microbial community but did not reverse diet-induced behavioural changes.

4.6.1 Effects of MINO on behaviour

MINO mediated two behavioural changes. Firstly, MINO treatment caused significantly longer latency to the first period of immobility in the FST. It appears that MINO might have mediated changes in locomotor activity rather than depressive-like behaviour, considering that duration of immobility was not affected by the treatment. Secondly, MINO significantly increased the time spent in the centre zone in HFD mice of the OFT. Given that MINO did not affect the distance travelled, duration of mobility or speed in the OFT, the increased time that MINO treated HFD mice spent in the centre region of the OFT indicates that the change may not be simply due to changes in locomotor activity, may be suggesting a possible reduction in anxiety-like behaviour. However, it has been criticised that the interpretation of activity in the centre zone in the OFT in terms of anxiety is oversimplification and may not valid due to its’ limited validity and lack of reproducibility of the OFT (Spruijt et al. 2014; Walsh and Cummins 1976). Thus, other behavioural tests which are more suitable to assess anxiety-like behaviour, such as the light/dark box or the elevated plus maze, may need to determine whether this behavioural change is related to anxiety-like behaviour in future investigation.

Antidepressant-like effects of MINO in other preclinical studies have shown that the therapeutic effects of MINO appear to be associated with the regulating inflammatory state.
For instance, Henry et al. (2008) showed that pre-treatment of MINO (50mg/kg via an intraperitoneal injection for three days) facilitated a recovery of depressive-like behaviour; anhedonia, after LPS challenge. The mice challenged by LPS exhibited anhedonic behaviour with significant increases in IL-6 and IL-1β mRNA expression in the hippocampus and cortex, whereas protection from anhedonic behaviour in MINO pre-treated mice was associated with reductions in the levels of IL-6 and IL-1β mRNA expression in the hippocampus and cortex (Henry et al. 2008). In addition, MINO treatment (30mg/kg by an intraperitoneal injection for 4 weeks) prevented development of chronic stress-induced depressive-like behaviour in mice (measured by the FST and the TST) and anhedonic behaviour in the SPT (Liu et al. 2015). This stress-induced depressive-like behaviour was concomitant with significant elevations of pro-inflammatory cytokines, TNFα, IL-1β and IL-18, and MINO treatment blocked the elevation of these mediators.

These studies suggest that MINO exerts anti-depressant effects on depressive-like behaviour via regulating florid inflammatory states. To support this premise, an acute injection of MINO in healthy mice did not evoke any anxiolytic or antidepressant effects on behaviours in the FST, the OFT, the elevated O-maze or the light-dark box, in contrast to the commonly prescribed antidepressants, diazepam and imipramine (Vogt et al. 2016).

Our findings indicated that HFD-induced depressive-like behaviour was not associated with a pro-inflammatory state in the periphery and CNS, yet it does not exclude the possibility that there might be subtle shifts towards a pro-inflammatory state or an increase in susceptibility to low-grade inflammation in HFD mice. This is only speculative given that MINO treatment increased the time spent in the centre zone of the OFT in HFD mice which may be indicative of behavioural changes due to a possible reduction of anxiety-like behaviour. At the same
time, however, a possibility remains that this behavioural change elicited by MINO may not be related to an anxiolytic effect, as other diet-induced behaviours in the OFT, FST and SPT were not reversed by MINO treatment. Instead, our study demonstrated depressive-like behaviour is not necessarily concomitant with florid inflammation.

4.6.1.1 Antidepressant-like effects of MINO: Current literature

Another possible therapeutic mechanism of action of MINO might be synergistic effects when used in conjunction with antidepressants. A clinical study assessing the effects of adjunctive MINO treatment on MDD patients reported a significant improvement of depressive symptoms (Miyaoka et al. 2012). Adjunctive MINO treatment also exerted antidepressant-like effects on the negative symptoms of patients with schizophrenia (Ghanizadeh et al. 2014; Khodaie-Ardakani et al. 2014). In addition, a double-blind, randomised and placebo-controlled clinical trial that assessed effects of adjunctive MINO treatment for MDD indicated positive effects on depressive symptoms (Dean et al., accepted by Australian & New Zealand Journal of Psychiatry, in press). Analyses using biological samples collected from this trial to identify underlying pathways contributing to the therapeutic effects of MINO are currently underway.

In line with this evidence, a preclinical study showed add-on effects of MINO in combination with antidepressants on depressive-like behaviour in the FST (Molina-Hernandez et al. 2008). Co-administration of MINO with sub-threshold doses of desipramine, mGluR1 antagonist EMQMGM, mGluR5 antagonist MTEP and NMDA receptor antagonist dizocilpine, exerted enhanced therapeutic effects on behavioural despair in the FST compared to single administration of these drugs.
In addition to anti-inflammatory effects shown in several preclinical studies, the antidepressant-like effect of MINO has been attributed to modulating the glutamatergic system (Pae et al. 2008) since MINO reduces glutamate neurotransmission (Morimoto et al. 2005), via inhibiting microbial activation (Riazi et al. 2015) and via enhancing glial glutamate transport (Darman et al. 2004; Nie, Zhang, and Weng 2010). Desipramine is an antidepressant that increases noradrenaline levels. Hence, the observation in Molina-Hernandez et al. (2008) that a significant decrease in immobility time in the FST might result from a synergistic effect of increased levels of noradrenaline elicited by desipramine and glutamate levels regulated by MINO. On the other hand, EMAMGM, MTEP and dizocilpine are glutamate antagonists and showed antidepressant-like effects on immobility time in the FST (Molina-Hernandez et al. 2008). Augmentation of MINO to these glutamate antagonists might exert neuroprotective effects on not only glial glutamate transporters but also pre- and post-synaptic glutamate neurotransmission, thus, it may result in a reduction of depressive-like behaviour in the FST. Interestingly, a serotonergic antidepressant fluoxetine did not interact with MINO on immobility time in the FST (Molina-Hernandez et al. 2008).

Taken together, therapeutic effects of MINO on behaviour might differ when used as a monotherapy as compared to adjunctive therapy. In our study, MINO was used as a monotherapy and our HFD mice did not exhibit florid inflammatory state nor dysfunction of the glutamatergic system. This implies that the aetiology of depressive-like behaviour in HFD mice may not be associated with pathways on which MINO can exert antidepressant-like effects and may suggest a subtype of MDD.
4.6.2 MINO as an antibiotic agent

4.6.2.1 Effects of MINO on gut microbiota as a broad-spectrum antibiotic

Our study showed significant shifts in the gut microbial population in response to MINO treatment, indicative of effects of MINO as a broad-spectrum antibiotic. MINO treatment resulted in a significant reduction of the number of species (Chao 1 richness index) and membership of abundant bacteria within the composition of the gut microbial community in the SD/MINO and HFD/MINO groups of mice.

Furthermore, analysis of weighted UniFrac PCoA analysis showed clear separation between the four treatment groups, indicating that both diet and MINO exerted significant effects on the microbial communities. Intriguingly, MINO mediated significant changes in membership of the abundant bacteria and the gut microbiome community profiles in both HFD and control animals, rather than restoring the profile of the HFD fed mice to the original state. These results were confirmed using AMOVA on weighted UniFrac distances, which indicated significant group differences in the microbial communities \((p = 0.001)\).

Weighted UniFrac distance accounts for not only absence and presence of taxa but also the number of times that each taxon was observed (Lozupone and Knight 2008). Thus, the profile shifts between the microbial communities in this study were driven by a combination of changes at the OTU-level of the community and shifts in the relative abundance of the taxa. The findings in our study highlighted significant effects of MINO on the gut microbiota community via changing gut microbial profile.
4.6.2.2 Effects of MINO on gut microbiota as a bacteriostatic agent

On the other hand, the effect of MINO on the relative abundances of the two major phyla were relatively similar within the SD groups (Bacteroidetes, vehicle versus MINO treated: 61% versus 60%; Firmicutes, vehicle versus MINO treated: 21% versus 17%) and in the HFD groups (Bacteroidetes, vehicle versus MINO treated: 63% versus 63%; Firmicutes, vehicle versus MINO treated: 22% versus 22%), indicating the absence of a straightforward shift in the structure of the community. However, MINO treatment significantly increased F/B ratio in the HFD/MINO group compared to the SD/MINO group. Our results suggest that MINO might have differential effects on SD and HFD fed gut microbiota.

The findings here suggest that different key populations in the SD and HFD fed gut microbiota might be affected by MINO treatment and in turn, may result in different alterations in the balance of the memberships of abundant bacteria between the SD and HFD fed gut microbiome communities. Our findings highlighted that MINO exerted a strong influence on the gut microbiota via changing memberships of abundant bacteria within the community. This intriguing finding needs to be further elaborated by what drives the differential effects of MINO, as well as evaluations of deeper levels of bacterial compositions, such as class or species levels, in future studies.

4.6.2.3 Effects of MINO on gut microbiome: Current literature

Surprisingly, despite the clinical use of MINO as an antibiotic, the specific effects of MINO on the gut microbiota has been documented in very few studies. MINO treatment (via intraperitoneal injection, 5mg/kg, 21 days) in parallel with chronic restraint stress prevented stress-induced depressive-like behaviour in the FST (Wong et al. 2016). Whereas F/B ratio did
not differ between each treatment group (i.e., saline x no stress, MINO x no stress, saline x stress and MINO x stress), patterns of the relative abundance in each treatment group were different from each other. This indicates that a prevention of stress-induced depressive behaviour by MINO was not necessarily associated with restoring the relative abundance of gut microbiota.

Interestingly, the pattern of the relative abundance in saline-treated stressed animals was similar to the pattern of the relative abundance in MINO-treated non-stressed animals. While saline-treated and stressed animals exhibited depressive-like behaviour, MINO-treated non-stressed animals did not exhibit depressive-like behaviour. The contrasting behaviour yet the similar relative abundance in these two differently treated groups in Wong et al. (2016) implies a complexity of this ecosystem and hence, relying solely on the F/B ratio and levels of relative abundance may potentially misguide the characterisation of the relationship between gut microbiome and behaviour, since abundance levels of bacteria within a community are not necessarily equal (Lozupone and Knight 2008). Our study showed that HFD and MINO induced changes in the gut microbiome via significant changes in Chao 1 index (alpha diversity), PCoA (beta diversity) and the F/B ratio.

On the other hand, we did not observe changes in relative abundance levels. Therefore, how differences in relative abundance levels reflect other measurements such as alpha diversity and beta diversity was unclear. Experimental and environmental differences in our study and Wong et al. (2016) also need to be taken into account such as dosage of MINO (5mg/kg in Wong et al. (2016) and 50mg/kg in our study), modes of drug delivery (intraperitoneal injection vs. oral gavage), duration of drug delivery (21 days vs. 28 days), timing of drug delivery (prevention vs. intervention), animal model used (chronic restraint stress vs high fat
diet feeding) and differences in animal facilities could contribute to the different findings. However, our study concurs with Wong et al. (2016) on two points, firstly MINO did not restore gut microbiome to the original state; and secondly, changes in the gut microbiome are not necessarily associated with changes in behaviour.

Yang et al. (2015) examined effects of MINO on the gut microbiome in an animal model of hypertension. The gut microbiota in hypertensive rats was characterised by a significant reduction of Chao1 alpha diversity and a significant increase in the F/B ratio compared with the control group. Chronic MINO treatment (50mg/kg via oral gavage, four weeks) significantly lowered blood pressure. This was associated with a significant decrease in the F/B ratio with significantly lower relative abundance of Firmicutes and higher relative abundance of Bacteroidetes, yet the Chao1 alpha diversity was unaffected.

In addition, PCoA analysis showed that a cluster of the gut microbiome in the MINO treated hypertension group appeared to be distant from the gut microbiome in the control and hypertension groups (Yang et al. 2015). The authors reported that MINO restored dysbiosis in hypertension, however, levels of relative abundance of Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria remained at same levels between the control and MINO treated hypertension groups. Similarly, levels of these bacteria in the hypertension group did not differ from the control group. Deeper levels of analysis indicated that the MINO treated hypertension group exhibited significantly higher abundance levels of Akkermansia (a member of Verrucomicrobia), Bacteroides (a member of Bacteroidetes), Enterorhabdus (a member of Actinobacteria) and Marvinbryantia (a member of Firmicutes) compared with the control and hypertension groups.
In line with Wong et al. (2016) and our study, Yang et al. (2015) showed that MINO treatment mediated a different gut microbiome profile, instead of restoring the original gut microbiome community.

Taken together, broad-spectrum bacteriostatic effects of MINO were not associated with restoring the original ecosystem. MINO exerts a significant impact on a wide range of bacteria, however, it appears that the bacteriostatic effect of MINO mediates a shift in a balance of the gut microbiota by which it might slow down growth of existing bacteria, which in turn may provide opportunities for other bacteria to grow. Hence, the gut microbiota elicited by MINO treatment is different from both healthy (control) and diseased states. This raises the question of which underlying pathways link the changes in the gut microbiome elicited by MINO with health and disease in the host, as well as behavioural changes. Neither Yang et al. (2015) and Wong et al. (2016) documented underlying pathways linking lowered blood pressure (Yang et al.) or a reversal of depressive-like behaviour (Wong et al.) with MINO-induced changes in the gut microbiome.

Our findings showed that there were four different microbial communities resulting from HFD and MINO treatment, as evidenced by the PCoA, Chao1 index and F/B ratio. Amongst these four different gut microbiome communities identified in our study, only the gut microbiome in the HFD/Veh mice was associated with the behavioural changes. In next section, we investigated what underlying signalling pathways may be involved in conferring changes in gut microbiome on physiological and behavioural changes.
4.6.3 Effects of MINO on plasma LBP levels

In our study, MINO treatment significantly reduced the elevation of plasma LBP levels elicited by HFD. Our statistical analysis indicated that the effect of MINO treatment on plasma LBP levels did not differ between SD and HFD mice (a main effect of treatment). In other words, MINO reduced plasma LBP concentrations in not only the HFD mice but also the SD mice. These results suggest that the modulatory effects of MINO on plasma LBP concentrations might be attributed to a common biological mechanism shared in the SD and HFD mice, possibly changes in the proportion of LPS producing bacteria in the gut. LBP levels can also fluctuate with body weight: levels of LBP increase with weight gain and decrease with weight loss (Gonzalez-Quintela et al. 2013). However, our findings indicate that MINO equally reduced LBP levels in both the SD and HFD mice, and MINO did not affect body weight between each treatment group. Therefore, changes in levels of LBP in our study appears to be due to the changes in gut microbial composition elicited by MINO.

4.6.4 Effects of MINO on soluble CD14 levels

Levels of plasma soluble CD14 were not affected by MINO treatment. Significant elevations of soluble CD14 levels have been documented in autoimmune disease such as multiple sclerosis (Laugerette et al. 2011) and severe inflammatory disease such as non-alcoholic/alcoholic fatty liver disease (Kitabatake et al. 2017; Su et al. 1998; Zuo et al. 2001), which was concomitant with elevations of pro-inflammatory mediators (Bas et al. 2004). Our observation suggests that there were no transient elevations of pro-inflammatory cytokines, IL-6 and TNFα, in the periphery in the HFD mice, indicative of an absence of florid inflammation as found in severe inflammatory disease. In addition, as discussed earlier, MINO
appears to exert anti-inflammatory efficacy in models which display marked elevations of levels of inflammatory mediators. In contrast, our HFD mice did not exhibit elevated levels of pro-inflammatory cytokines. This may correlate with the lack of effect of MINO on soluble CD14 levels.

4.6.5 Effects of MINO on inflammatory state

Our findings indicated that the levels of IL-6 and TNFα in all treatment groups were in the normal range, and insufficient sensitivity of our methods precluded us from comparing levels of these pro-inflammatory mediators between treatment groups. Therefore, whether MINO subtly affects levels of IL-6 and TNFα remains unclear. Whilst the HFD-induced elevation of plasma LBP levels was ameliorated by MINO, which may be indicative of gut microbial signalling affecting a pro-inflammatory state, unchanged soluble CD14 levels indicates the interpretation of inflammatory state in these mice is not straightforward.

On the other hand, our study found that MINO induced a significant downregulation of hypothalamic GFAP expression, an indicator of astrocyte activation, in the SD/MINO mice, while a microglial activation marker, Iba1 expression, was not affected. This downregulation of GFAP expression in the hypothalamus indicates a potentially MINO-induced astrocyte functional deficit that may correlate with possibly morphological changes, cell loss or hypoactivity. However, this is only speculative as we do not have supportive evidence. In the hippocampus, on the other hand, GFAP and Iba1 expression were not affected by MINO. Our findings indicated that the SD/MINO mice did not exhibit behavioural changes that might associate with this downregulation of hypothalamic GFAP expression. Therefore, not yet
clear what underlying pathways are responsible for this downregulation and how this condition affects behaviour.

An intriguing recent study, however, might shed light on these somewhat contradicting findings in our study. Rothhammer et al. (2016) showed that metabolites from dietary tryptophan by the commensal bacteria regulate neuroinflammation via the ligand-activated transcription factor aryl hydrocarbon receptor (AHR) expressed on astrocytes in mice using an animal model of multiple sclerosis. The tryptophan-derived AHR antagonist, indoxyl-3-sulfate, synthesised through complex biochemical pathways catalysed by ampicillin-sensitive but vancomycin-resistant bacteria including *Lactobacillus reuteri*, regulated CNS inflammation by limiting NF-κB activity in a SOCS2-dependent manner (Rothhammer et al. 2016). This study indicates that diet and the gut microbiota could have modulatory effects on glial cell functions via its metabolites, and suggesting the importance of balanced commensal gut bacteria on central immune function.

In our study, MINO induced significant shifts in the profiles of gut microbiota and memberships of abundant bacteria in both the SD and HFD mice. Ampicillin and vancomycin are both bactericidal antibiotics, contrary to MINO as a bacteriostatic antibiotic. While vancomycin is a narrow-spectrum antibiotic, ampicillin is a broad-spectrum antibiotic which shares the same mechanism of action with MINO. Rothhammer et al. (2016) did not document how ampicillin and vancomycin treatment resulted in changes in the gut microbiota, however, one could speculate that MINO-induced changes in the balance of commensal bacteria via supressing growth of specific bacterial strains that are susceptible to a broad-spectrum antibiotics and a resultant reduction of metabolites from the bacterial strains may contribute to the significant downregulation of *GFAP* expression in the MINO
Our study showed that the significant reduction of Chao1 richness index, indicative of significant reductions of abundant bacterial memberships in the SD/MINO, HFD/Veh and HFD/MINO microbiota compared to the SD/Veh gut microbiota, which may be supportive of this speculation as it follows a similar trend to the downregulation of the hypothalamic GFAP gene expression in the SD/MINO, HFD/Veh and HFD/MINO mice compared to the SD/Veh mice. MINO also reduced microbial signalling, indicated by LBP levels, indicative of changes in a balance of gut microbiome community. However, these conclusions are only speculative, and additional methodologies will be required to elucidate this MINO-induced GFAP downregulation in future investigations.

### 4.7 Conclusions

Our study highlighted effects of MINO that significantly altered gut microbial profiles and relative abundances of bacterial phyla. MINO altered the balance of the gut microbiome in SD/Veh and HFD/Veh mice via reductions of bacterial abundance profiles and increases in the F/B ratio, and resultant profiles of the gut microbial community were significantly different from the original states. However, the MINO induced alterations of the gut microbial community were not associated with a reversal of depressive-like behaviour induced by a HFD. Our findings indicate that “change” per se in this complex ecosystem does not necessarily correlate with behavioural outcomes. Instead, certain populations within the gut microbiome may be of particular significance with regards to influencing CNS function and therefore behaviour. We need to develop a more in-depth understanding of exactly which elements of gut microbiota are critical and how these populations exist in balance in order to understand how the microbiome can shape mood. Our findings of no reversal of diet-induced depressive
behaviours by MINO does not preclude the beneficial effects of MINO on psychiatric disorders including MDD (Ghanizadeh et al. 2014; Khodaie-Ardakani et al. 2014; Miyaoka et al. 2012). Given that there is a discrepancy between clinical and preclinical studies regarding antidepressant effects on MINO, future studies need to take into account the synergistic effects of MINO with antidepressants. It also suggests the necessity of considering effects of MINO on the gut microbiota when developing new therapeutics and adjunctive treatment.
Chapter 5

Concluding discussion
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5.1 Overview

This project focused on investigating the underlying pathophysiological mechanisms of MDD with respect to diet, gut microbiome and inflammation. In addition, two novel therapeutic agents, NAC and MINO were examined in terms of their capacity to modulate high fat diet-induced changes in behaviour, gut microbiome and inflammation.

In the clinical component of this project, we evaluated therapeutic effects of adjunctive NAC treatment on inflammatory markers, IL-6 and CRP, and a neurogenesis marker, BDNF, were evaluated in a clinical MDD population. While adjunctive NAC treatment significantly improved depressive symptoms, this was not associated with alterations in the levels of IL-6, CRP and BDNF, indicative of a disconnect between these markers and depressive symptoms. This is suggestive of alternate mechanisms of action of NAC.

In the preclinical component of this study, we demonstrated that high fat diet consumption can drive depressive-like behaviour/mood, and that this was associated with diet-induced changes in the gut microbiome. Interestingly, this diet-induced depressive-like behaviour was not associated with elevations of pro-inflammatory mediators, instead peripheral and central inflammatory markers were within normal ranges.

On the other hand, NAC and MINO did not reverse diet-induced depressive-like behaviour nor affect the inflammatory state. Whereas NAC induced subtle changes in the gut microbiome, MINO treatment resulted in significant changes in the gut microbiome and subsequently attenuated microbial signalling. Our findings suggest that MINO and NAC might not be optimal choices to regulate the mechanisms underpinning HFD-induced depressive mood.
5.2 Diet effects on the gut-brain axis

A strong association between poor dietary patterns and an increased risk of developing MDD symptoms has been indicated by numerous epidemiological studies. However, there is a paucity of studies investigating the specific mechanisms linking poor diet quality and depressive mood. Our study demonstrated that high fat diet consumption can be a driving factor in developing depressive-like behaviour, and highlighted the importance of diet in modulating the gut microbiome community. Intriguingly, the significant changes in the microbial community elicited by the HFD were not associated with florid inflammation, despite the resultant depressive mood. This suggests that there are alternate signalling pathways linking HFD-induced changes in the gut microbiome and the behavioural changes.

Dietary contents are digested by gut microbiota in the intestine and in turn, produce bacterial metabolites as a result of bacterial fermentation of resistant starch (Jacobasch et al. 1999). A main product of bacterial metabolites is short chain fatty acids (SCFAs), such as butyric acid, propionic acid and acetic acid (Kimura et al. 2011), and these SCFAs have been shown to influence behaviour (Stefanko et al. 2009; Vecsey et al. 2007). Hence, dietary manipulation can potentially exert effects on behaviour via altering the availability of nutrients for the gut microbiota. For example, mice fed with a diet containing lean ground beef exhibited a greater diversity of the gut microbiome compared with those receiving standard chow and presented less anxiety-like behaviour (Li et al. 2009).

Therefore, we can speculate that HFD feeding might have altered the composition of bacterial metabolites and subsequently predisposed to depressive-like behaviour. However, the gut-brain axis is comprised of the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic pituitary adrenal (HPA) axis (Carabotti et al. 2015; Kelly et al.
It is also possible that other pathways may have played a role in HFD-induced depressive-like behaviour in our study, for instance, via the vagus nerve. Some evidence suggests that the gut microbiome communicates with the brain through the vagus nerve, by demonstrating that neurochemical and behavioural effects were not present in vagotomised mice (Bravo et al. 2011; Bercik et al. 2011).

Nevertheless, our study highlights the connection from the gut to the brain and depressive mood using HFD feeding. Although HFD feeding in this project is an extreme example of dietary manipulation that may not be fully representative of the complicated dietary patterns in humans, this project supports the premise that healthy diets are important for mental health, and implies that establishing healthy dietary patterns could serve as a prevention tool for mood disorders.

### 5.3 MDD and inflammation

Our findings indicated that the reciprocal relationship between inflammation and MDD might not encompass all types of MDD. In the NAC and MDD trial, we showed that the improvement of depressive symptoms did not necessarily coincide with reductions in the levels of the pro-inflammatory mediators, CRP and IL-6, in our clinical population. This indicates that levels of pro-inflammatory markers are not necessarily correlated with the severity of depressive symptoms, and implicates that a disconnect between depressive mood and inflammatory state can exist in MDD.

Raison and Miller (2011) also concur with this premise by pointing out that there are individual differences in immune reactivity in response to inflammatory stimulation, such as IFN α. Whereas some individuals are equipped with an immune system which is resilient to the development of depressive symptoms in response to inflammatory signals, others have
an immune system which confers susceptibility to the development of depressive symptoms in response to even low levels of inflammatory stimuli (Raison and Miller 2011), suggesting an inflammatory subtype of MDD may exist. Furthermore, Dowlati et al. (2010) reported that the degree of elevations of pro-inflammatory mediators found in MDD were far more modest than transient elevation in these mediators found in patients with autoimmune or infectious diseases.

During the process of the biological data analysis in the NAC and MDD trial, we also conducted thorough analyses to assess whether levels of IL-6 and CRP were associated with factors which could potentially contribute to pro-inflammatory status, such as body mass index and comorbidities including cardiovascular, endocrine, gastrointestinal and musculoskeletal conditions in the MDD population we studied. However, we did not observe any association between these conditions and levels of CRP and IL-6. Small sample sizes in the population mean that these analyses were relatively underpowered, however.

In addition, a challenge to identify the association between indicators of inflammatory activity and MDD was that the study population was selected solely based on the clinical diagnosis. It has widely been hypothesised that there exists significant variation in the underlying pathophysiology of MDD, so that clinical heterogeneity is underpinned by possibly even broader heterogeneity of aetiology (Barchas and Brody 2015; Berk et al. 2013; Haroon, Miller, and Sanacora 2017; Nollet and Leman 2013; Lamers et al. 2013; O'Keane, Frodl, and Dinan 2012; Raison and Miller 2011). There currently exists only limited means by which clinical populations can be stratified by aetiology. In the case of our study, this means that some MDD patients may exhibit elevated pro-inflammatory mediators, whereas levels in others might not differ from healthy individuals. Averaging biological samples from this heterogeneous population of MDD patients might preclude us from identifying subtle changes in
inflammatory mediators. Therefore, our study implicated that we need to develop alternate approaches to identify the association between inflammation and MDD, rather than solely relying on concentrations of inflammatory mediators. As such, the crucial involvement of the gut microbiome in depressed mood, as evidenced by our project and others, may suggest a potential use of microbial signalling pathways as an indicator of the pathophysiology of MDD.

5.4 Future studies

The importance of the gut-brain axis in MDD has attracted growing interest, as increasing evidence suggests the gut microbiome plays a crucial role in both health and disease states. Our study provided empirical evidence to support the concept of communication from the gut to the brain, and subsequent effects on behaviour following HFD feeding. This growing body of evidence raises the question of whether diet can be used as an intervention tool to modulate pathways from the brain to the gut and subsequently alter behaviour and mood. A recent study using a dietary improvement program as a novel intervention answered this question by reporting that a 12-week dietary intervention program improved depressive symptoms in the treatment group (Jacka et al. 2017). Effects of this dietary intervention on underlying pathways, including gut microbiome, to modulate depressive symptoms are as yet unknown, however this study indicates that dietary interventions are a potential novel therapeutic strategy for MDD.

To optimise the efficacy of dietary intervention in MDD, investigations to identify how different dietary factors have differential effects on the gut-brain axis, including gut microbiome and behaviour, are warranted. In our study, we examined how fat content affects the gut-brain axis and behaviour. However, there are a wide range of fat types such as saturated fat and unsaturated fat in our diets, and different lipid compositions have
differential effects on the body. For instance, Laugerette et al. (2012) reported that feeding a diet contains different types of lipid such as palm oil, milk fat, rapeseed oil and sunflower oil resulted in differential peripheral inflammatory profiles in mice. This study showed that consumption of palm oil resulted in an increase in susceptibility to low grade inflammation in the host compared with the inflammatory state of mice fed diets containing other oils. However, the differential effects of these oils on gut microbiome and behaviour were not examined.

Other studies have shown that mice fed high fat diets rich in safflower oil, containing a high proportion of omega-6 polyunsaturated fatty acids (PUFA), exhibited reduced abundance of a dominant bacteria, Bacteroidetes, while enriching the populations of Firmicutes, Actinobacteria and Proteobacteria (de La Serre et al. 2010; Turnbaugh et al. 2008). However, how these changes in the gut microbiome community elicited by PUFA affect behaviour is not known. Therefore, we are currently investigating how different dietary fat compositions affect the neurobiological pathways linking gut microbiome and behaviour in collaboration with Professor Felice Jacka at IMPACT SRC, Deakin University. In this project, we are examining effects of different fat types which are commonly used in human diets including olive oil, coconut oil, soy bean oil and ghee on biological pathways comprising the gut-brain axis, and anxiety and depressive-like behaviour.

The second study currently underway is to investigate effects of a diet containing high levels of resistant starch on the gut-brain axis and behaviour. As discussed earlier, SCFAs, butyric acid, propionic acid and acetic acid are produced by the gut bacteria as a result of bacterial fermentation of resistant starch (Jacobasch et al. 1999). SCFAs are reported to exert positive effects on memory and cognitive functions in mice (Stefanko et al. 2009; Vecsey et al. 2007) but also indirectly regulate glial cell function in mice (Erny et al. 2015). In addition, one SCFA,
butyric acid, plays an important role in colonic mucosal growth and epithelial proliferation (Jacobasch et al. 1999; Venkataraman et al. 2016) and in a balanced gut microbiome community in an elderly population (Tachon et al. 2013). Thus, through this study, we aim to identify whether a diet containing high resistant starch ameliorates the effects of high fat diet induced changes in gut microbiome and behaviour. Taken together, these studies currently underway will provide empirical support for how different dietary factors exert therapeutic effects on mood through the gut-brain axis, and essential information for the development of effective dietary prevention and intervention programs.

In parallel with these studies, analyses using biological samples collected from a double-blind, randomised and placebo-controlled clinical trial that assessed effects of adjunctive MINO treatment for MDD are currently underway. In this clinical trial, the add-on MINO treatment exhibited positive effects on depressive symptoms (Dean et al., accepted, in press). Several signalling markers which are indicative of inflammatory and gut microbial activities will be assessed to evaluate underpinning mechanisms of therapeutic effects of MINO on the study population.

5.5 Gut microbiome and behaviour

Our study indicated that high fat diet mediated depressive-like behaviour was associated with both changes in the gut microbiome and elevation of LBP levels. However, the link between these factors was not straightforward. Our study showed that MINO-induced changes in the gut microbiome and attenuation of LBP levels were not associated with a reversal of diet-induced depressive-like behaviour. This suggests that alterations in the gut microbiome and its signalling system are not necessarily concomitant with behavioural change, and hence, changes in gut microbiome “per se” do not exert effects on behavioural changes.
For instance, a recent study by Bharwani et al. exemplified this premise. Oral administration of a single bacterial strain *Lactobacillus rhamnosus* (JB-1) resulted in a partial reversal of anxiety-like behaviour in mice induced by chronic stress (Bharwani et al. 2016). Whilst the chronic stress mediated significant changes in the gut microbiome including reduction of abundant bacteria and alteration of diversity, the administration of JB-1 failed to reverse the stress-induced disruption of the gut microbiome. Bharwani et al. (2016) showed the beneficial effects of administration of single bacteria strain on anxiety-like behaviour, in the absence of a recovery of broader stress-induced dysbiosis; and that recovery from anxiety-like behaviour can occur in the presence of stress-induced changes in the gut microbiota. This study showed that certain bacterial populations may exert stronger effects on behaviour than others. Hence, it suggests that changes in the gut microbial community need to be profiled in more detail in order to advance our understanding of the key bacterial populations and their communicating pathways.

The gut microbiome is a complex ecosystem which has extensive networks with other systems such as the endocrine and immune systems, and dysfunction in these systems are also implicated in the pathophysiology of MDD. To evaluate underlying mechanisms connecting the gut microbiome and behaviour, therefore, is an enormous challenge, however, our findings at least highlight the importance of diet in modulating the connection between the gut microbiome and behaviour in the context of MDD.

5.6 Antidepressant effects of NAC and MINO

Despite antidepressant effects of NAC on depressive symptoms (Berk et al. 2014; Hasebe et al. 2017), the underlying therapeutic pathways were not associated with inflammatory activity as measured by levels of IL-6 and CRP in our study population (Hasebe et al. 2017).
Our preclinical study also indicated that the antidepressant effects of NAC and MINO were not associated with HFD-induced depressive-like behaviour. Thus, the findings from our study suggest that the therapeutic mechanisms of NAC and MINO might involve alternate pathways; and that the significant shifts in the gut microbial community elicited by MINO may not be associated with antidepressant effects on HFD-induced MDD.

5.7 Conclusion

Throughout this PhD project, importance of diet on health and disease states of the body and the brain in the context of MDD were identified and provided empirical evidence to support the epidemiological premise that poor dietary pattern can precede MDD. Dietary factors directly change key microbial populations in the gut, which may result in transforming the healthy gut microbial population into a disease inducing organism, which is implicated in the pathophysiology of MDD.


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