The role of the gut microbiome in Major Depressive Disorder.

The role of the gut microbiome in Major Depressive Disorder in a gnotobiotic murine model.

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TITLE: The functional role of the gut microbiome in Major Depressive Disorder in a gnotobiotic mouse model.

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Abstract

The aetiology of major depressive disorder (MDD) is poorly understood. Current evidence suggests immune activation and gut microbiota may play a role. Recent studies demonstrated that behavioural traits can be transferred through microbiota transplantation into germ-free (GF) mice. Here we study whether microbiota from patients with MDD can induce depressive-like behaviour.

Methods: GF NIH Swiss mice were colonized with stool microbiota from a patient with MDD with elevated faecal β-defensin 2, or a healthy donor (HC). After three weeks, behaviour was assessed using standard tests. Expression of neuroimmune markers was assessed in the gut and brain using gene expression profiling and immunohistochemistry. Microbiota composition was assessed by 16S rRNA sequencing.

Results: Microbiota profiles differed between the two groups of mice (p=0.001). Mice colonised with microbiota from a single characterised MDD patient (MDD1), exhibited lower preference for sucrose (p=0.002) and more emotionality (p=0.003) than mice with HC microbiota, however other MDD mice did not display abnormal behaviour. Abnormal MDD1 behaviour was associated with lower BDNF expression in the dentate gyrus of the hippocampus (p=0.02). Mice colonised with another characterised MDD patient (MDD4 mice) did not have differences in BDNF expression in the same region (p=0.20). MDD1 and MDD4 mice had altered hippocampal and gut gene expression for genes associated with the immune and nervous system. In summary, GF mice colonized with MDD1 microbiota exhibit depression-like behaviors. This appears to be accompanied by changes in intestinal permeability and neuroimmune function. These results suggest that gut microbiota has the capacity to influence the expression of MDD in some patients.

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I want to highlight the difficulties and ironies I have faced researching Major Depressive Disorder for two years, whilst simultaneously suffering from Major Depressive Disorder every day. Without the support and understanding of Premek, I surely would have been unable to persevere against the constant eroding force of my illness. I would like to acknowledge the research being done in MDD field, which has not only enriched me academically but allowed me to understand my own disease at the deepest level and given me hope for a brighter future.

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List of abbreviations

BBB	Blood brain barrier
BDNF	Brain-derived Neurotrophic Factor
BDEF2	Human beta defensin 2
BDEF3	Mouse beta defensin 3
CNS	Central nervous system
CUS	Chronic Unpredictable Stress
CUS	Chronic Unpredictable Stress
CVS	Chronic variable stress
DASS	Depression, Anxiety and Stress Scale
ELISA	Enzyme-linked Immunosorbent Assay
ENS	Enteric Nervous System
EGC	Enteric glial cell
GABA	Gamma aminobutyric acid
GAD	Generalised anxiety disorder
GAD1	Glutamate decarboxylase 1
GDNF	Glial Derived neurotrophic factor
GF	Germ Free
GIT	Gastrointestinal tract
HAMD	Hamilton depression scale
HC	Healthy Control
HDAC	Histone deacetylase
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
IL	Interleukin
IFN	Interferon
LPS	Lipopolysaccharide

MAASLIN	Multivariate association with linear models
MDD	Major Depressive Disorder
MGBA	Microbiota gut brain axis
NAcc	Nucleus accumbens
NFkB	Nuclear factor kB
PSWQ	Penn state worry questionnaire
OTU	Operational Taxonomic Unit
SSRIs	Selective Serotonin-Reuptake Inhibitors
SCFA	Short chain fatty acid
SI	Small intestine
TNF-α	Tumour necrosis factor-alpha

Declaration of academic achievement

This thesis research project was part of a larger study initiated by the Bercik-Collins lab and the Surette lab. All experiments for this thesis were conducted by Marc P. Louis-Auguste with assistance from Jun Lu and Dr. Giada De Palma. Data analysis and interpretation was conducted by Marc P. Louis-Auguste under the supervision of Dr. Bercik and Dr. De Palma. Other key figures in molding the project included Dr. Collins, Dr. Surette and Dr. Mishra who formed part of the committee supervising this project.

1. Introduction

1.2 An overview Major Depressive Disorder

Major Depressive Disorder (MDD) is a debilitating mental illness affecting 10% of the USA population, and is the fourth leading cause of disability globally (Vos T et al. 2015). It is characterised by episodes of low mood, anhedonia, reduction in concentration, abnormal sleep patterns and suicidal thoughts (World Health Organisation 1992). MDD is highly heterogeneous in its presentation and potential pathophysiological mechanisms, and despite the availability of pharmaceutical and psychotherapeutic treatments, one third of patients with MDD are resistant to conventional therapies (Rush AJ et al. 2006). Due to our lack of understanding of the pathogenesis of the disease in general, there are not many alternative treatments either, calling for a deeper understanding of MDD pathologies.

MDD has 3.9% prevalence in Canada as of 2012; it also has a higher prevalence in younger age groups and is co morbid with anxiety in 24.9% of cases (Patten S.B., et al. 2012). In Canada suicide is the leading cause of death among people aged 10-35 after unintentional accidents (Statistics Canada, 2016). In addition to this 60% of suicides in Canada are committed by people suffering from depression, highlighting that MDD is a major contributor to mortality in people aged 10-35 in Canada (Cavanagh JT et al. 2003, Lesage AD et al. 1994; Patten S.B., et al. 2012). The economic burden of depression in the USA was $83.1 billion in 2000, and rose to $210.5 billion in 2010, highlighting a growing need to tackle the disease from an economic standpoint (Greenberg PE et al.

2015). These statistics underline the socio-economic impact of MDD and the necessity for thorough investigation of MDD pathophysiology and new treatment modalities.

1.3 Structural associations with Major Depressive Disorder in the brain

The brain is at the centre of MDD pathogenesis, with observable dysfunctions in multiple regions and networks. One of the most intensely studied networks is the limbic system, which refers to a set of brain structures that include the prefrontal cortex, nucleus accumbens, amygdala, and hippocampus. The limbic system is central to emotional regulation, processing reward, motivation and other cognitive functioning in mammals (Mogenson G.J., et al. 1980).

As mentioned previously, MDD is a mood disorder characterised by abnormal emotional and motivational behaviours, suggesting strong links with dysfunction in the limbic system. The limbic system is well preserved evolutionarily between mammals, meaning it is well suited for study in rodent models (Reep R. L. et al. 2007).

The hippocampus is located in the midbrain and is an important site of adult neurogenesis, processing of spatial memory in the dorsal region, and processing of emotional responses in the ventral region. Changes to this region could therefore alter these functions. Reductions in hippocampal volume are found in some MDD patients and this correlates with the frequency and duration of depressive episodes (Videbech P., et al. 2004, Campbell S., et al. 2004). On a molecular level, a decrease in neurogenesis in the dentate gyrus of the hippocampus has been found in both MDD patients and

animals subjected to chronic unpredictable stress (CUS). MDD patients and CUS animals treated with antidepressants have increased neurogenesis in the dentate gyrus, and this neurogenesis is essential for antidepressant action in mice (Malberg JE., et al. 2000; Santarelli L., et al. 2003; Boldrini M., et al 2009, 2013).

The amygdala is essential for regulating negative emotions such as fear, and social behaviour. Reduced amygdala volume has been found in some patients with MDD, indicating this region may contribute to MDD pathology (Bellani, M., et al. 2011).

The nucleus accumbens is central to reward-motivation pathways in the brain. Therefore, MDD symptoms such as anhedonia and lack of motivation have a root in this region of the limbic system (Ikemoto, S. et al. 1999). Studies have found that people suffering from MDD have significantly reduced responses to reward stimuli in the nucleus accumbens, implicating a dysfunction in this region in MDD patients (Pizzagalli D., et al. 2009). The nucleus accumbens has also been found to be involved in the pathophysiology of chronic stress in mice. Chronic stress exposure decreases blood-brain barrier integrity specifically in the nucleus accumbens of mice exposed to stress, and this is accompanied by immune infiltration of the parenchyma and depressive-like behaviours (Russo S., et al. 2018).

The prefrontal cortex is located in the forebrain and is responsible for higher level cognitive functioning along with processing negative emotional stimuli within the limbic network (Etkin A., et al. 2011). Patients with MDD have reduced glial cell density and neuronal size/density in the prefrontal cortex (Cotter D., et al. 2002). Chronic social

stress has been found to inhibit glial cell proliferation in the prefrontal cortex of rats, which produces an asymmetrical pattern of cytogenesis between the left and right hemispheres. However, this was abolished by treatment with fluoxetine (Czéh B., et al. 2007).

1.4 The role of Brain Derived Neurotrophic Factor in Major Depressive Disorder

Studies have established that features of cerebral MDD/CUS pathology include decreased neurogenesis and neuronal density in limbic regions (Cotter D., et al. 2002; Malberg J.E., et al. 2000; Santarelli L., et al. 2003). A key mediator of neuronal proliferation and survival is brain derived neurotrophic factor (BDNF); BDNF is a neurotrophin that promotes neuronal survival and proliferation in the central nervous system (CNS). BDNF has been found to be abnormally low in the serum of patients with untreated depression, and is normalised with antidepressant treatment (Sen S., et al. 2008). Reduction of BDNF levels in the hippocampus of rats is associated with decreased neurogenesis in the dentate gyrus and depressive-like behaviour. These trends have also been found in post mortem analysis of patients with depression (Tailiaz D., et al. 2010; Ray MT et al. 2011). The picture is not so simple however: in other regions of the limbic system BDNF has detrimental effects, as it has been found that inhibiting BDNF activity in the nucleus accumbens has an antidepressant effect (Eisch A.J. et al., 2003). It is apparent, however, is that BDNF is a key playmaker in the neurobiology of depression.

1.5 The monoamine theory of Major Depressive Disorder

Along with neurotrophic factors, neurotransmitters have also been implicated in MDD aetiology. Deficiencies of neuroactive amines in regions of the brain, such as the limbic system, are core drivers of MDD pathology (Delgado, P. L., 2000). Reserpine was used in the 20th century as an antihypertensive drug and functioned by blocking the intracellular absorption of monoamines, exposing them to monoamine oxidase metabolism; it was discovered that depressive symptoms were a side effect of reserpine monoamine depletion (Brodie, B.B., et al. 1957). This discovery was complemented by findings that monoamine oxidase inhibitors and tricyclic antidepressants bolster the effects of monoamines at the neural synapse, and were the treatment of choice for MDD until the advent of more selective agents (Coppen A., 1967). Development of pharmacological agents targeting monoamine metabolism began under the assumption that increasing levels of monoamines in the brain would ameliorate the symptoms of MDD. Selective serotonin, noradrenaline/dopamine reuptake inhibitors (SSRI, SNRIs) such as fluoxetine, escitalopram, and bupropion have been used to successfully treat MDD patients for many years now (Brodie, B.B., et al. 1957). As mentioned previously, studies examining hippocampal neurogenesis found that SSRIs stimulate neurogenesis and that this was essential for the antidepressive effects of SSRIs. Selective uptake inhibitors block the reabsorption of monoamines through the presynaptic terminal, thus potentiating their synaptic action for longer. The monoamine hypothesis in its modern form has divided the diverse symptomatology of MDD into serotonin and

noradrenaline/dopamine related subsets. Serotonin deficiency appears to be related more to anxiety, obsessive/compulsive symptoms whereas noradrenaline/dopamine deficiency is associated with symptoms of low of motivation, low concentration and diminished cognitive ability (Marin H., et al. 2005). This is consistent with anatomical data showing that serotonergic neurons innervate regions of the limbic system such as the amygdala, which is associated more with anxious behaviour. Instead, dopaminergic/noradrenergic neurons innervate the nucleus accumbens, which has a role in motivation/reward behaviours (Stahl S.M., et al 2008; Bocchio, M., et al. 2016).

1.6 The genetic and environmental pathogenesis of Major Depressive Disorder

The pathophysiological heterogeneity that characterises MDD supports a strong genetic component and heritability to the disease. Twin studies have shown 37% heritability of MDD, with individual novel environmental risks contributing significantly to the onset of MDD (Lohoff FW., et al. 2008). Family studies have shown a two to three fold risk of developing MDD among close relatives, with the most heritable phenotype being early onset, and high recurrence of disease (Weissman MM., et al. 1993). Taken together, these studies suggest that MDD is highly heritable, but what are the specific inherited changes which contribute to MDD pathogenesis? To answer this question studies have examined genetic polymorphisms in MDD patients in genes related to serotonin. For example the L allele of the serotonin transporter gene SLC6A4 (5-HTTLPR promoter region) has been associated with better response to SSRI treatment (Serretti A., et al.

2007). However other studies have contrasted with these findings, positing that HTR2A (serotonin receptor) has more influence on treatment outcome (McMahon F.J., et al. 2006). The inconclusive nature of these polymorphism studies highlights the fact that genetics can only be seen as a component of MDD pathogenesis, as there is no single gene that can cause MDD. It is very likely that the combination of multiple small genetic differences contribute to susceptibility. Linkage studies have shown that some chromosomal regions in particular are associated with MDD and other psychiatric disorders (Hamilton SP., et al 2003; McGuffin P., et al. 2005). We know the pathogenesis of MDD likely has an environmental component from twin/population studies along with animal models used to study MDD: exposing rodents to exogenous chronic or acute stressors generates a behavioural phenotype which mimics many aspects of MDD (Willner P., 1997). To complement this, studies in humans have found that stressful life events are significantly associated with the onset and severity of MDD. This provides further legitimacy to a strong environmental pathological driver for MDD and for stress-based animal models of depression (Kendler K.S. et al. 1999; Schmitt A., et al. 2014).

A possible reason that there is such strong interfamilial heritability but no distinct allelic component to MDD is that environmental triggers create epigenetic vulnerabilities in MDD patients and susceptible individuals (Bohacek J., 2013). Epigenetics describes the regulation of gene expression through controlled changes to chromatin, often in response to environmental changes. Epigenetic modifications in gametes can be

inherited, and have recently been posited to be the 'missing heritability' aspect of genetic inheritance (Trerotola M., et al. 2015).

1.6.2 Epigenetics and Major Depressive Disorder

Histones are a group of structural proteins that form chromatin when combined with DNA and non-histone proteins (Allan, J., et al. 1986). Histone and DNA modifications are conducted by enzymes that either ligate or remove methyl and acetyl groups to specific residues in the DNA/histone molecule. DNA methylation is associated with genetic silencing, whereas histone methylation and acetylation promotes gene expression (Strahl B.D. et al. 2000; Zhang T.Y., et al. 2010; Jaenisch R., et al. 2003).

There is growing evidence for the involvement of epigenetic processes in MDD pathophysiology, with many studies focusing on inhibition of certain histone deacetylases (HDACs) to alter rodent behaviour. Histone deacetylase inhibitors (HDACi) can produce antidepressive effects when administered to chronic/acute stress animal models systemically or intracerebrally, which shows that epigenetic changes in the brain could play a role in the pathology of certain depressive-like behaviours (Schroeder F.A., et al. 2007; Tsankova N.M., et al. 2006; Covington 3rd H.E., et al. 2011). In the limbic system, histone acetylation decreases then abruptly increases in the nucleus accumbens after chronic social defeat stress (CSDS), in correlation with HDAC2 levels. Furthermore, infusion of HDACi sodium butyrate into the NAcc was shown to have potent antidepressive effects, demonstrating that alterations to histone acetylation may

putatively be involved in the pathogenesis of depressive-like behaviours in the CSDS model. Later, the significance of microbially derived butyrate and a potential role for microbially guided epigenetic alterations will be discussed (Covington 3rd H.E., et al. 2009).

Increased acetylation, however, is not always correlated with antidepressant outcomes, and highly depends on the brain region studied; this is conceptualised when looking at HDAC5 expression in the NAcc vs. the hippocampus. CSDS mice exhibit increased HDAC5 expression in the NAcc when given the antidepressant imipramine. Mice lacking HDAC5 in the NAcc present with increased depressive-like behaviours, implying that HDAC5 is involved in the regulation of behaviour through the NAcc. Other studies have found the expression of HDAC5 to be increased in the hippocampus of rats undergoing chronic variable stress. It was also discovered that a HDAC5 specific inhibitor was sufficient to reverse anhedonia in these rats, highlighting that HDAC expression has differential impacts on behaviour depending on the brain region it is expressed in (Renthal W., et al. 2007; Benton C.S., et al. 2011; Covington 3rd H.E., et al. 2011). These studies show that epigenetic changes in the brain can drive aspects of MDD. It also appears that HDAC activity can impact the antidepressive effects and expression of neurotrophins in the context of MDD. For example, in the hippocampus, it has been found that CSDS induces epigenetic changes to BDNF promoters, corresponding with decreases in hippocampal *Bdnf* mRNA (Tsankova NM., et al. 2006). Given what we know about the importance of BDNF in the pathophysiology of MDD, it is certainly interesting that environmental

triggers can impact BDNF expression through epigenetic mechanisms. This further highlights the potential of investigating other epigenetic mechanisms in the context of MDD.

1.7 The role of the immune system in Major Depressive Disorder pathophysiology.

There are many theories implicating the immune system and inflammatory processes in the pathophysiology of MDD. It has been well established that the immune system is receptive to canonical neurotransmitters such as serotonin, GABA, dopamine and acetylcholine, the latter of which is implicated in the anti-inflammatory reflex. This presents a rapid way for the nervous system to crosstalk with the immune system (Mössner R., et al. 1998; Tracey K. J. 2002; Bjurstöm H., et al. 2008). It is also known that the nervous system is dependent on cytokine secretion for nociception and higher level cognitive functions such as sociability, memory and learning in mice. Thus it would be logical that immune aberrations could result in alterations to these physiological processes and alter behaviour (Filiano A.J., et al. 2016; Derecki N. C., et al. 2010).

1.7.2 Microglia in the pathogenesis Major Depressive Disorder

The brain is generally free from immune infiltration, however, microglia are immune cells that reside in the brain and partake in many physiological processes. Microglia are of myeloid origin and migrate to the brain early in embryonic development (Gomez Perdiguero E., et al. 2013). Microglia play an important role in surveilling the CNS for

pathogen and danger signals similar to macrophages in the periphery, and also function in synaptic pruning, brain development, and cognition (Hong S., et al. 2016). Elevated microglial density has been found in patients with depression and other psychiatric disorders, potentially implicating microglial activation in the pathogenesis of MDD (Steiner J., et al. 2008). In addition to this microglial activation markers such as translocator protein (TSPO) have been found to be associated with MDD duration in humans (Setiawan E., et al. 2015; 2018). These studies used positron emission tomography (PET) to assess TSPO distribution volume in the brain of MDD patients. Microglial activation as assessed by TSPO volume was found to be increased significantly in untreated MDD patients and patients with long term MDD (Setiawan E., et al. 2015; 2018). These studies provide strong evidence of a role for microglial activation in MDD pathophysiology. Microglia have also been directly associated to depressive-like behaviour in chronic stress models where deficits in working memory were related to increases in activated microglia density in stress sensitive brain regions. In conjunction minocycline, a microglia inhibitor and antibiotic, has been found to reduce these deficits, implicating microglia in the expression of depressive-like behaviour (Hinwood M. et al. 2012). Microglia can respond to environmental stimuli such as stress and traditional immune stimulants; their intimate role in normal brain physiology and cognition means any pathological activation of microglia can result in direct aberrant changes in important brain regions associated with MDD.

1.7.3 Sickness behaviour, cytokines and Major Depressive Disorder

Cytokines are proteins secreted primarily by immune cells and have a range of local and systemic effects. Cytokines can interface with the nervous system and induce changes which can promote certain behaviours beneficial to fighting infection. Sickness behaviour is mediated by pro-inflammatory cytokines in response to infection by a pathogen (Vollmer-Conna U., et al. 2004). It provides an evolutionary advantage to social animals by inducing conservation withdrawal, which is characterised by anhedonia, loss of appetite, reduced social interaction, and fatigue, saving energy to fight infection and arguably benefits a social group by reducing the probability of disease transmission (Raison C. L., et al. 2013). There are many similarities between symptoms of sickness behaviour and MDD symptoms; mice exposed to lipopolysaccharide (LPS) express sickness behaviour, and these symptoms can be ameliorated by administering conventional antidepressants. This suggests that some of the behavioural changes that define sickness behaviour and MDD are enacted via similar pathways (Yirmiya R., et al. 2001).

The similarities between MDD and sickness behaviour suggests that they share similar immune pathways that induce behavioural changes. Evidence for this includes the presentation of other cardinal signs of an elevated immune response in MDD patients, for example MDD patients have been found to have a consistently elevated body temperature in the range that confers protection to pathogens (Rausch J.L., et al. 2003). This implies that some patients with MDD have immune activation sufficient to induce

fever, and this is likely mediated by proinflammatory cytokine secretion which in turn could alter behaviours in patients. Further evidence for the role of cytokines in MDD comes from cancer patients treated with IFN-α and IL-1. These patients have shown an increased susceptibility to MDD post treatment, with IFN-α treatment producing a 50% increase in MDD prevalence alone. However MDD onset can be prevented by antidepressant prophylaxis, which implies that the pathways the cytokines are activating to alter behaviour are responsive to reuptake inhibitors and so must have some homology with those activated in MDD pathophysiology (Raison C.L., et al. 2006; Musselman D.L., et al. 2001). Some MDD patients have high levels of inflammatory biomarkers such as TNF-α and anti-serotonin antibodies in their circulation, implying an atypical state of immune activation in these patients (Maes M., et al. 2012; Miller A.H., et al. 2016; Dowlati Y. et al., 2010). In summary, acute inflammatory responses are sufficient to alter behaviour in a physiological infection control setting, but also during cytokine therapy. Chronic inflammatory diseases provide another window with which to explore the impact of the immune system on MDD prevalence and pathophysiology.

1.7.4 The comorbidity of Major Depressive Disorder with inflammatory diseases

Patients that suffer from inflammatory diseases commonly have comorbid MDD; this has been found to negatively impact the efficacy of treatment in cases of Rheumatoid Arthritis and Crohn's disease. This implies that chronic immune activation can influence psychiatric disorders and in turn psychiatric condition can affect or predict inflammatory

disease treatment outcome, possibly through a neuroimmune axis (Guloksuz S., et al. 2013; Matcham F., et al. 2016; Kurina L.M., et al. 2001; Persoons P., et al. 2005). Blocking TNF-α using Etanercept has been found to improve symptoms of depression by 50% in patients with psoriasis, and 41% in patients with Crohn's disease, strongly implicating TNF-α in the pathophysiology of MDD in these patients. In addition to this work, the Bercik lab has found that infliximab can ameliorate anxiety like behaviour in mice, highlighting the importance of inflammation in possibly mediating abnormal behaviours. This also opens the door to antibody therapy to treat psychiatric illnesses in some cases (Tyring S., et al. 2006; Persoons P., et al. 2005).

1.8 The human microbiome

The human gastrointestinal tract harbours a diverse community of microorganisms which outnumber host cells and generate a vast range of gene products that can interface with the host (Ley R.E., et al. 2006). Generally a healthy microbiome consists mostly of four major phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria,* where *Firmicutes* and *Bacteroidetes* phyla are the most abundant and the ratio of these phyla differs from person to person (Human Microbiome Project Consortium., 2012; Lozupone C.A., et al. 2012).

The human microbiota evolved alongside the host in a mutualistic fashion and both are entangled in a beneficial symbiotic relationship that in healthy individuals has a range of benefits for the host. Humans are colonised at birth by the maternal microbiome; these

founder communities play a vital role in the development of the immune system and central nervous system (Mueller N.T., et al. 2015; Sommer F., et al. 2013). To interface with the host, gut microbiota can secrete a wide range of gene products and metabolites which can influence immune responses in the GIT and beyond. However, a loss of homeostasis can result in a detrimental microbiotal influence (Singh N., et al. 2014; Chang P., et al. 2014).

Dysbiosis is a broad term which attempts to conceptualise negative microbiome-host interactions. Some diseases are linked to an altered microbiota; patients suffering from inflammatory bowel diseases, for example, have been found to have lower bacterial diversity, with decreased *Firmicutes* and increased *Proteobacteria* abundance however these finding are not consistent (Manichanh, C et al. 2006; Frank D.N., et al. 2007; Gophna U., et al. 2006; Tamboli C.P., et al. 2004). Lower diversity however is not an indicator in itself of dysbiosis and the concept of dysbiosis itself is not widely accepted in the scientific community. We define and will use dysbiosis as microbiota that alter homeostasis with the host.

1.8.2 Links between the microbiome and Major Depressive Disorder

Interest in linking psychiatric disorders to the microbiome has yielded studies that have attempted to link changes to microbiotal composition to disease. Two studies have analysed the microbiota of patients with MDD and found that MDD patients had an

altered microbiome, in comparison to healthy controls (Naseribafrouei, A., et al. 2014; Jiang H., et al. 2015). MDD patients were found to have *Bacteroides, Actinobacteria* and *Proteobacteria* over-represented in their microbiomes, a feature seen previously in studies examining altered microbiota and IBD. On a species level, *Faecalibacterium prausnitzii* levels were found to be consistently reduced in MDD patients and these reductions correlated negatively with depressive symptoms, implying this bacterium could have a protective role against MDD (Jiang H., et al. 2015). Studies have also attempted to use MDD microbiota to colonise rodents and assess behaviour changes. It was found that colonisation of antibiotic-depleted SPF rats with microbiota pooled from a cohort of patients with MDD can induce a depressive phenotype, implicating the microbiota more directly in MDD pathology. However the methodologies used in these studies such as pooling microbiota and using antibiotic depleted microbiota in rodent models make it difficult to draw specific mechanistic conclusions from data.

1.9 The microbiota-gut-brain axis

The microbiota-gut-brain axis (MGBA) refers to the bidirectional communication pathways between the microbiota, the gut, and the brain. Gut microbes can secrete a range of metabolites that can directly modulate the enteric nervous system (ENS) and the activation state of gut immune cells; these metabolites can also penetrate the blood brain barrier to interface with glia and neurons (Lyte M., 2014; Braniste V., et al. 2014).

In line with the general symbiotic nature of the host-microbiota relationship the immune system can induce changes directly to microbial populations through the activation of certain inflammasomes and bacterial sensors. These pathways can alter processes such as antimicrobial peptide secretion, gastrointestinal motility, and mucous secretion to change the GI environment to favour certain bacteria, which in turn modifies the milieu of secreted bacterial metabolites interacting with the host (Muller, P. A., et al. 2014; Ramanan D., et al. 2014; Wlodarska M., et al. 2014).

1.9.2 The microbiome and the brain

Recently, the interest has been focused on the effect of microbial dysbiosis on the human brain. Clinical use of antibiotics and laxatives to treat the cognitive symptoms of hepatic encephalopathy highlights that the microbiome can have far reaching effects on the brain, a seemingly distant organ from the gut (Bercik P., et al. 2012). Evidence has been found for microbiome induced immune alterations that in turn can alter brain physiology in studies looking at the outcome of ischaemic stroke in mice. The study found that antibiotic treatment impacted γδ T cell trafficking from the gut to the meninges and this in turn altered the recovery time in antibiotic treated mice highlighting that the microbiome can affect immune and CNS physiology on a systemic level (Benakis C., et al. 2016). Studies exploring the T-cell trafficking to the brain have found that T cell activity in the meninges can affect cognitive function through the secretion of cytokines such as IL-4 and IFN-y in mice (Filiano A.J., et al. 2016). We can

postulate that T-cells trafficked from the gut to the meninges through the MGBA can also induce these behavioural changes as it has already been highlighted that the microbiome can impact the trafficking of T-cells, and the GIT harbours high densities of T-cells which can interface with the microbiome. As mentioned previously, inflammatory bowel diseases are often comorbid with psychiatric illnesses such as MDD and GAD, and it is also very apparent that dysbiosis contributes heavily to the chronicity, severity and pathophysiology of IBD (Kurina L.M., et al. 2001). With what we know about microbiotal interactions with the immune system in the gut, and the potential systemic consequences, we can speculate that the impact of the microbiota on IBD pathophysiology may also extend beyond the gut and into the psychiatric aspects of the disease. Adding to this further, dysbiosis has also been linked to primary psychiatric developmental disorders such as autism, with autistic patients receiving faecal transplants exhibiting improvements in cognitive and gastrointestinal symptoms which persisted eight weeks after treatment (Kang D.W., et al. 2017). Unpublished data from the Bercik & Collins laboratory has shown that microbiota from generalised anxiety disorder patients can induce anxiety like behaviour in colonised germ free mice. Taken together, this evidence suggests the microbiota has a role in the pathophysiology of a range of psychiatric and developmental disorders, and that the mechanistic aspect of this relationship likely takes place through the MBGA.

1.9.3 Bacterial metabolites and the microbiota-gut-brain axis

The microbiome secretes many neuroactive peptides and metabolites including classical neurotransmitters, such as serotonin, GABA, dopamine and noradrenaline (Yunes R.A., et al. 2016; Pokusaeva K., et al. 2017; Lyte M., 2014). Patients with MDD have been found to have decreased levels of microbiota derived metabolites compared to healthy controls potentially implicating the metabolic output of the microbiota with MDD (Zheng P, et al. 2012). Stress can disrupt the vaginal microbiome of mice, and this in turn can affect the metabolic profile of the offspring through alterations in the transmitted microbiome. These studies reaffirm that the microbiome is susceptible to alteration via stress with stress also being a major risk factor for MDD (Jašarević R., et al. 2015). Some of the most studied bacterially derived metabolites are short chain fatty acids (SCFAs) which are produced predominantly by *Firmicutes* bacteria from dietary fibre, and have been found to be beneficial to health. SCFAs can alter the functional aspects of the immune system, and have been found to promote anti-inflammatory states in the gut by altering T_{REG} cell populations through SCFA receptors, with beneficial outcomes in models of colitis (Smith P.M., et al. 2013). SCFAs can also permeate the gut and travel in the circulatory system to the blood brain barrier (BBB) where they have been theorised to induce changes to BBB permeability, angiogenesis, neurogenesis and cognitive functions (Michel L., et al. 2016). Butyrate is a well-studied SCFA that is secreted solely by the microbiome; studies mentioned previously explored epigenetic changes in CSDS

by using sodium butyrate as an HDACi. Butyrate in this model alleviated depression-like behaviour in CSDS mice through epigenetic mechanisms providing a potential functional role for a microbiota derived product in MDD.

The complex nature of MDD pathophysiology and the heterogeneity of its clinical, and likely mechanistic presentation, is analogous to the diverse range of microbiomes in our populations, with each having a potentially unique systemic impact. It is also apparent that the microbiome may have a role in the pathophysiology of MDD; however this is likely to be restricted to a subgroup of patients. We hypothesise that these patients have a microbiome which can alter gut/neuroimmune homeostasis and through this can impact brain physiology in a pathological manner to contribute to the overall clinical presentation of MDD.

1.10 Hypothesis and expected outcomes

This project will examine the functional relationship between the microbiome and Major Depressive Disorder using a humanised gnotobiotic mouse model. We hypothesise that colonising gnotobiotic mice with microbiota from individual MDD patients will induce behavioural changes resembling MDD in addition to neurochemical changes in limbic structures of the colonised mice. Any changes will be compared using mice colonised with microbiota from single healthy donors (HC), which will serve as the control groups in the project. This project will be focusing on the gut brain axis as the putative mechanistic driver of MDD like behaviour in the mice. We will be probing the

small intestine and colon for neural and immune changes as we also hypothesise that MDD microbiota will promote pro-inflammatory gene expression and changes to ENS composition.

1.10.2 Experimental approach

NIH Swiss gnotobiotic mice (n=90, 40 males, 50 females) were colonised with microbiota from individual MDD patients or an age/sex matched healthy donor. MDD donors were chosen based on age, DASS score, lack of comorbid conditions and concentrations of faecal B-defensin 2 (BDEF2). After three weeks mouse behaviour was assessed through a series of standardised tests over 10 days; mice were euthanized at the end of behaviour testing. Brains were processed for immunohistochemistry and limbic structures isolated for gene expression analysis, colon and small intestine were processed for gene expression analysis. Cecum and small intestine samples were sent for 16S rRNA gene sequencing and microbiota profiles were generated from the obtained sequences.

2. Methods

2.1 Analysis of samples from human participants

Patients and healthy controls analysed in this project were derived from a clinical study initiated by Dr. Rebecca Anglin in 2016. All patients donated faecal and blood samples for analysis. MDD patients were screened for inflammatory biomarkers in the stool and serum alongside age/sex matched healthy donors. Enzyme linked immunosorbent assays (ELISA) were used to assess concentrations of faecal and serum inflammatory biomarkers. Psychometric tests were used to assess disease severity among the participants, the tests included the Hamilton depression scale (HAMD), the Penn state worry questionnaire (PSWQ) and the Depression Anxiety and Stress Scale (DASS) which was used as the main indicator of depression in this study.

2.1.2 Subject selection

The subjects were selected based on a variety of parameters such as depression severity on the DASS score, age, weight, medical history and diet. Patients were diagnosed with MDD by Dr. Anglin using the Mini-International Neuropsychiatric Interview (MINI) diagnostic interview. All MDD donors used in this study were diagnosed as having current MDD.

Faecal beta defensin-2 (BDEF2) concentrations were elucidated using the beta defensin 2 ELISA kits (Immunodiagnostik, DE). BDEF2 concentrations were used to further differentiate the MDD patient pool, with high scoring patients (>70 ng/ml) used as

indicators of immune reactivity towards gut microbiota. ROME-III diagnostic criteria were assessed to rule out potentially confounding gastrointestinal abnormalities. Faecal samples collected from the donors were then used to colonise the subject mice.

Experiment 1

MDD1 was selected as the first donor, and was age/sex matched with healthy donor HC1. MDD1 is a 25 year old male with a depression score of 42 (extremely severe >28), and an anxiety score of 20 (extremely severe >20) on the DASS scale. MDD1 was taking antipsychotic and antidepressant medication. MDD1 had high levels of faecal beta defensin 2 (114 ng/ml). HC1 had a depression score of 0 (normal 0-8), and an anxiety score of 2 (normal 0-7). HC1 had normal levels of beta defensin 2 (37.7 ng/ml).

Experiment 2

MDD2 was selected as the second donor and was matched with healthy donor HC2. MDD2 is a 42 year old female with a depression score of 12 (mild 10-13) and an anxiety score of 8 (mild 8-9) on the DASS scale beta defensin 155. Despite the low DASS score MDD2 had a high depression score on the Hamilton depression scale of 36 (very severe depression) HC2 is a 42 year old female and had a depression score of 2 (normal), and an anxiety score of 4 (normal). HC2 had normal levels of faecal beta defensin 2 (4 ng/ml).

Experiment 3

MDD3 was selected as the third donor and was matched with healthy donor HC4. MDD3 is a 38 year old woman with a depression score of 42 (severe) and anxiety score of 8 (mild). MDD3 had normal faecal beta defensin 2 levels (53 ng/ml). HC3 is a 35 year old female with a depression score of 4 (normal) and an anxiety score of 0 (normal). HC3 had normal beta defensin 2 levels (0.23 ng/ml).

Experiment 4

MDD4 was selected as the fourth donor and was matched with healthy donor HC4. MDD4 is a 19 year old male with a depression score of 36 (severe) and an anxiety score of 6 (normal). MDD4 had normal levels of faecal beta defensin 2 (34 ng/ml). HC4 is a 22 year old male and had a depression score of 2 (normal) and an anxiety score of 0. HC4 faecal beta defensin levels were normal (48 ng/ml).

2.1.3 Donor characteristics and experiment size

MDD donor/ HC donor	DASS depression score	BMI	Sex	DASS anxiety score	BDEF2 levels ng/ml	Mice colonised	Age
MDD1/HC1	42/0	17/23.1	M	20/2	114/37	14/14	25/24
MDD2/HC2	12/2	27.1/23.5	F	8/4	155/4	10/11	42/42
MDD3/HC3	42/4	21.2/?	F	8/0	53/0.23	10/10	38/35
MDD4/HC4	36/2	18.4/21.2	M	6/0	34/48	11/10	19/22

2.2 Animals

Germ-free NIH Swiss mice (n=90, 35 males, and 55 females) (6-12 weeks old) were obtained from the Axenic Gnotobiotic Unit of McMaster University. Mice were housed in individually ventilated cages, on a 12 hr: 12 hr light/dark cycle with ad libitum access to TEKLAD breeder diet 7904 (irradiated) and sterile water. Handling occurred in ultra clean level rooms or sterilised laminar flow hoods to minimise bacterial contamination. All experiments were performed in accordance with McMaster University animal utilization protocols, with approval from the McMaster University Animal Care Committee, and conducted under the Canadian Council on Animal Care Guidelines.

2.2.1 Microbiota transfer

Human donor faecal samples were stored at -80◦C and aliquoted under anaerobic conditions. To prepare the samples for gavage we combined 0.2 g of frozen faeces with 2 ml of anaerobic saline under anaerobic conditions in sterile micro-centrifuge tubes. Mice were gavaged with 200 µl of the diluted sample and were monitored over 48 hours and then left undisturbed for three weeks to allow the microbiota to stabilise.

2.2.2 Behavioural tests

Mouse behaviour was assessed three weeks post colonisation under controlled conditions. Mice were rested at least 24 hours between tests to allow time for recovery the tests were carried out in order of least stressful to most stressful, to reduce the possible interference of acute stress on other experiments.

Open field test

Mice are placed in the test box for 10 minutes and movements were detected automatically by motion sensors built into specially constructed chambers. Time in centre is calculated by ActivityMonitor software by adding together time spent ambulatory, resting and vertical in the centre of the field in seconds. The open field test is used as a measure of anxiety like behaviour in psychiatric literature (Prut L. & Belzung C., 2003).

Three chamber social preference assay

This assay is used to measure sociability in rodents. It consists of three chambers of equal size, with sealable exits on both sides of the middle chamber (Moy SS., et al. 2004). A test mouse is placed in the open chamber and left to habituate for ten minutes. The flanking chambers each contain a cage, and after habituation and restriction of the test mouse to the central chamber the cages are occupied by either a novel mouse or inanimate object/nothing (Moy SS., et al. 2004). Once the cages are occupied the exits to the flanking chambers are unblocked and the mouse is allowed to freely move between both for ten minutes whilst its movements are recorded both by video and manually by the operator. As mice are innately social animals they should show a preference towards a novel mouse over an inanimate object (Moy SS., et al. 2004).

Tail suspension test

The TST is a dry analogue to the forced swim test, and as it requires less aftercare it can be conducted quickly and efficiently to measure behavioural despair (Cryan JF., et al.

2005). It involves securing the mouse's tail to a clamp and leaving the mouse suspended in the air for six minutes, during which bouts of immobility are recorded. Long periods of immobility are abnormal and indicative of a loss of motivation, and helplessness (Cryan JF., et al. 2005). As this test is very stressful for the mice, it is conducted near the end of the testing period to prevent stress induced changes which could interfere with results.

Sucrose preference test (SPT)

The SPT was conducted last in behavioural experiments due to possible confounding changes to the microbiota on repeated exposure to sucrose. The first phase of the experiment involves singly caging and habituating the subject mice to the presence of two sipper tubes in their environment, this period can last from 1-4 days (Eagle AL, et al. 2016). After the habituation period new sippers containing coloured water and 2% sucrose solution replace the habituation sippers. The sucrose intake is then measured for four days, with intake measured daily as changes to sipper tube weight. The sippers also switch position daily to prevent preference to a particular side (Eagle AL, et al. 2016). After the test period, sucrose intake results will be compared between the MDD and healthy control group.

Emotionality z scoring

To investigate the overall emotional behaviour of the test mice we combined z scores from the behaviour tests highlighted above utilising the same methods that have been reported previously (Guilloux JP., et al. 2011). To calculate z scores for individual tests we subtracted subject mouse values from the mean HC mice value and divided by the

HC mice standard deviation to generate a z score for the subject mouse. When z scores from multiple tests are combined and divided by the number of tests, it is an indicator of emotional behaviour, with scores deviating negatively from 0 considered to be abnormal.

2.3 Immunohistochemistry

Brains were frozen in cold isobutane and coronal sections of the prefrontal cortex, nucleus accumbens, dorsal (bregma -1.46 mm) and ventral hippocampus (bregma -2.54 mm) were obtained at 10um thickness using a microtome and transferred to apex coated microscope slides, slides were stored at -80^{o}C for long term storage. Slides were fixed with cold methanol washed with PBS and permeabilised with 0.025% tween. Slides were then blocked with 10% FBS in 1% BSA for two hours and incubated with primary antibodies overnight, anti-BDNF (Novus biosystems 1:500). Slides were washed with triton-x100 0.025% and incubated with secondary antibodies conjugated to Alexa488 (FITC) fluorophores (Invitrogen 1:1000) for one hour. Slides were washed with TBS and preserved with anti-fade DAPI reagent and coverslips sealed with nail polish. Slides were imaged at 20x magnification with consistent exposure; using fluorescent DAPI and FITC fluorescent camera filters. Analysis of images was conducted on Nikon NIS Basic Research analysis software. We set consistent DAPI and FITC thresholds to analyse slides, and calculated the mean DAPI and FITC area at that threshold in a specified region of interest (ROI).

2.4 Gene expression analysis

30 mg of colon and small intestine tissue, emptied from all content, was stored in RNAlater solution (Thermo fisher, USA) and stored at -80◦C. RNA was then extracted using the Qiagen RNAeasy mini kit (Qiagen, Toronto, ON, Canada), according to manufacturer's instructions. Extracted RNA was stored at -80◦C and prepared for Nanostring gene expression analysis (NanoString Technologies, Seattle, WA, USA) or reverse transcription for probe based Real Time (RT) qPCR. RNA was probed using a custom Nanostring (NanoString Technologies, Seattle, WA, US) code-set developed in house, including 72 genes of interest, related to gastrointestinal, immune and nervous system pathways. Expression ratios were generated and analysed using nSolver 4.0 (NanoString Technologies, Seattle, WA, US). QPCR based gene expression analysis was conducted using BIORAD Evagreen SSO Fast qPCR reaction mix (BIORAD, CA, USA) and BIORAD thermocyclers, specific Quantitect primers were purchased at Qiagen (Qiagen, Toronto, ON, Canada): GABAB1 (NM_019439), GABAB2 (NM_001081141), Occludin (NM_008756), GAD1 (NM_008077), HDAC3 (NM_010411), BDEF-3 (NM_013756) . For brain gene expression analysis 200 µm coronal sections of the prefrontal cortex (bregma 2.96 mm), nucleus accumbens (bregma 0.98 mm), dorsal (bregma -1.46 mm) and ventral hippocampus (bregma -2.54 mm) were cut on a microtome and stored in 350 µl of Qiagen mini RNA lysis buffer (Qiagen, Toronto, ON, Canada). RNA extraction was performed using the Qiagen RNAeasy mini kit (Qiagen, Toronto, ON, Canada) according manufacturer's instructions. Relative expression was calculated with the 2–DDCT

method correcting for different amplification efficiencies for each target and reference gene tested (Pffafl method) (Pfaffl MW. 2001). PCR efficiency was established by including a standard curve for each reaction, and efficiencies between 97-103% were accepted. The result obtained is the fold change of each target gene in each test sample relative to the calibrator sample (control population), all previously normalized to the expression of a reference gene. For normalisation of relative expression, PGK1 (Qiagen NM_008828) and GADPH (Qiagen NM_001256799) were used as reference genes, for cerebral and gut gene expression respectively.

2.5 Microbiome profiling

Cecal and jejunal content samples were collected and snap frozen at -80◦C immediately. Genomic DNA was isolated as previously described (Whelan F.J., et al. 2014). Amplification of the V3 region of the 16S rRNA gene, and Illumina based sequencing was performed as previously described (Bartram A.K., et al. 2011; Whelan F.J., et al. 2017). Sequencing data was processed by an in-house bioinformatics pipeline which incorporated quality filtering (Whelan F.J., et al. 2017).Taxonomic assignments used the RDP classifier with the Greengenes (gg2013) training sets (Wang Q, et al. 2007; DeSantis T.Z., et al. 2006). Analyses were done using either quantitative insights into microbial ecology (QIIME) or Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) (Whelan F.J., et al. 2017; Caporaso J.G., et al. 2010; Morgan X.C., et al. 2015;). Bacterial richness (chao1) and diversity (Simpson, Shannon)

was calculated in QIIME, as well as beta diversity using weighted and unweighted unifrac metrics. We used PICRUST to predict the functional composition of microbiota metagenomes using marker gene data and a database of reference genomes as described by Langille et al. (2013). The graphical representation of PICRUST results was done in GraphPad Prism Version 6 (La Jolla, CA, USA); statistical analysis of PICRUST results was done in STAMP (v2, Beiko) using White's non-parametric t-test.

In the cecum we had a total of count: 3,791,294 reads from 61 samples (summary: Min: 28856.0 Max: 89907.0 Median: 62357.000 Mean: 62152.361) and 975 OTUs after quality trimming (an average of 179.3 OTUs per sample with a Min: 109 and Max: 255 OTUs per sample). Quality trimming involved filtering out OTUs that appeared less than two times in the table. The trimmed OTU table was then rarefied to a depth of 25,000 reads in accordance with minimum cecum read count. In the small intestine we had a total of 1,988,963 reads from 45 samples (Min: 2211.0 Max: 133327.0, Median: 37317.000, Mean: 44199.178) and 821 OTUs after quality trimming (an average of 183.15 per sample and a Min: 86 Max: 277 OTUs per sample). Quality trimming involved filtering out OTUs that appeared less than two times in the table. The trimmed OTU table was then rarefied to 2,000 reads in the small intestine in accordance with the minimum small intestine read count. Rarefied OTU tables were then used for alpha and beta diversity analyses.

2.6 Statistical analysis

Statistical analyses for mouse behaviour, human ELISAs, and qPCR were performed using GraphPad Prism Version 6 (La Jolla, CA, USA). Figures of group comparisons were presented as box plots displaying the 10^{th}-90^{th} percentile of data variance. Two groups comparisons were done using the unpaired Student t-test or Mann-Whitney test where appropriate. STAMP bioinformatics software was used for the statistical analysis of PICRUST data; we used non parametric tests and constructed graphics using the relative mean frequency metrics provided by STAMP.

3. Results

3.1 Fecal beta defensin-2 levels elevated in MDD patients vs. HC donors

We measured fecal beta defensin-2 (BDEF2) level in MDD patients and HC donors using ELISA (Methods 2.1.2). MDD patients had significantly higher BDEF2 levels in stool vs HC donors, (Fig 1), (p=0.01). We found that the MDD group had a subset of patients with high BDEF2 levels. MDD1 and MDD2 were selected from this subset; MDD3 and MDD4 were selected from patients with normal BDEF2 levels (Methods 2.1.3).

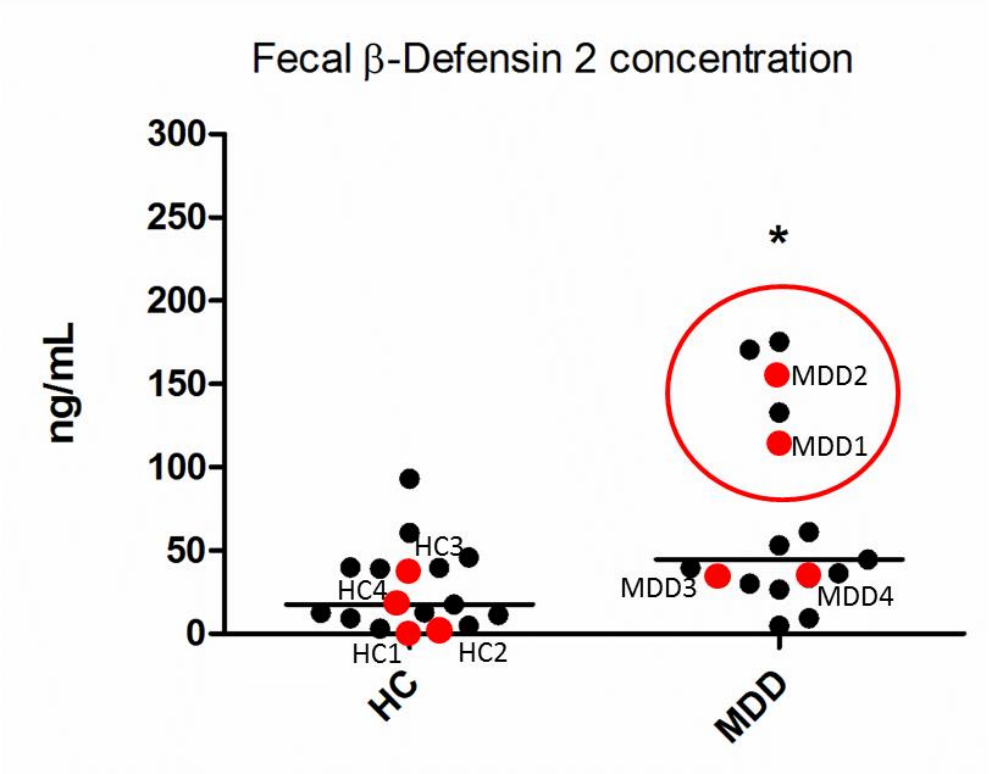


Figure 1) **Fecal concentrations of human Beta defensin 2 differ between MDD patients and HCs.** Fecal β-defensin 2 concentration is significantly elevated in MDD patients when compared to healthy controls (MDD n= 30, HC n= 30; Mann-Whitney test, p=0.01.)

3.2.1 MDD mice spent similar time in the centre of the open field to HC mice.

The open field test is used to measure anxiety like behaviour in the colonised mice (Prut L. & Belzung C., 2003; Methods 2.2.2). Some MDD donors in this study showed co-morbid anxiety and so we wanted to investigate whether this behaviour was transferred to colonised MDD mice. MDD1 (n=14), MDD2 (n=10), MDD3 (n=10) and MDD4 (n=11) mice spent similar time in the centre of the open field to HC1 (n=14), HC2 (n=11), HC3 (n=10) and HC4 (n=10) mice (Fig 2a i), implying that MDD mice did not show anxiety like behaviour in this test. Pooled MDD (n=45) mice spent similar time in the centre of the field to pooled HC mice (n=45) (Fig 2a ii).

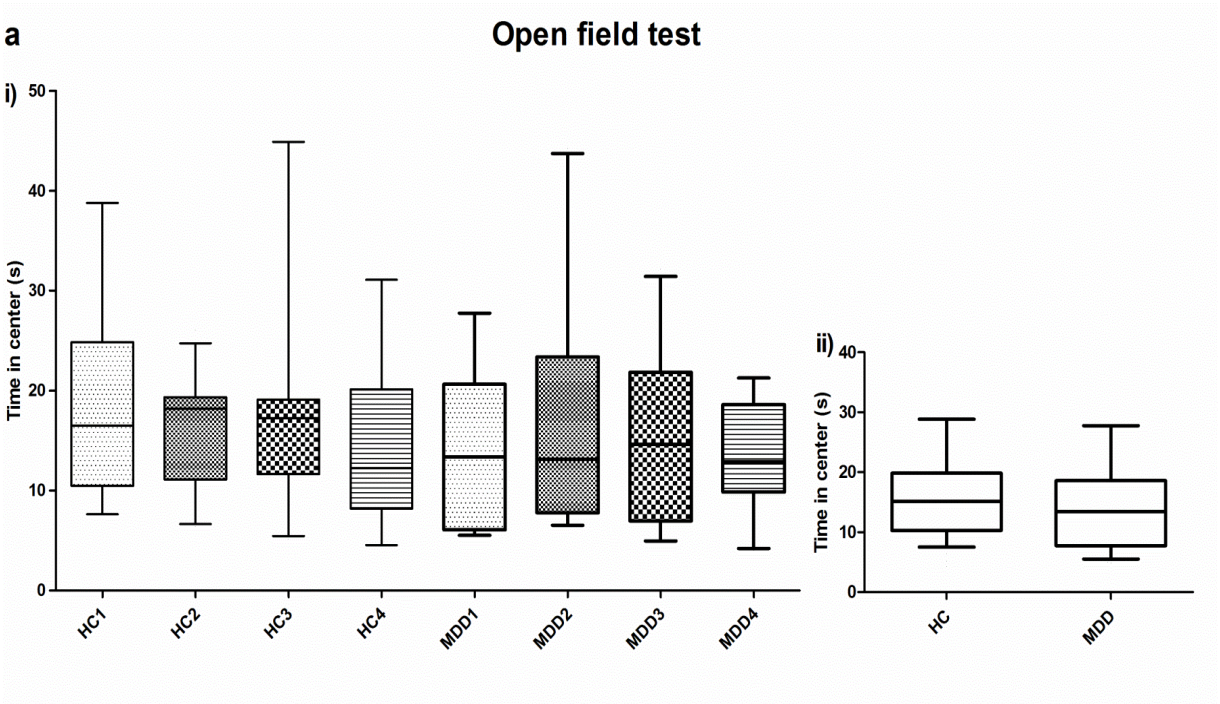


Figure 2 a) **Total time in centre of open field (time resting + time ambulatory + time stereotypic). MDD mice spend similar amount of time in centre of field relative to HC mice**.

i) No significant differences in time spent in centre of field between MDD mice and HC mice by donor, Mann-Whitney U-test: MDD1 n=14 vs HC1 n=14 p=0.26, MDD2 n=10 vs HC2 n= 11 p=0.69, MDD3 n=10 vs HC3 n=10 p=0.73, MDD4 n=11 vs HC4 n=10 p=0.91). Whiskers show 10^{th} and 90^{th} percentile.

ii) No significant difference in time spent in centre of field between pooled MDD mice and HC mice. Unpaired t-test: MDD mice n=45 vs HC mice n=45, p=0.38

3.2.2 MDD1 mice showed abnormal social behaviour in the three chamber sociability assay.

Social behaviour is measured in animal models of depression as abnormal social behaviour is a component of depressive behaviour (Russo S., et al. 2018). The three chamber sociability assay is used to test for abnormal social behaviour in MDD mice (Methods 2.2.2). MDD1 mice spent less time with novel mice than HC1 mice ($p=0.04$) (Fig 2b i), suggesting that MDD1 mice had abnormal social behaviour. MDD2, MDD3 and MDD4 mice did not show differences in social behaviour vs HC2, HC3 and HC4 respectively (Fig 2b i). Pooled MDD mice had trends for abnormal social behaviour compared to pooled HC mice ($p=0.09$) (Fig 2b ii).

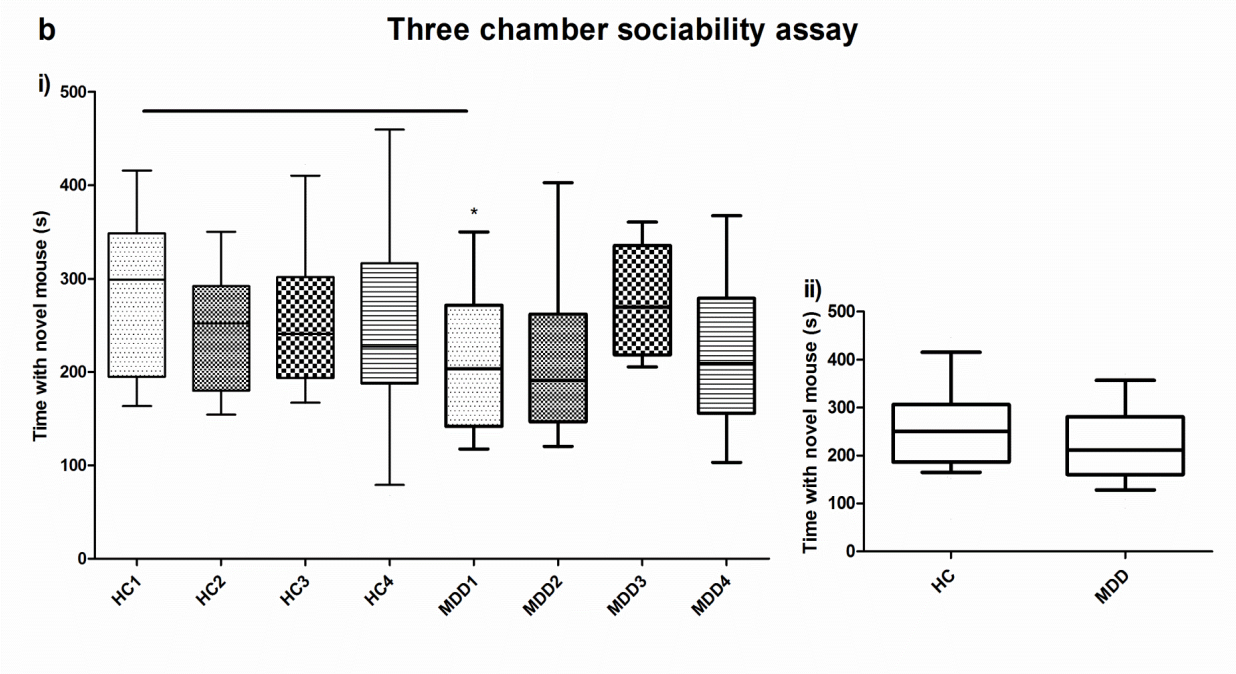


Figure 2 b) **Social behaviour of MDD and HC mice in the three chamber sociability assay**.

i) MDD1 mice have abnormal social behaviour compared to HC1 mice; other mice show no significant abnormal trend. Mann-Whitney U-test: MDD1 n=14 vs HC1 n=13 p=0.04, MDD2 n=10 vs HC2 n= 11 p=0.21, MDD3 n=10 vs HC3 n=10 p=0.09, MDD4 n=11 vs HC4 n=10 p=0.50 Whiskers show 10^{th} and 90^{th} percentile.

ii) Pooled MDD mice and HC mice have no significant differences in social behaviour. Unpaired t-test: MDD n=45 vs HC n=45, p=0.09. Whiskers show 10^{th} and 90^{th} percentile.

3.2.3 MDD mice did not show behavioural despair in the tail suspension test vs. HC mice

We used the tail suspension test to measure behavioural despair in the colonised mice; this was quantified as seconds spent immobile over six minutes, immobility in the tail suspension test is a sign of depressive-like behaviour (Cryan JF., et al. 2005) (Methods 2.2.2). MDD mice showed similar behaviour in the tail suspension test compared to HC mice (Fig 2c i). Pooled MDD mice did not show altered behavioural despair compared to pooled HC mice (Fig 2c ii).

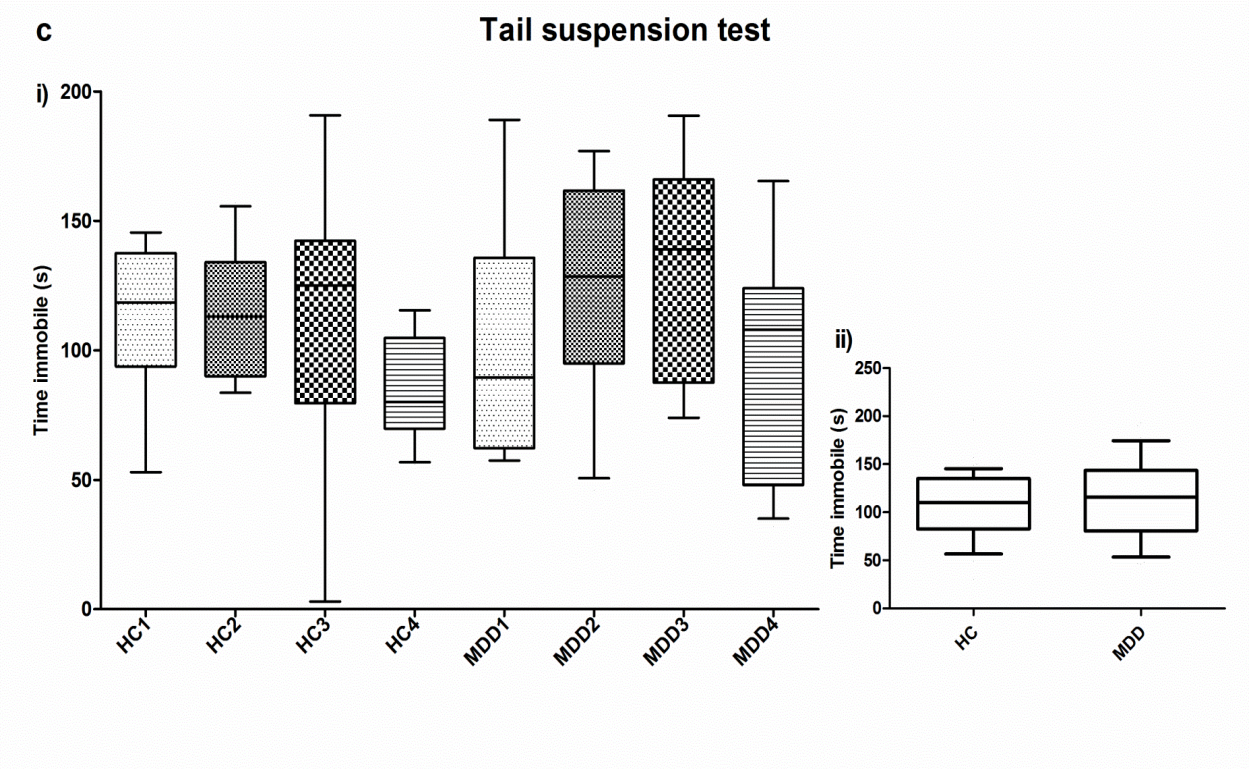


Figure 2 c) **Tail suspension tests, time spent immobile.**

i) No significant differences in time spent immobile between MDD mice and HC mice by donor.
Mann-Whitney U-test: MDD1 n=14 vs HC1 n=14 p=0.43, MDD2 n=10 vs HC2 n=11 p=0.37, MDD3 n=10 vs HC3 n=10 p=0.52, MDD4 n=11 vs HC4 n=10 p=0.35, Whiskers show 10th and 90th percentile.

ii) No significant difference in time spent immobile between pooled MDD mice and HC mice.
Unpaired t-test: MDD mice n=45 vs HC mice n=45, p=0.41 Whiskers show 10th and 90th percentile.

3.2.4 MDD1 mice showed features of anhedonia in the sucrose preference test vs. HC1 mice.

The sucrose preference test is used to measure for anhedonia, and is the gold standard measure of depressive-like behaviour in mouse models of depression (Eagle AL, et al. 2016; Methods 2.2.2). MDD1 mice showed abnormal sucrose preference compared to HC1 mice ($p=0.0016$) (Fig 2d i). MDD 2, MDD3 and MDD4 mice showed normal sucrose preference compared to HC mice. Pooled MDD mice had trends for lower sucrose preference than pooled HC mice, however this was not significant ($p=0.12$) (Fig 2d ii).

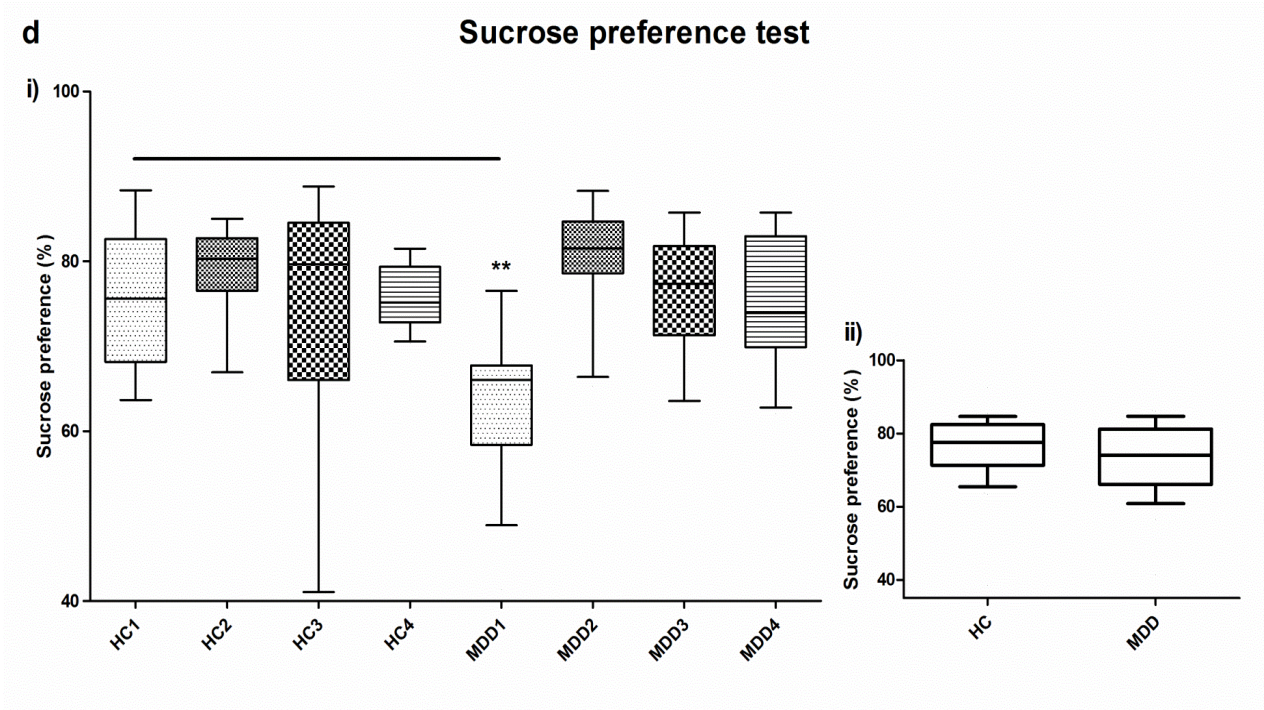


Figure 2 d) **Sucrose preference test. Percentage of sucrose consumed over 48 hours used to measure sucrose preference.**

i) MDD1 mice had significantly lower sucrose preference than HC1 mice. Mann-Whitney U-test: MDD1 n=14 vs HC1 n=14 p=0.0016, MDD2 n=10 vs HC2 n=11 p=0.41, MDD3 n=10 vs HC3 n=10 p=0.85, MDD4 n=11 vs HC4 n=10 p=0.75, whiskers show 10th and 90th percentile.

ii) No significant difference in sucrose preference between pooled MDD mice and HC mice.
Unpaired t-test: MDD mice n=45 vs HC mice n=45 p=0.12, whiskers show 10th and 90th percentile.

3.2.5 MDD1 mice show more negative emotionality than HC1 mice

The emotionality metric is used to compile behaviour tests into one score which can inform us on the overall emotional behavioural state of the mice, and is used to summarise depressive-like behaviours in models of depression (Guilloux JP., et al. 2011; Methods 2.2.2). MDD1 mice showed significantly altered emotional behaviour compared to HC1 ($p=0.02$) (Fig 2e i). MDD2, MDD3 and MDD4 mice did not have significantly altered emotional behaviour compared to HC mice. Pooled MDD mice had trends for altered emotional behaviour compared to pooled HC mice (Fig 2e ii).

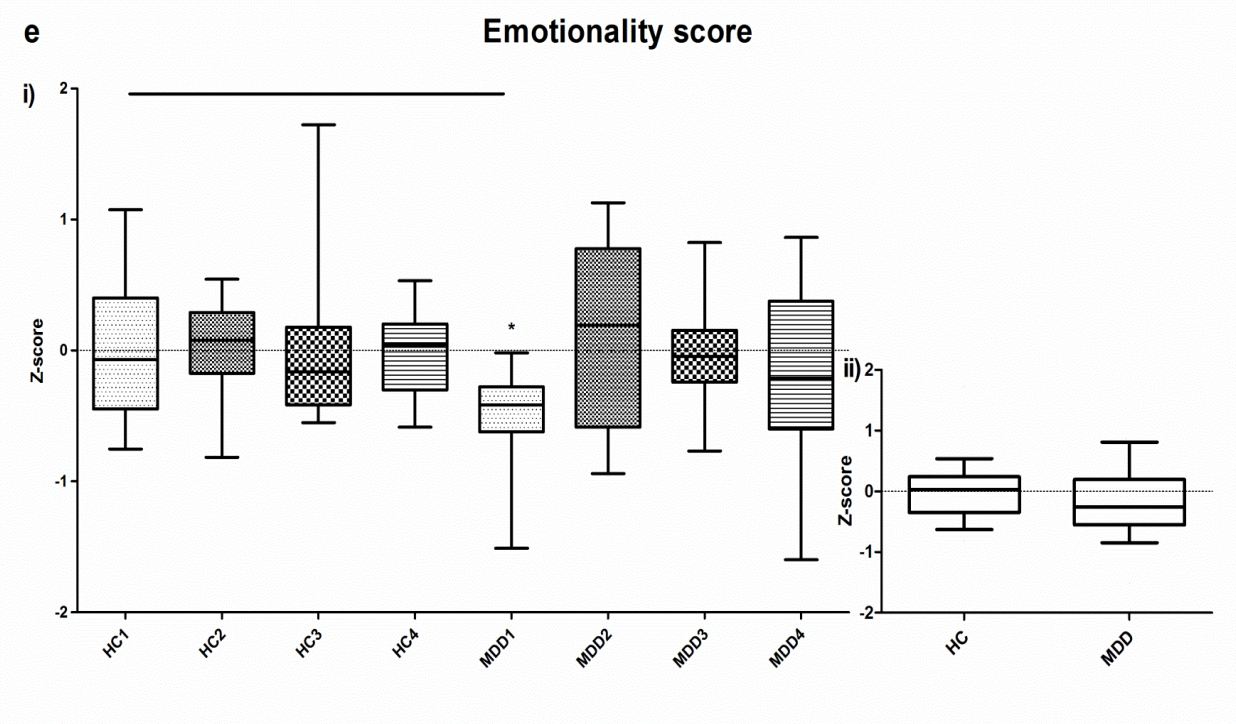


Figure 2 e) **Behaviour tests were z-scored and collated to generate an emotionality metric, with a lower score indicative of abnormal behaviour.**

i) MDD1 mice show significantly abnormal emotionality scores compared to HC1 mice Mann-Whitney U-test: MDD1 n=14 vs HC1 n=14 p=0.02, MDD2 n=10 vs HC2 n=11 p=0.69, MDD3 n=10 vs HC3 n=10 p=0.63, MDD4 n=11 vs HC4 n=10 p=0.54 whiskers show 10^{th} and 90^{th} percentile.

ii) No significant difference in emotionality scores between pooled MDD mice and HC mice.
Unpaired t-test: MDD mice n=45 vs HC mice n=45 p=0.11, whiskers show 10^{th}

3.3 MDD1 mice show altered occludin and beta defensin-3 expression in the small intestine and colon

Beta defensin-3 (BDEF3) is the mouse homologue for human BDEF2 (Bals R., et al. 1999). We wanted to investigate whether MDD1 microbiota colonisation was sufficient to induce the upregulation of BDEF3 expression in MDD1 mice. We analysed the relative gene expression of BDEF3 in the colon and small intestine of MDD1 and HC1 mice as described in Methods 2.4. BDEF3 expression was elevated in MDD1 mice (n=8) small intestine (Fig 3a) ($p=0.008$), and colon ($p=0.04$) vs. HC1 mice (n=6). Occludin is a tight junction protein in the gut epithelium which contributes to gut barrier integrity (Mir H., et al. 2016). We hypothesise that proinflammatory microbiota could disrupt the gut barrier and alter gut occludin expression. We analysed the relative gene expression of occludin in the gut of MDD1 and HC1 mice as described in Methods 2.4. MDD1 mice showed lower occludin expression in the small intestine vs. HC1 mice (Fig 3b) ($p=0.008$) but not in the colon.

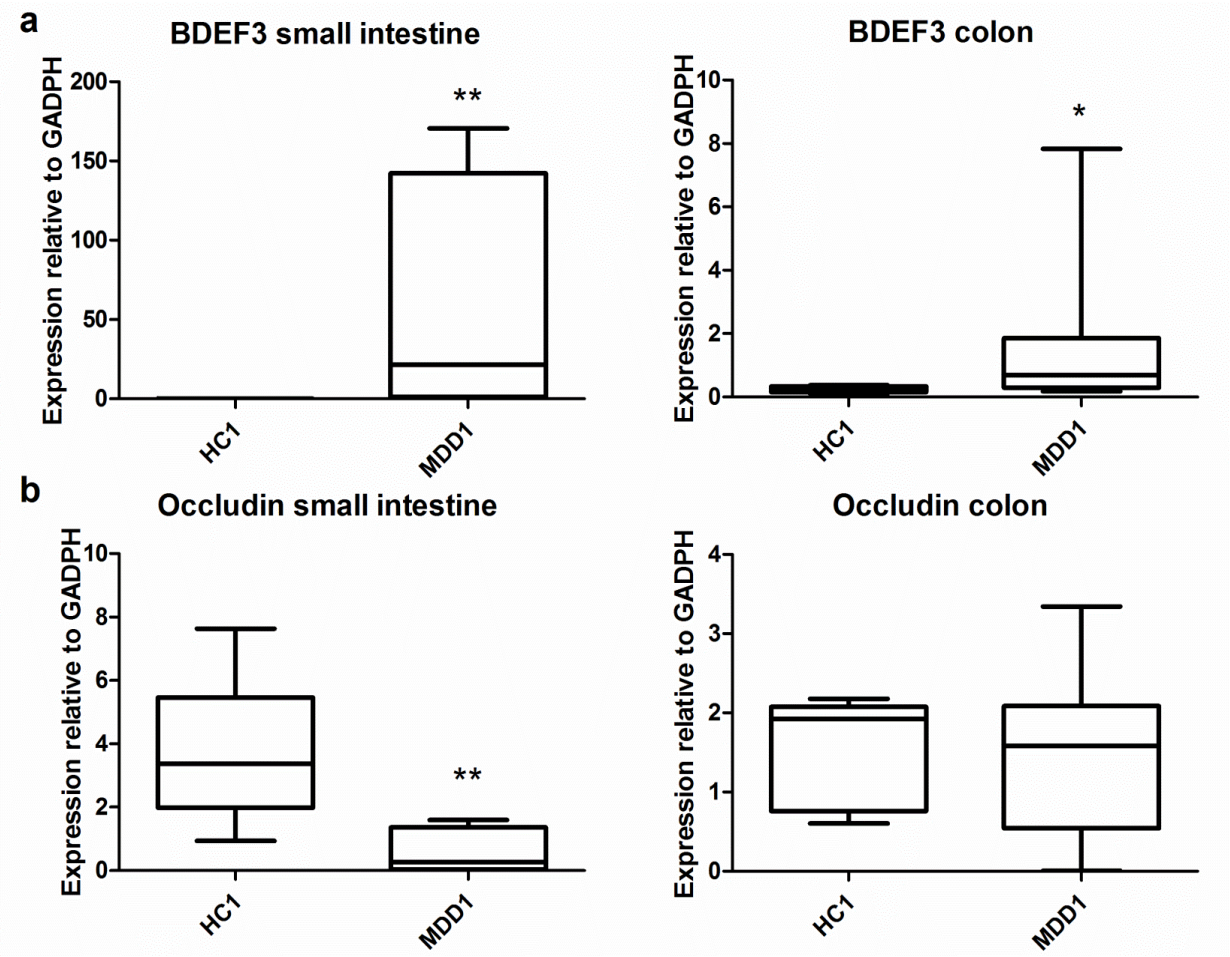


Figure 3 **Relative expression of pro-inflammatory and barrier function genes in the colon and small intestine of MDD1 and HC1 colonised mice**. Mann-Whitney U-test: MDD1 n=8 vs HC1 n=6

a) Expression of BDEF3 in MDD1 mice colon is significantly higher than in HC1 mice p= 0.04, Expression of BDEF3 in MDD1 mice small intestine is significantly higher than in HC1 mice p=0.008, whiskers show 10th and 90th percentile.
b) Expression of occludin in MDD1 mice colon p= 0.85 similar to HC1 mice, Expression of occludin in MDD1 mice small intestine significantly lower than in HC1 mice p= 0.008, whiskers show 10th and 90th percentile.

3.4 MDD1 mice show altered GABA related gene expression in the small intestine vs. HC1 mice

GABAergic neurons have been previously implicated in MDD pathology and the microbiota-gut-brain axis (Gilabert-Juan J., et al. 2013; Tripp A., et al. 2011; Bravo J.A., et al. 2011). We analysed the relative expression of GABAergic markers GAD1 (GABA transporter 67), and $GABA_{B1\&2}$ receptors in the small intestine and colon of MDD1 and HC1 mice as described in Methods 2.4. MDD1 mice had increased expression of GAD1 ($p=0.02$) (Fig 4c) and $GABA_{B2}$ (Fig 4b) ($p=0.0007$) in the small intestine vs. HC1 mice. We saw similar expression of $GABA_{B2}$ and GAD1 in the colon of MDD1 and HC1 mice (Fig 4b, c). We saw no alterations of $GABA_{B1}$ in the small intestine or colon (Fig 4a).

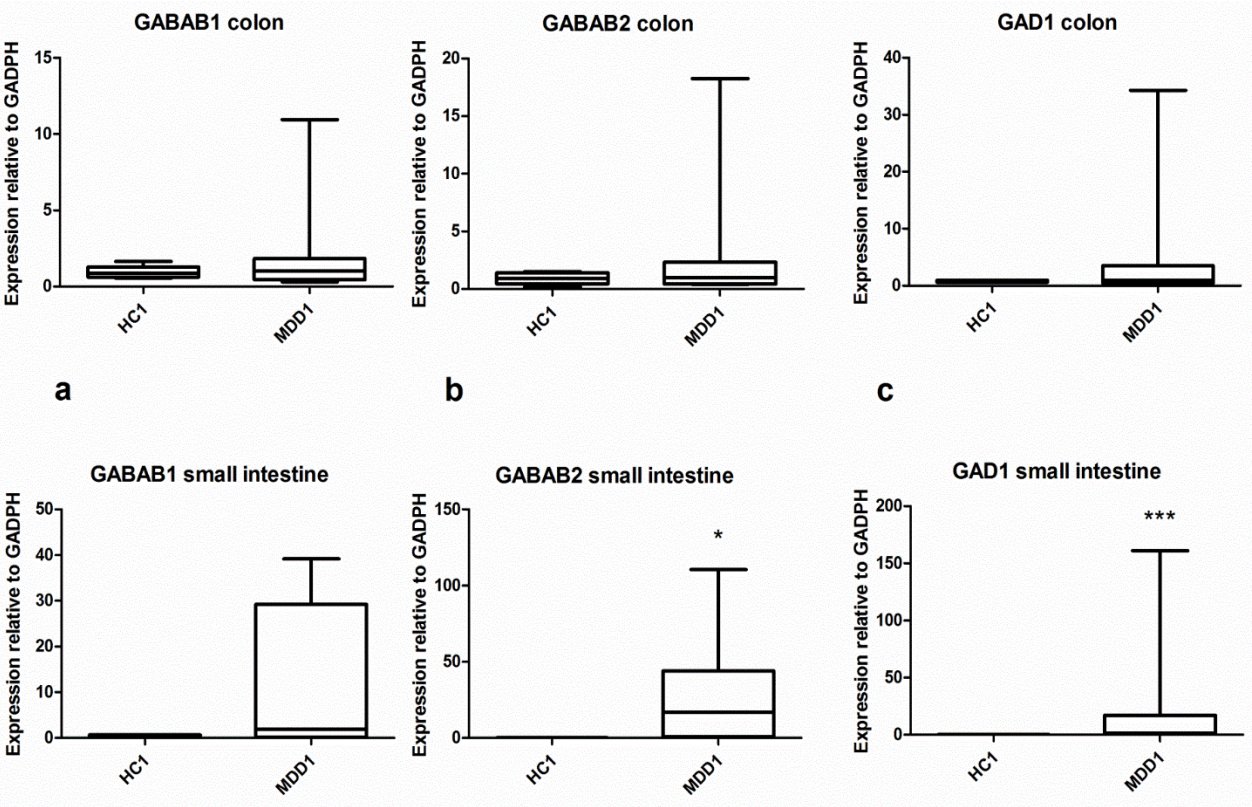


Figure 4 **Relative expression of GABA related genes in colon and small intestine of MDD1 and HC1 colonised mice.** Mann-Whitney U-test: MDD1 n=8 vs HC1 n=6

a) Expression of GABAB1 in MDD1 mice colon is similar to HC1 mice p= 0.94, Expression of GABAB1 in MDD1 mice small intestine is similar to HC1 mice p= 0.49, whiskers show 10th and 90th percentile.
b) Expression of GABAB2 in MDD1 mice colon is similar to HC1 mice p= 0.66, Expression of GABAB2 in MDD1 mice small intestine significantly higher than in HC1 mice p= 0.02, whiskers show 10th and 90th percentile.
c) GAD1 in MDD1 mice colon is similar to HC1 mice p= 0.57, GAD1 in MDD1 mice small intestine is significantly higher than in HC1 mice p=0.0007, whiskers show 10th and 90th percentile.

3.5 MDD1 mice show altered occludin, GABAergic and epigenetic gene expression in the nucleus accumbens and prefrontal cortex respectively.

Occludin forms part of the blood brain barrier (BBB), downregulation to occludin expression can alter the BBB and influence mouse behaviour (Huber J.D., et al. 2001). We investigated whether MDD1 microbiota could alter relative occludin expression in the prefrontal cortex of MDD1 mice as described in Methods 2.4. We found that MDD1 mice have decreased relative occludin expression in the prefrontal cortex ($p=0.008$) (Fig 3a) vs. HC1 mice. GABAergic neurons have been previously implicated in MDD pathology and the microbiota-gut-brain axis (Gilabert-Juan J., et al. 2013; Tripp A., et al. 2011; Bravo J.A., et al. 2011). We analysed the relative expression of GABAergic markers GAD1, and $GABA_{B1}$ in the nucleus accumbens of MDD1 and HC1 mice as described in Methods 2.4. We found that the relative expression of $GABA_B$ (Fig 3b) ($p=0.01$) and GAD1 (Fig 3d) ($p=0.015$) was increased in the nucleus accumbens of MDD1 mice vs HC1 mice. HDACs are associated with depressive-like behaviour in chronic stress mice, so we wanted to investigate whether MDD1 mice had alterations in HDAC expression in the limbic system (Covington 3rd H.E., et al. 2009). We investigated the relative expression of HDAC3 in the nucleus accumbens of MDD1 mice and found HDAC3 to be increased in the nucleus accumbens of MDD1 mice (Fig 3c) ($p=0.03$) vs. HC1 mice.

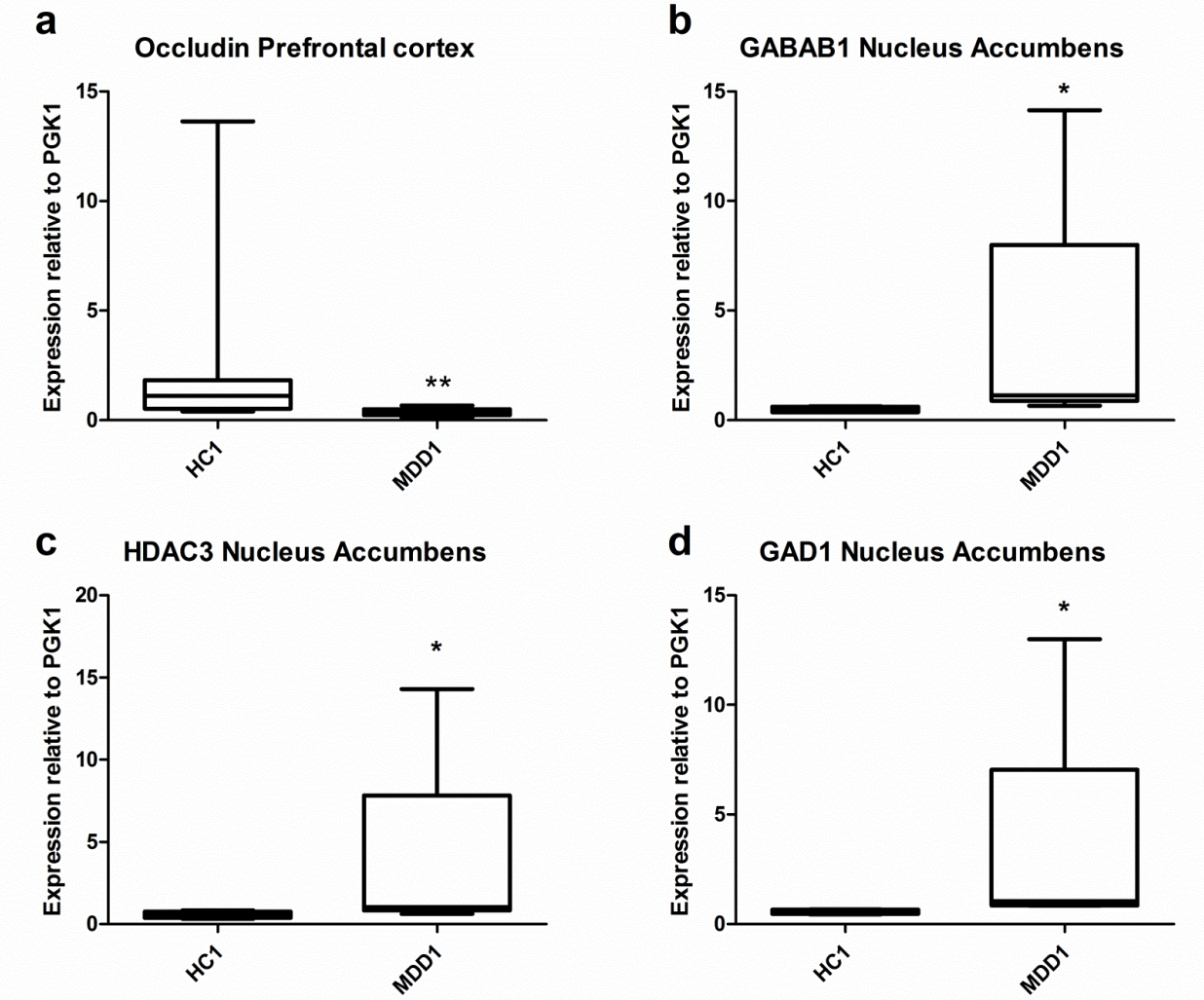


Figure 5 **Relative expressions of GABAB1, HDAC3 and GAD1 in the Prefrontal cortex and Nucleus accumbens of MDD1 mice and HC1 mice.** Mann Whitney U-test

a) Occludin expression is significantly lower in MDD1 mice (n=6) PrF than in HC1 mice (n=7) p= 0.008, whiskers show 10th and 90th percentile.
b) GABAB1 expression is significantly higher in MDD1 mice (n=5) in the NAcc than in HC1 mice (n=4) p= 0.01, whiskers show 10th and 90th percentile.
c) HDAC3 expression significantly higher in MDD1 mice in the NAcc p= 0.03, whiskers show 10th and 90th percentile.
d) GAD1 expression is significantly higher in MDD1 mice in the NAcc than in HC1 mice (n=4) p= 0.015, whiskers show 10th and 90th percentile.

3.6 MDD1 and MDD4 mice have altered gene expression vs. HC1 and HC4 mice in the small intestine, colon and hippocampus.

We wanted to assess differences in RNA expression in MDD1, MDD4, HC1 and HC4 mice in the small intestine, colon (MDD1 n=8 vs. HC1 n=6; MDD4 n=5, HC4 n=6), and hippocampus (MDD1 n=6 vs. HC1 n=4; MDD4 n=5 vs. HC4 n=5) as we hypothesise that MDD microbiota can induce alterations to neural and immune gene expression along the gut-brain axis. We extracted RNA from the tissues of interest and used a custom Nanostring code set to assess gene expression (Methods 2.4). We generated expression ratios for MDD mice vs. HC mice in the tissues of interest and used the mean expression of genes in MDD and HC mice in Table 1 to illustrate whether expression was significantly higher (green) or lower (red) in MDD/HC mice. We found a range of neuroimmune markers altered in MDD1 (Table 1A) and MDD4 mice (Table 1B) vs HC1 and HC4 mice.

Gene	Accession #	HC1	MDD1	p-value
Small intestine				
C3	NM_009778.2			0.001
CCR2	NM_009915.2			0.0044
P75	NM_033217.3			0.0071
CHAT	NM_009891.2			0.0079
GATA-3	NM_008091.3			0.0085
Cldn2	NM_016675.3			0.0119
GABAB	NM_019439.3			0.0232
Nod2	NM_145857.2			0.0256
CCR6	NM_001190333.1			0.0278
CXCR3	NM_009910.2			0.0301
Tlr2	NM_011905.2			0.0306
Casp8	NM_009812.2			0.0336
Cldn1	NM_016674.3			0.0356
nNOS	NM_008712.2			0.0451
Rae1	NM_175112.5			0.0483
Colon				
Myd88	NM_010851.2			0.001
S100BETA	NM_009115.3			0.0132
Gpr44	NM_009962.2			0.0158
Ocln	NM_008756.2			0.0191
CALRET	NM_007591.3			0.0349
F2rl1	NM_007974.4			0.0445
Hippocampus				
P75	NM_033217.3			0.043351

Gene	Accession #	HC4	MDD4	p-value
Small intestine				
Tlr4	NM_021297.2			0.031336
PGP9.5	NM_011670.2			0.048708
TAAR4	NM_001008499.1			0.048708
CD11b	NM_001082960.1			0.04931
Colon				
CCR6	NM_001190333.1			0.000409
Hif1a	NM_010431.2			0.001324
Il23r	NM_144548.1			0.001649
MMP9	NM_013599.2			0.003579
SEMAPHO	NM_011352.2			0.004936
5HT	NM_010484.2			0.005597
CD11C	NM_021334.2			0.008911
Il22ra2	NM_178258.5			0.019831
Cxcr4	NM_009911.3			0.021121
trpv1	NM_001001445.1			0.021662
Cd86	NM_019388.3			0.024583
Mapk1	NM_011949.3			0.025055
CXCR3	NM_009910.2			0.027143
Tlr2	NM_011905.2			0.028957
Cldn1	NM_016674.3			0.035478
Rae1	NM_175112.5			0.035796
C3	NM_009778.2			0.036733
GDNF	NM_010275.2			0.038107
Lyz1	NM_013590.3			0.03975
Tollip	NM_023764.3			0.044696
Hippocampus				
SUBP	XM_006505028.1			0.01426
Il22ra2	NM_178258.5			0.02505
Ocln	NM_008756.2			0.032789
GABAA	NM_010252.4			0.034679

Table 1 **Nanostring gene expression analysis of MDD1, MDD4, HC4 and HC1 mice.**
Green indicates higher expression, red indicates lower mean expression to illustrate MDD1/MDD4:HC1/HC4 expression ratios.

A) A range of genes associated with the immune and nervous system were found to be significantly altered in the gut (MDD1 n=8 vs. HC1 mice n=6) and hippocampus (MDD1 n=6 vs. HC1 mice n=4) of MDD1 mice and HC1 mice.

B) Genes associated with the immune and nervous system were found to be significantly altered in the gut (MDD4 n=5 vs HC4 n=6) and hippocampus (MDD4 n=5 vs HC4 n=5) of MDD4 and HC4 mice. Neural, immune, and BBB associated genes were significantly altered in the hippocampus of MDD4 mice.

3.7 MDD4 mice show no alterations in relative gene expression of neural and inflammatory markers in the colon vs. HC4 mice.

We analysed the relative gene expression of BDEF3 in the colon of MDD4 (n=5) and HC4 mice (n=6) as we wanted to ascertain whether the higher BDEF3 expression seen in MDD1 mice was a result of MDD1 microbiota colonisation or a by-product of bacterial colonisation. MDD4 and HC4 donors had normal BDEF2 levels (Methods 2.1.3), so we expected no difference in BDEF3 expression in MDD4 and HC4 microbiota colonised mice. We saw no alterations to relative BDEF3 expression in MDD4 mice vs. HC4 mice (Fig 7b). In addition to this we also assessed relative $GABA_B$, occludin and NFkB expression as we wanted to see if there were any common expression patterns between MDD1 and MDD4 mice. We saw no differences in $GABA_B$ (Fig 3c), Occludin (Fig 3a), or NFkB (Fig 3d) expression in MDD4 mice vs. HC4 mice.

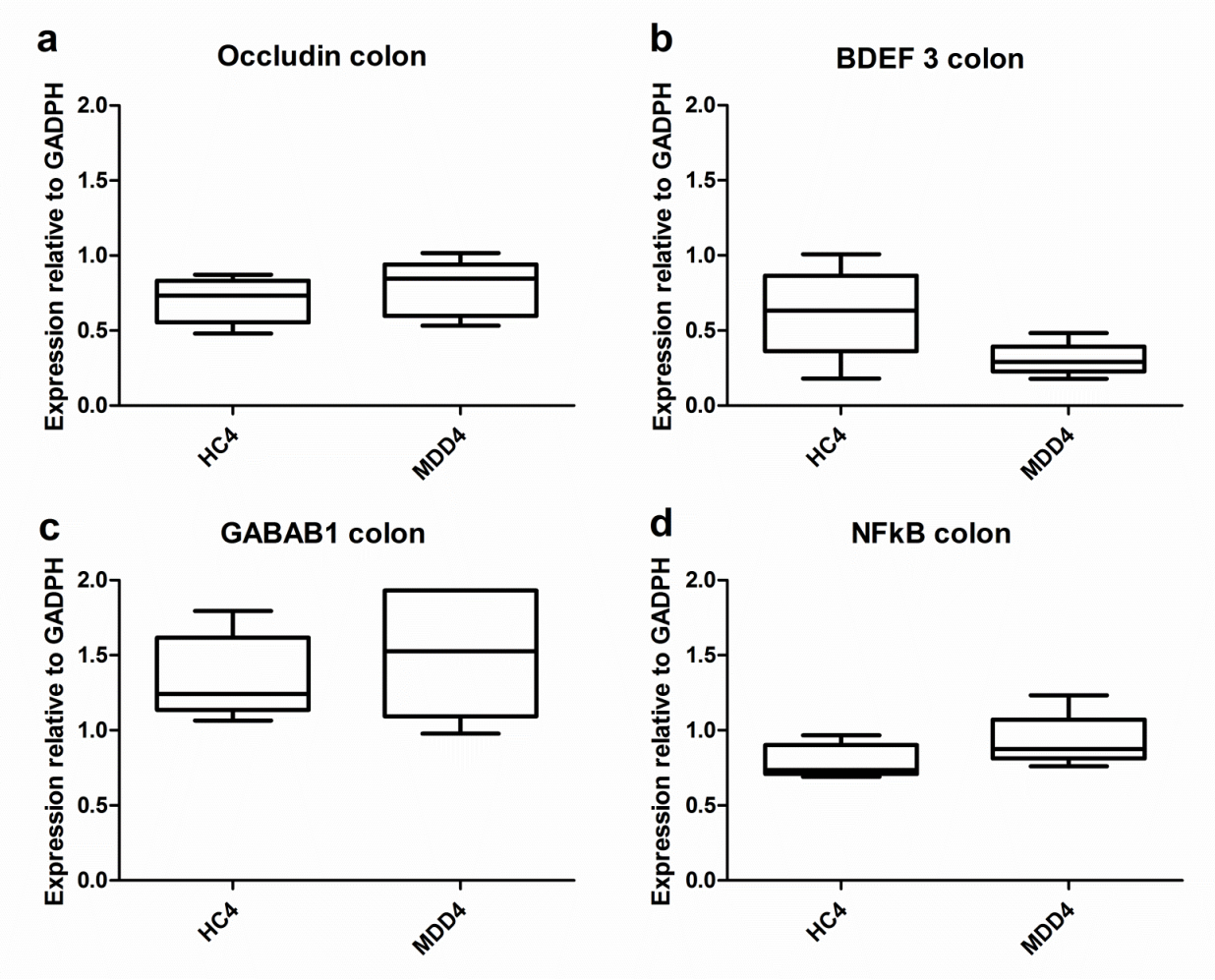


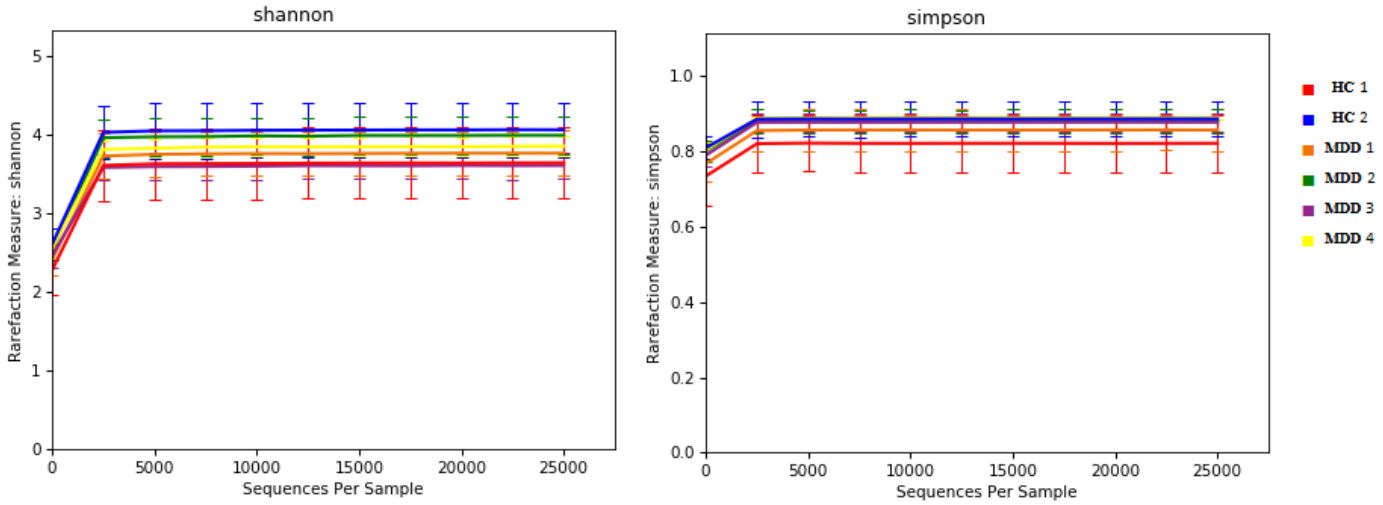
Figure 7 **Relative expression of GABAB1, BDEF3, NFkB, and occludin in the colon of MDD4 and HC4.** (Mann-Whitney U-test: MDD4 n=5 vs HC4 n=6)

a) Occludin expression in MDD4 mice colon is similar to HC4 mice. p= 0.53, whiskers show 10th and 90th percentile.
b) BDEF3 expression in MDD4 mice colon trends to be lower than in HC4 mice but not significantly p= 0.08, whiskers show 10th and 90th percentile.
c) GABAB1 expression in MDD4 mice colon not significantly different than in HC4 mice p= 0.11, whiskers show 10th and 90th percentile.
d) NFkB expression in MDD4 mice colon not significantly different than in HC4 mice p= 0.17, whiskers show 10th and 90th percentile.

3.8 Alpha diversity of MDD1, MDD2, MDD3, MDD4, HC1 and HC2 mice cecum and small intestine microbiota

We wanted to assess the diversity of species in MDD and HC mice small intestine and cecum microbiota, as microbiome species diversity has been implicated in MDD previously (Background 1.8.2). We saw no difference in cecum species diversity between MDD1 (n=16), MDD2 (n=7), MDD3 (n=5), MDD4 (n=5), HC1 (n=20), HC2 (n=8) mice in Shannon and Simpson diversity indices (Fig 8a). Pooled cecum MDD (n=33) and HC microbiota (n=28) showed no difference in cecum diversity (Fig 8b). We saw no difference in small intestine species diversity between MDD1 (n=12), MDD2 (n=7), MDD3 (n=5), MDD4 (n=5), HC1 (n=8), HC2 (n=8) mice in Shannon and Simpson diversity indices (Fig 8c). Pooled SI MDD and HC microbiota showed no difference in small intestine diversity (Fig 8d).

a



b

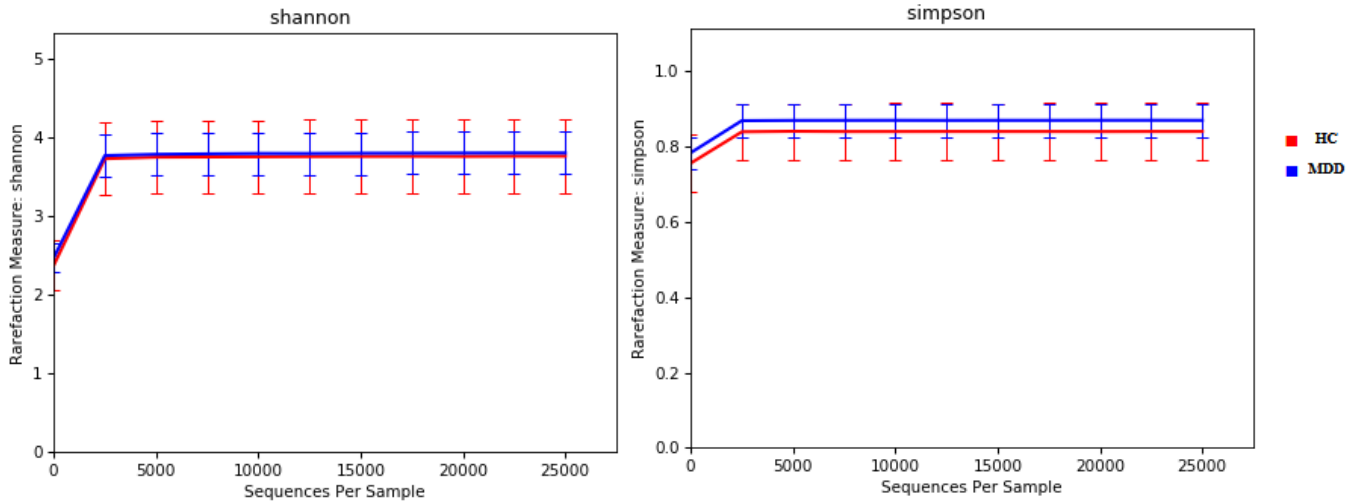


Figure 8 **Alpha diversity analysis of MDD and HC mice cecum microbiota.**

a) There was no difference in cecum microbiota diversity between MDD1 (n=16), MDD2 (n=7), MDD3 (n=5), MDD4 (n=5), HC1 (n=20), HC2 (n=8) mice groups as assessed by the shannon and simpson index, unpaired t-test $p>0.05$.

b) There was no difference in cecum microbiota diversity between pooled MDD mice n=33 and HC mice n=28 as assessed by the shannon and simpson index, unpaired t-test $p>0.05$.

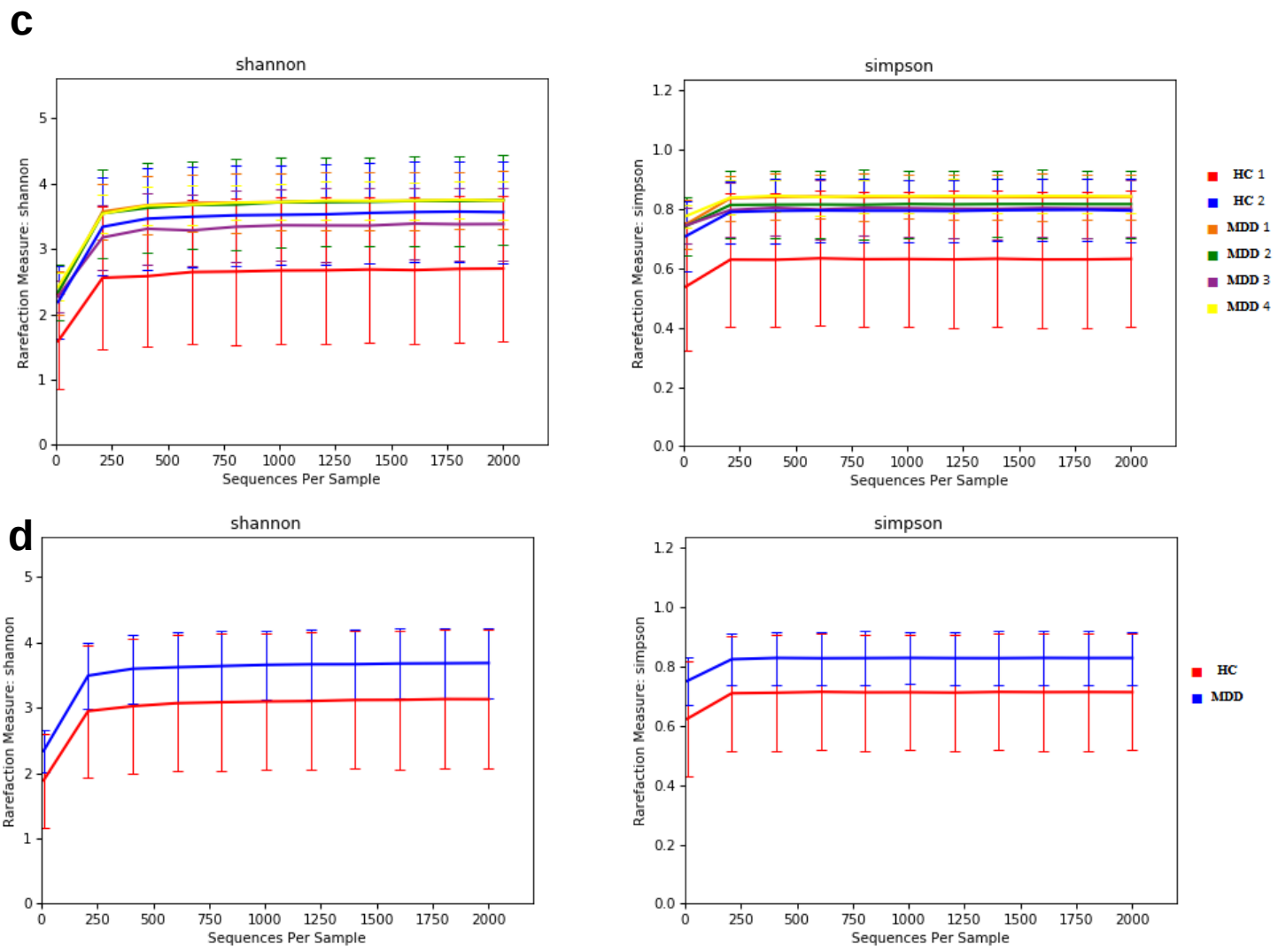


Figure 8 **Alpha diversity analysis of MDD and HC mice small intestine microbiota.**

c) There was no difference in small intestine microbiota diversity between MDD1 (n=12), MDD2 (n=7), MDD3 (n=5), MDD4 (n=5), HC1 (n=8), HC2 (n=8) mice groups as assessed by the shannon and simpson index, unpaired t-test p>0.05.

d) There was a significant difference in SI microbiota diversity between pooled MDD mice (n=29) and HC mice (n=16) as assessed by the shannon and simpson index, p=0.021; p=0.005.

3.9 Beta diversity of MDD1, MDD2, MDD3, MDD4, HC1 and HC2 mice cecum and small intestine microbiota.

We wanted to see the similarity between MDD mice vs. HC mice small intestine and cecum microbiota as we wanted to ascertain whether the alterations to mouse behaviour and gene expression could be explained by significant dissimilarity of MDD1 mice microbiota from HC1 mice microbiota. We hypothesised that cecum MDD1 (n=16), MDD2 (n=7), MDD3 (n=5), MDD4 (n=5), HC1 (n=20), and HC2 (n=8) microbiota would cluster distinctly from each other in addition to small intestine MDD1 (n=12), MDD2 (n=7), MDD3 (n=5), MDD4 (n=5), HC1 (n=8), HC2 (n=8) microbiota. We used unweighted and weighted unifrac metrics to assess the similarity between microbiota and constructed principle coordinate analysis plots with these metrics (Fig 9). We found that MDD1, MDD2, HC1, HC2 mice cecum and SI microbiota were significantly dissimilar when analysed with unweighted unifrac (Fig 9a, c) (p=0.001). Weighted unifrac analysis showed significant dissimilarity in MDD1 vs HC1 mice SI microbiota (Fig 9d) (p=0.008) but not in cecum microbiota (Fig 9b) (p=0.1). Weighted unifrac Dissimilarity in MDD2 vs HC2 microbiota was significant in the cecum (Fig 9b) (p=0.025) but not in the small intestine (Fig 9d) (p=0.067).

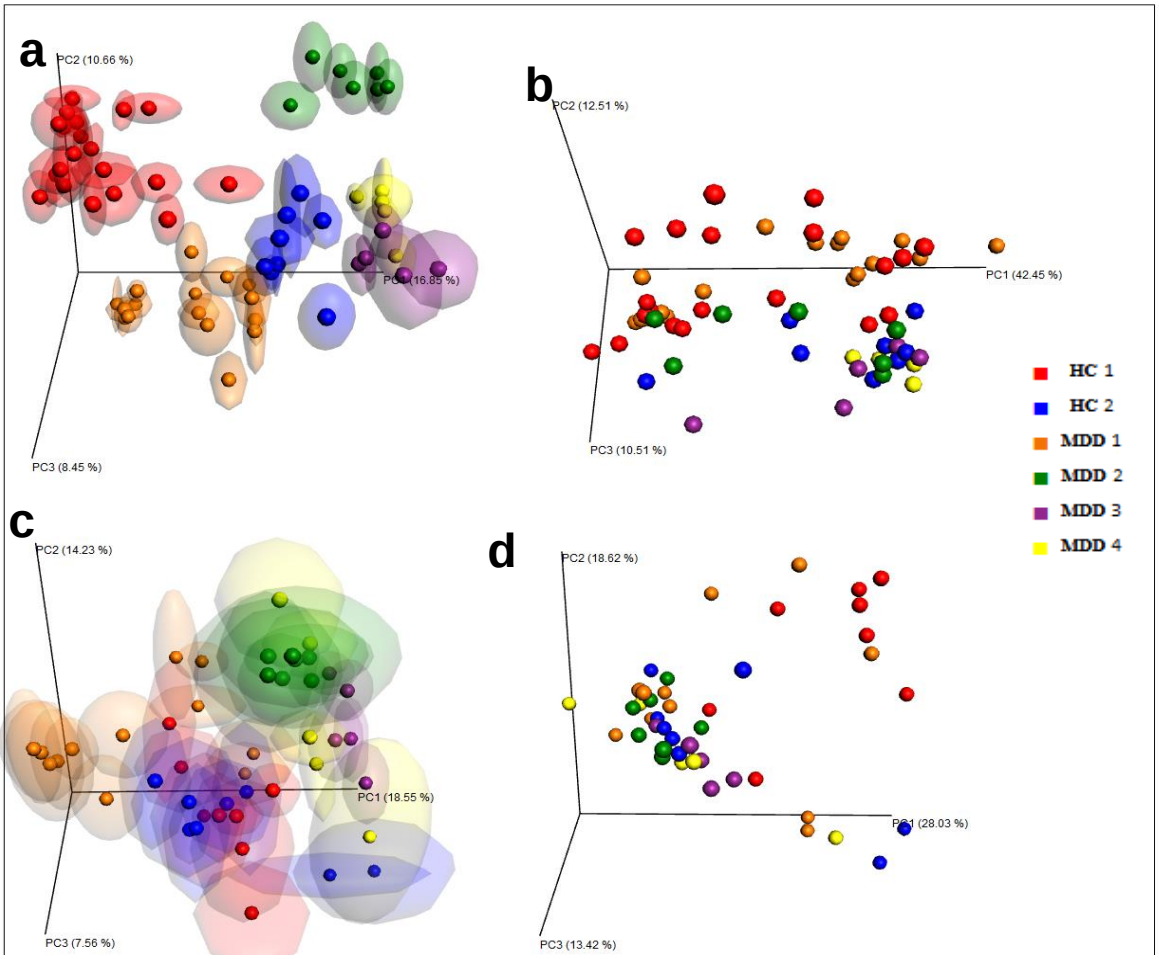


Figure 9 **Jackknifed beta diversity analysis of MDD and HC mice microbiota in the small intestine and cecum, visualised through PCoA plots, halos represent interquartile range from jackknife resampling.**

a) i) Cecum microbiota of MDD1 n=16 and HC1 n=20 cluster distinctly according to the unweighted unifrac similarity metric, ANOSIM p=0.001, ii) Microbiota of MDD2 n=7 and HC2 n=8 mice cluster distinctly, unweighted unifrac ANOSIM p=0.001. iii) Cecum microbiota of MDD mice n=33 HC mice n=28 (all donors) and HC mice (all donors) cluster distinctly, unweighted unifrac ANOSIM p=0.001

b) i) Cecum microbiota of MDD1 and HC1 mice do not cluster distinctly according to the weighted unifrac metric, ANOSIM p=0.1 ii) Cecum microbiota of MDD2 and HC2 mice cluster distinctly according to the weighted unifrac metric, ANOSIM p=0.025. iii) Cecum microbiota of MDD mice (all donors) and HC mice (all donors) cluster distinctly, weighted unifrac ANOSIM p=0.005

c) Small intestine microbiota of MDD1 n=12 and HC1 n=8 mice cluster distinctly, unweighted unifrac ANOSIM p=0.001 ii) SI microbiota of MDD2 n=7 and HC2 n=8 mice cluster distinctly, unweighted unifrac ANOSIM p=0.001, iii) SI microbiota of pooled MDD mice (n=29) and pooled HC mice (n=16) cluster distinctly, weighted unifrac ANOSIM p=0.001

d)Small intestine microbiota of MDD1 and HC1 mice cluster distinctly, weighted unifrac ANOSIM p=0.008 ii) SI microbiota of MDD2 and HC2 do not cluster distinctly, weighted unifrac ANOSIM p= 0.067. iii) SI microbiota of MDD mice (all donors) and HC mice (all donors) cluster distinctly, weighted unifrac ANOSIM p=0.008.

3.10 Predicted metabolic pathways altered in MDD1 small intestine microbiota.

We wanted to investigate the metabolic output of MDD1 and HC1 microbiota as we know microbiota metabolites are key in mediating microbiota-gut-brain interactions (Background 1.9.3), and we saw significant alterations to MDD1 mice behaviour and gene expression along the gut brain-axis. We used PICRUST to predict the metabolic pathways of MDD1 and HC1 microbiota as previously described (Caporaso J.G., et al. 2010; Morgan X.C., et al. 2015). We found that MDD1 small intestine microbiota (n=12) had a higher proportion of sequences associated with glutamate (Fig 10 d) and glutamatergic metabolism (Fig 10 e), than HC1 small intestine microbiota (n=8) (p=0.03, p=0.0009). We did not see this trend in the cecum microbiota of MDD1 (n=16) and HC1 mice (n=20) (Fig 10h). We saw increased biosynthesis of monoamine neurotransmitters and LPS in MDD1 vs. HC SI microbiota (p=0.0009; p=0.018) (Fig 10f, a). We did not see alterations to LPS biosynthesis in the cecum microbiota of MDD1 mice (Fig 10i) (p=0.33). SCFA metabolism was lower in MDD1 vs. HC SI microbiota (Fig 10b, c) (p=0.04, p=0.02). We did not see this trend in MDD1 vs. HC1 cecum microbiota (Fig 10g) (p=0.08).

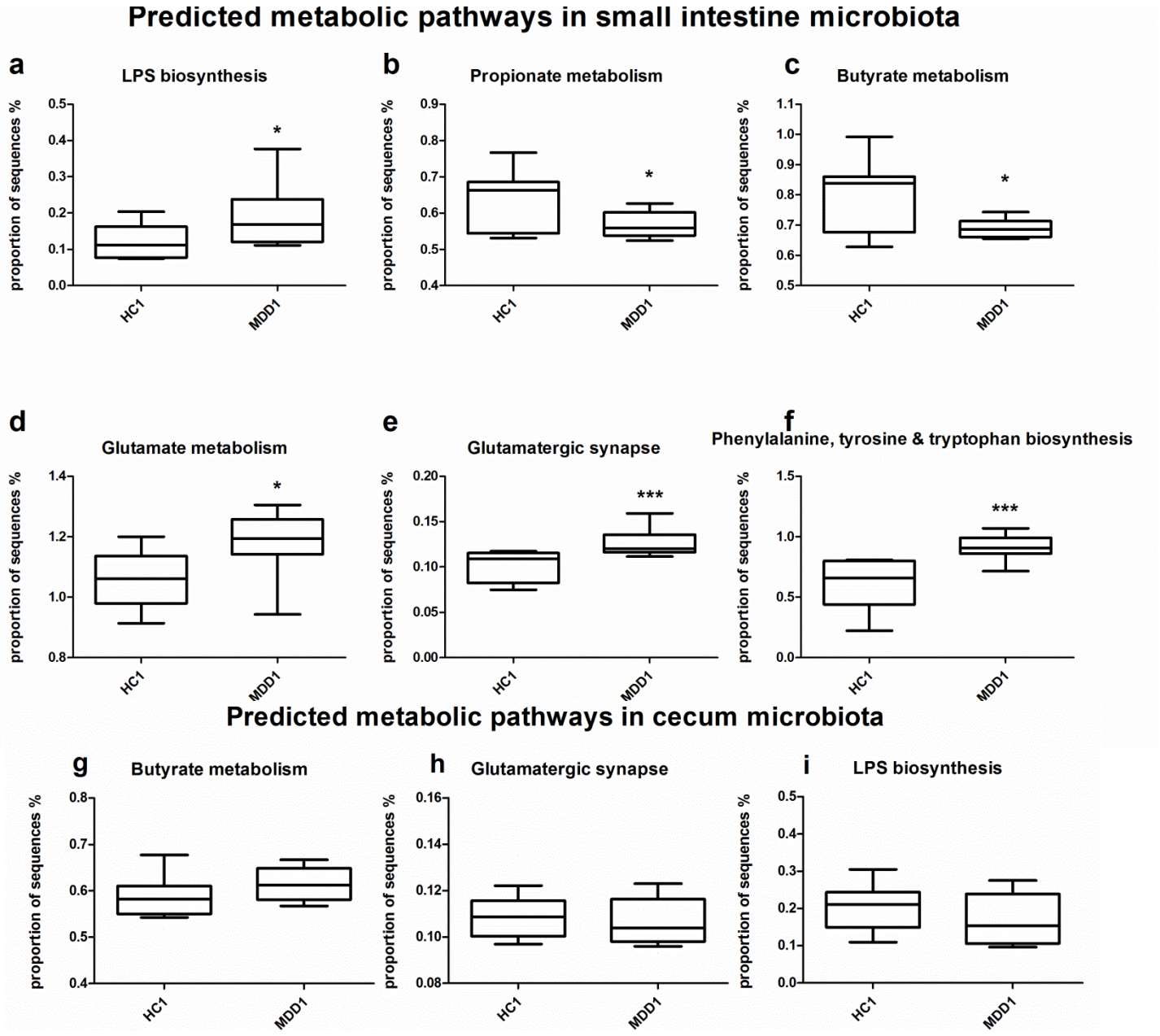


Figure 10) **Predicted metabolic pathways of MDD1 and HC1 gut microbiota**: Small intestine MDD1 n=12 vs HC1 n=8, cecum MDD1 n=16 HC n=20, Welch's non parametric test of % proportion of sequences, whiskers show 10th and 90th percentile.

a) LPS biosynthesis is elevated in MDD1 SI microbiota vs HC SI microbiota, p=0.018

b) Propionate metabolism lower in MDD1 SI microbiota vs HC SI microbiota, p=0.04

c) Butyrate metabolism lower in MDD1 SI microbiota vs HC SI microbiota, p=0.02

d) Glutamate metabolism higher in MDD1 SI microbiota vs HC SI microbiota, p=0.03

e) Glutamatergic synapse associated metabolism higher in MDD1 SI microbiota vs HC SI microbiota, p=0.0009

f) Monoamine biosynthesis higher in MDD1 SI microbiota vs HC SI microbiota, p=0.0009

g) Butyrate metabolism similar in MDD1 cecum microbiota vs HC cecum microbiota, p=0.08

h) Glutamatergic synapse associated metabolism similar in MDD cecum microbiota vs HC cecum microbiota p=0.9

i) LPS biosynthesis similar in MDD1 cecum microbiota vs HC cecum microbiota, p=0.33

3.11 BDNF expression was decreased in the dentate gyrus of MDD1 mice vs HC1 mice.

Decreased BDNF and neurogenesis in the dentate gyrus of the hippocampus is associated with depressive-like behaviour in mice and MDD in humans (Tailiaz D., et al. 2010; Ray MT et al. 2011). We hypothesised that MDD1 mice and MDD4 mice would have decreased BDNF in the dentate gyrus as assessed by immunofluorescence compared to HC1 and HC4 mice (Methods 2.3). We found that MDD1 mice (n=6) had decreased BDNF in the dentate gyrus vs HC1 mice (n=7) (p=0.02) (Fig 11a), MDD4 mice (n=5) did not show a significant decrease in BDNF in the dentate gyrus vs HC4 mice (n=5) (Fig 11b).

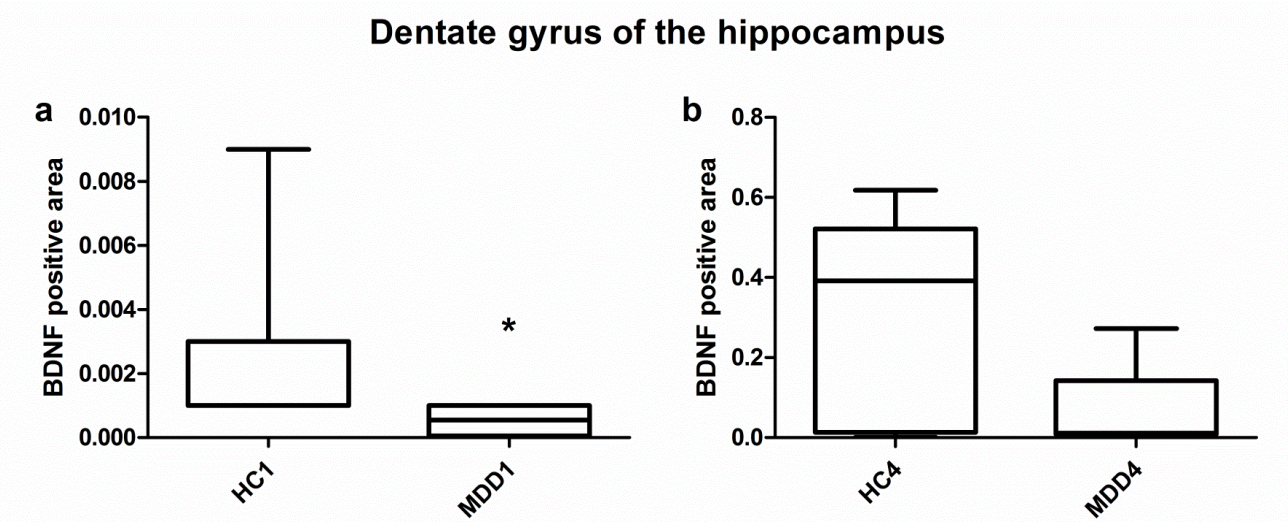


Figure 11 **BDNF expression in the dentate gyrus of the hippocampus.**

a) BDNF expression was significantly lower in MDD1 colonised mice than HC1 mice. Mann Whitney U test: MDD1 n=6 vs HC1 n=7, p=0.02, whiskers show 10th and 90th percentile.
b) BDNF expression was not significantly different between MDD4 and HC4 mice. Mann Whitney U test: MDD4 n=5 vs HC4 n=5, p= 0.20, whiskers show 10th and 90th percentile.

4. Discussion

This study highlights the heterogeneous nature of MDD and a potential role for the microbiome in some cases of MDD. Mice colonised with microbiota from well characterised MDD donors had variable behavioural profiles particularly one donor which successfully transferred depressive-like behaviour. MDD1 mice had altered expression of markers associated with intestinal permeability and BBB integrity, along with decreased expression of BDNF in the hippocampus. These results suggest MDD microbiota have the capacity to induce depressive-like behaviour, however we also show not all MDD microbiota can induce abnormal behaviour in gnotobiotic mice. Previous studies attempting to explore the role of microbiota in MDD have pooled microbiota from multiple MDD patients to colonise rodent models (Kelly JR., et al. 2016). Such an approach ignores the unique nature of individual microbiomes, the heterogeneous nature of MDD and the potentially contrasting effects these factors can have on a systemic level. By colonising our mice with well characterised microbiota from individual human patients we were able to induce distinct behavioural phenotypes in gnotobiotic mice with microbiota from different MDD patients and associate systemic changes directly to the individual transplanted microbiome.

4.1 Beta defensin 2 as a biomarker for Major Depressive Disorder

The donor selection for the colonization of germ-free mice was conducted based on age and the inflammatory biomarker B-defensin 2 (BDEF2) (Methods 2.1.3). BDEF2 is an

antimicrobial peptide secreted in the lumen of the gut by a wide range of epithelial and immune cells in response to inflammatory stimuli (Schröder J.M., et al. 1999). The inducible nature and specific antimicrobial activity of BDEF2 is useful for informing us on the status of the immune system of the host, assuming that abnormally elevated levels of faecal BDEF2 may indicate a loss of homeostasis between the host gut and the microbiome.

Faecal BDEF2 levels were measured in MDD and healthy control (HC) subjects, and it was found that MDD patients had significantly higher BDEF2 levels than HC subjects (Fig 1). MDD BDEF2 levels were divided into distinct subpopulations, with some MDD patients having BDEF2 levels similar to HC subjects, and some clustering above the normal range of BDEF2 levels (Fig 1). The distinct clustering of a subset of donors complements what we know about the heterogeneous nature of MDD, and suggests that BDEF2 is a potentially useful biomarker for distinguishing between subsets of MDD patients. We know the microbiome can modulate the gut immune system and literature has implied that patients with MDD and other psychiatric disorders have an altered microbiota composition compared to healthy volunteers; perhaps in some of these patients this is also concurrent with altered immune activation in the gut (Jiang H., et al. 2015; Naseribafrouei A., et al. 2014).

We selected the donor for Experiment 1 and 2 from the high faecal BDEF2 cluster as it suggests these patients have an altered gut homeostasis, and the link between inflammation and MDD would incline us towards a donor whose microbiota has elicited

a proinflammatory response in the gut (Smith P.M., et al. 2013; Kurina L.M., et al. 2001). We found that MDD1 microbiota transfer was sufficient to induce alterations to BDEF3 expression (Fig 3a), the mouse homologue for BDEF2 in MDD1 mice. This suggests that we also transferred a potentially abnormal microbiota mediated immune profile to MDD1 mice. Donor MDD4 had normal BDEF2 levels and this was also reflected in MDD4 mice BDEF3 expression, suggesting that the alterations to BDEF3 expression is mediated by differences in the microbiota, rather than an artefact of bacterial colonisation.

4.1.2 Overview of Major Depressive Disorder microbiota transfer

By assessing the behaviour of colonised mice we ascertained that donor MDD1 was able to transfer depressive-like behaviour in multiple tests, MDD2 mice showed social deficits but otherwise behaved normally in other tests (Fig 2b). This may be due to the relatively low depression scores seen in donor MDD2, but we would require more high BDEF2 donors to understand if there is a definitive association with MDD and the fact we saw an element of depressive-like behaviour in MDD2 mice is promising (Fig 2b). Donor MDD1 had the most severe psychiatric profile along with evidence of elevated GI immune activation (Methods 2.1.3), this was mirrored in MDD1 mice. Donors MDD3 and MDD4 had severe MDD presentation but normal BDEF2 levels (Methods 2.1.3), in addition there was no behavioural transfer to recipient mice suggesting donors MDD3 and MDD4 are phenotypically distinct from donors MDD1 and MDD2. This further

compounds the evidence against pooling microbiota of MDD donors in studies, as we show distinct phenotypes between MDD donors, and the colonised MDD mice in our study.

4.2 Microbiome profiles of colonised mice

We conducted alpha and beta diversity analysis of MDD and HC mice colon and small intestine (SI) microbiota to assess the broad similarities and differences in microbiota profiles between the experimental groups. We saw no clear distinction between cecum microbiome diversity between groups or pooled MDD and HC microbiomes (Fig 8a, b). In the small intestine we saw no differences between groups (Fig 8c), however when we pooled the groups together we saw a significant decrease in HC mice diversity compared to MDD mice (Fig 8d). This is consistent with published data reporting lower diversity in some MDD patients; however other studies have reported contradictory information (Jiang H., et al. 2015). Alpha diversity metrics are too broad to make any truly meaningful conclusions about any potential microbiotal roles in MDD, which is highlighted by the contradictory results from studies exploring the microbiome and MDD.

4.2.2 Gut beta diversity of colonised mice

We show that cecum and SI microbiota profiles cluster closely by group when using unweighted metrics: MDD1 and MDD2 mice microbiota profiles cluster separately from HC1 and HC2 mice profiles; however, resampling showed samples had low robustness

suggesting large numbers of rare OTUs are dictating similarity (Fig 9 a, c). When taking abundance into consideration, through weighting, we see an increase in similarity between the cecum microbiota of MDD1 mice vs HC1 mice (Fig 9b), suggesting that core, abundant, OTUs making up the cecum microbiomes of MDD1 and HC1 mice increase the similarity between them. Rarer bacterial species may be key in dictating the changes observed in this study between MDD1 and HC1 mice. Other studies have noted that rarer bacterial species tend to be transient in a microbiome over time, and in the future it will be interesting to study whether changes to the microbiome temporally correlate with changes to emotional symptoms in MDD patients (Caporaso J.G., et al 2011). SI microbiota profiles between MDD1 mice and HC1 are significantly different with both metrics (Fig 9c, d). We also distinct gene expression profiles in the SI (Table 1a) between MDD1 mice and HC1 mice with more altered gene expression in the SI than in the cecum. This may suggest that MDD1 microbiota have their greatest impact in the SI; however we must examine the metabolic profiles in these compartments, as we know bacterially derived metabolites are key to interactions along the MGBA, and metabolomic profiles can vary greatly among closely related microbiota profiles (De Palma G., et al. 2017).

4.2.3 Predicted metabolic profiles of MDD and HC mice small intestine microbiota

Predicted metagenome analysis of MDD1 and HC1 microbiota sequences informed us of the predicted metabolic output of MDD1 microbiota and contrasts with HC1 microbiota.

In the small intestine we found an altered proportion of genes associated with the glutamatergic synapse and glutamate metabolism in MDD1 mice (Fig 10d, e); this suggests a strong microbiotal influence on GABA related gene expression in the SI, as glutamate is a GABA precursor. We did not see the same differences in cecum microbiota (Fig 10h), which is also concurrent with GABA related gene expression in the colon of MDD1 mice (Fig 4), and gives further evidence for the microbiota underpinning alterations to GABA related gene expression in the SI in MDD1 mice. Interestingly we found alterations to monoamine biosynthesis in MDD1 SI microbiota (Fig 10f), implying the MDD1 microbiota affects the availability of a range of substances relevant to the enteric nervous system (ENS). We know the microbiota is key in maturing and regulating the ENS, and the differences in metabolic pathways related to the nervous system between MDD1 mice and HC1 mice suggests MDD1 microbiota is strongly interfacing with the ENS and possibly the CNS (Goldstein AM., et al. 2013).

We saw a decrease of SCFA metabolism in MDD1 SI microbiota (Fig 10b, c), which could suggest that MDD1 mice have either lower or higher availability of butyrate and propionate, which in turn could alter immune and epigenetic functions in colonised mice, depending on what kind of SCFA metabolism is occurring (Introduction 1.6.2; 1.9.3). The proinflammatory immune profile of MDD1 mice in the SI suggests there may be decreased SCFA availability (Table 1). It is also known that SCFAs can alter BBB permeability and induce epigenetic changes/behavioural modulation through the limbic system (Introduction 1.6.2; 1.9.3). We saw alterations to BBB permeability and

epigenetic gene expression in areas of the limbic system in MDD1 mice (Fig 5a, c), which may be related to the altered availability and metabolism of SCFAs in MDD1 microbiota. Interestingly we did not see significant alterations in SCFA metabolic pathways in the cecum, where most fermentation of carbohydrates occurs (Fig 10g). These findings highlight the potential importance of carbohydrate fermentation in the SI. We cannot make concrete conclusions from the predicted data shown in Fig 10, we will elucidate the concentrations of SCFAs in the gut of MDD1 and HC1 mice by mass spectrometry in future experiments.

Finally we found that LPS biosynthesis was higher in MDD1 SI microbiota (Fig 10a), suggesting MDD1 microbiota have higher LPS availability which in turn could alter mouse physiology between MDD1 and HC1 mice on a systemic level. We know LPS can induce depressive-like behaviour in mice (Introduction 1.7.3), and alter gut and BBB permeability (Mass M., et al. 2008). Our gene expression data indicates MDD1 mice have decreased SI barrier and BBB integrity, along with increased expression of innate immune components (Table 1; Fig 3; Fig 5a) which also is concurrent with altered behaviour (Fig 2e), and increased LPS biosynthesis in MDD1 mice. We need to confirm whether MDD1 mice have increased serum LPS to make firm conclusions from this data, but the changes seen in MDD1 mice suggest the predicted increased LPS biosynthesis is relevant to what we saw.

4.3 MDD microbiota effect on mouse behaviour

In this pilot project, MDD1 donor microbiota was able to induce abnormal behavioural profiles in recipient mice (Figure 2a-e), suggesting that microbiota can influence emotional behaviour in some cases of MDD. Abnormal behaviour was also accompanied by alterations in neuroimmune gene expression along the gut-brain axis and altered microbiota metabolomic profiles. Our results affirm that the microbiome likely contributes to MDD behaviours in a subset of patients; this is in line with the heterogeneous nature of MDD pathophysiology.

4.3.2 Social deficits in MDD1 and MDD2 mice

We were interested to see if MDD microbiota could alter social behaviour in mice as social withdrawal is a characteristic of MDD in humans (World Health Organisation, 1992). Social behaviour assays have recently been used to measure depressive-like behaviour in rodents (Russo S., et al. 2018). Reduced social interaction of subject mice with a novel mouse is indicative of a social deficit or sickness behaviour as mice are naturally social animals (Filiano A.J., et al. 2016; Raison C. L., et al. 2013). Using the three chamber sociability assay we found that MDD1 colonised mice spent significantly less time with novel mice than HC1 colonised mice, implying MDD1 mice have abnormal social behaviour (Fig 2b). This finding was mirrored in MDD2 mice who spent significantly less time with novel mice than HC mice (Fig 2b). MDD1 and MDD2 donors had high faecal BDEF2 levels (Fig 1), and MDD1 mice had high BDEF-3 expression in the

gut, implying some elements of the donor GI immune profile were mirrored in the recipient mice (Fig 3a). MDD1 mice also had decreased expression of proteins associated with gut barrier and BBB integrity (Fig 3b; Fig 5a). A compromised BBB has also been shown to induce social deficits in models of depression (Raison C. L., et al. 2013; Russo S., et al. 2018). LPS biosynthesis was shown to be increased in the SI of MDD1 mice (Fig 10a), which could increase the concentration of circulating LPS in these mice and alter barrier permeability systemically. This may increase leakage of bacterial components such as LPS into the blood, which has been shown to induce sickness behaviour, which includes social withdrawal, in mice (Yirmiya R., et al. 2001).

MDD1 mice also had increased expression of T-cell related genes in the gut, especially in the SI, where we saw increased expression of CXCR3 and CCR6 (Table 1a). These two chemokine receptors are required for the migration of T-cells to CNS compartments (Sporici R., et al. 2010). Changes to T-cell migration in MDD1 mice could lead to an altered immune profile in compartments such as the meninges, which may alter social behaviour (Filiano A.J., et al. 2016). To validate this hypothesis we would need to have more detailed information about the immune profile of both the gut and the meninges, which can be undertaken in future experiments.

4.3.3 Signs of anhedonia in MDD1 mice

Anhedonia is another component of MDD in humans, and is characterised by low motivation and desire to engage in pleasurable activities (World Health Organisation,

1992). Anhedonia is strongly associated with decreased activity in the nucleus accumbens in response to reward, suggesting a dysfunction in reward-motivation pathways is central to the pathophysiology of anhedonia (Wacker J., et al. 2009). In animal models of depression anhedonia can be measured through the sucrose preference test (SPT). Consumption of sucrose below 75%, is indicative of a dysfunctional reward motivation pathway and anhedonia (Eagle A.L., et al. 2016). We found that the mean HC, MDD3, MDD2 and MDD4 mice sucrose preference was over 75% indicating these mice did not have symptoms of anhedonia (Fig 2d). MDD1 mice, however, showed abnormal sucrose preference compared HC mice, implying these mice have anhedonia (Fig 2d).

Previous studies have shown that reduced sucrose intake is associated with altered gene expression in the limbic system; including alterations to GABA related gene expression (Der-Avakian A., et al. 2012). Our molecular probing found alterations to GABA related gene expression along the gut-brain axis in MDD1 mice, including in the nucleus accumbens; a region heavily implicated in anhedonic behaviour (Fig 4; 5b, d). Experimental mice in all groups had a similar weight gain which suggests the anhedonic behaviour seen in MDD1 mice is not associated with nutrition or appetite. Rather, it indicates a deficiency in the reward-motivation pathways in the limbic systems of these mice (Der-Avakian A., et al. 2012).

Recent studies have shown that activating gut vagal neurons can induce reward behaviour in mice (Han W., et al. 2018). Activation of these neurons induced an increase

of dopamine in the limbic reward pathway, glutamatergic/GABAergic neurons were found to be the main neurons regulating this activity. Our studies show that MDD1 mice display anhedonia, along with alterations to GABA related gene expression in the gut (Fig 4), and the brain (Fig 5b, d), in addition to increased glutamatergic/glutamate metabolism in MDD1 SI microbiota (Fig 10 d, e). Our findings are in complement to those made by Han W et al., and suggest that MDD1 microbiota are influencing vagal glutamatergic/GABAergic neurons and in turn altering reward pathways to induce anhedonia in MDD1 mice. Other studies have used antibiotics to deplete mice microbiota and found that antibiotic treatment resulted in altered sensitivity to cocaine induced reward pathways (Kiraly D.D., et al. 2016). These changes were associated with altered BDNF expression in the nucleus accumbens and increased sensitivity to cocaine in antibiotic treated mice. This study suggests a role for the microbiota in mediating gut-brain reward pathways and complements what we found in the sucrose preference test and alterations to gene expression in the nucleus accumbens.

Studies have shown that pooled MDD microbiota can induce some aspects of depressive-like behaviour; however, we are the first to show that a single characterised donor can transfer anhedonic behaviour to mice (Fig 2di). Had the donors been pooled we may have lost this signal of anhedonia, as it is apparent not all donor MDD microbiota were able to induce abnormal sucrose preference, and when we pooled MDD mice together we saw no difference in sucrose preference vs pooled HC mice (Fig 2dii).

4.3.4 MDD1 mice have altered emotional behaviour

By z scoring all behaviour tests we were able to generate emotionality scores to inform us on the overall emotional behaviour of the mice (Methods 2.2.2). The further a value negatively deviates from 0, the more abnormal the mouse behaviour is relative to the control (Guilloux J. P., et al. 2011).

There was a contrast of emotional profiles among the four groups of MDD colonised mice, with MDD4, MDD2 and MDD3 mice displaying similar emotional behaviour to HC mice (Fig 2e). MDD1 mice displayed significant negative emotional behaviour in comparison to HC1 mice and HC mice (Fig 2e).

This implies that MDD1 colonised mice have an abnormal emotional behaviour profile which may be attributed to the microbiota influencing brain physiology and cognitive functioning through the gut-brain axis. The MDD1 mice emotional profiles were consistent with literature examining depressive-like behaviour using the same scoring method (Guilloux J. P., et al. 2011). The overall reduction of normal emotional behaviour in MDD1 mice strongly indicates that MDD1 microbiota is able to modulate behaviour through unknown mechanisms, and induce depressive-like behaviour through the MGBA.

In addition this metric highlights that different MDD microbiota can induce distinct behavioural phenotypes in gnotobiotic mice, whereas different HC microbiota induced similar behaviour. When we pooled MDD emotionality scores (Fig 2e), we saw no

difference between MDD and HC mice, highlighting the importance of using single donors in these studies, and the heterogeneity of MDD microbiota-host interactions.

Apart from the SPT and sociability assay, the open field test was also included in the emotionality scoring in order to assess anxiety like behaviour in the mice. The open field test is based on the rodents' conflict between exploratory behaviour, and aversion towards open areas (Prut L. & Belzung C., 2003). We found that HC mice spent similar amounts of time in the centre of the field to MDD mice (Fig 2a). There was no difference between different MDD groups in the open field test, although the data suggests there are subtle trends for anxiety like behaviour. However, this data used in conjunction with the results from the other tests may provide a better picture of the overall emotional profile of the MDD colonised mice. Similarly, our other metric for assessing depressive-like behaviour, the tail suspension test, yielded inconclusive results which were also used to generate the informative emotionality scores described above (Fig 2c, e). MDD1 mice spent less time immobile than the other MDD and HC mice groups (Fig 2c). Conducting multiple behaviour tests is vital to understanding the overall emotional state of the test animal, and mice may not show consistent behaviour across different tests, perhaps due to the microbiota differences.

4.3.5 Potential behavioural mechanisms at play in MDD1 mice

Previous studies in our lab have indicated that emotional behaviour regulation by the microbiome is mediated by the vagus nerve in some cases. The anxiolytic effects of the

probiotic *B. longum* on colitis induced anxiety like behaviour can be blocked by vagotomy (Bercik P., et al. 2011). Analysis of MDD1 SI microbiota taxonomical abundance showed that *B. longum* abundance in MDD1 mice is significantly lower compared to HC1 mice, we can hypothesise based on previous studies, that *B. longum* deficiency in MDD1 mice may have contribute to their abnormal behaviour. Our studies also indicate that GABA gene expression is altered along the gut-brain axis (Fig 4; Fig 5a; Table 1a), in concurrence with the presentation of abnormal behaviour in MDD1 mice. This mirrors the precedent set by Bravo et al., where *L. rhamnosus* was found to alter exploratory behaviour and GABA related gene expression in the brain, with these changes also blocked by vagotomy (Bravo J. A., et al. 2011). The well-established precedent of LPS involvement in gut barrier dysfunction, sickness behaviour and immune activation presents us with simple explanation for the abnormal behaviour seen in MDD1 mice, as we saw a range of evidence of increased LPS expression in MDD1 mice (Fig 10a; Table 1A), which could in turn induce behavioural changes as has been noted previously in the literature (Introduction 1.7.3).

4.4 MDD microbiota effect on gut gene expression

4.4.1 Evidence of increased immune activation in gut of MDD1 mice

We hypothesised that MDD microbiota would induce a pro-inflammatory environment in the gut and changes to neural gene expression in the enteric nervous system (ENS). Compared to HC1 mice, MDD1 mice had significantly higher levels of BDEF3, the mouse

homologue for human BDEF2, in both the colon and SI, implying that MDD1 microbiota was sufficient to induce immune changes throughout the gut. MDD4 mice had similar BDEF3 expression to HC4 mice (Fig 7b), which mirrors the physiological levels of faecal BDEF2 found in the MDD4 donor and suggests that BDEF2 secretion depends on microbiota composition impacting immune states rather than an artefact of bacterial colonisation. Nanostring gene expression analysis found that there were increases of immune markers in the gut of MDD1 mice, (Table 1), suggesting that MDD1 microbiota colonisation was sufficient to alter the immune profile of MDD1 mice in the gut. MDD4 mice used for gene expression analysis displayed components of depressive-like behaviour, and had a similar upregulation of immune related genes in the colon, along with genes for antimicrobial peptides. This suggests MDD4 microbiota can also elicit changes to the expression of genes related to pro-inflammatory states (Table 1b). The contrast in expression profiles between MDD1 and MDD4 mice also suggests that the microbiota of these mice are interacting with the host via different mechanisms.

4.4.2 Decreased expression of gut barrier components in MDD1 mice.

MDD1 mice were found to have decreased expression of tight junction proteins such as occludin in both the SI and colon (Fig 3b; Table 1), and claudin 2 in the SI only, implying these mice had decreased gut barrier integrity. This could lead to leakage of bacterial products into the circulatory system and across the blood brain barrier to induce the activation of pro-inflammatory pathways (Huber J.D., et al. 2001). MDD4 mice had no

difference in occludin expression when compared to HC4 mice (Fig 7a). MDD1 SI microbiota had increased biosynthesis of LPS compared to HC1 microbiota (Fig 10a). It has been found that LPS can alter gut permeability and induce sickness like behaviour in mice through leakage into the circulatory system, making this a possible pathway for the behavioural changes seen in MDD1 mice (Mass M., et al. 2008). MDD patients have also been found to have higher levels of circulating LPS which implies that an altered gut barrier state is a feature in some MDD patients, providing some parity with animal studies (Mass M., et al. 2008).

4.4.3 Alterations in GABA related gene expression in MDD1 mice.

GABA can be synthesised both by the microbiome and mammalian cells. Neurons expressing GABA related genes are known as GABAergic neurons, and have inhibitory properties in the nervous system (Pokusaeva K., et al. 2017). GABA related gene expression was found to be altered in the SI of MDD1 mice, with increases in GABA receptor $GABAB_2$, and GABA transporter GAD67 found (Table 1; Fig 5b). These markers are classically used to characterise GABAergic neurons in the CNS, therefore, we can speculate that upregulation of GABA related genes mirror an increase of GABAergic density in the ENS of MDD1 mice.

The consequences of altered GABAergic expression/density have not been characterised in the gut. Alterations to GABA related gene expression has been found in the brain of mice exposed to *L. rhamnosus* probiotics, suggesting that the microbiota can alter GABA

related gene expression in the CNS (Bravo J.A., et al. 2011). The precedent set in the Bravo J.A. study makes it possible for us to speculate that some microbiota can impact GABAergic expression in the ENS.

We found that MDD1 SI microbiota had a higher association with pathways involving the glutamatergic synapse and glutamate metabolism (Fig 10 d, e). It is likely that MDD1 SI microbiota participate in the biosynthesis of GABA and can directly impact GABAergic neurons through other processes. This complements the expression data we have obtained from the gut, and implies that the microbiota can directly alter GABAergic density in the ENS. Altered GABAergic expression was reflected in the limbic system (Fig 5). The involvement of GABAergic neurons in MDD and chronic stress pathophysiology makes these findings particularly interesting, as reduced GABAergic density in limbic structures is often associated with MDD and chronic stress (Gilabert-Juan J., et al. 2013; Tripp A., et al. 2011). Our data suggests that depressive-like behaviour is accompanied by altered GABA related gene expression in the gut as well as the brain in MDD1 mice (Fig 4; 5b). This is likely mediated by increased involvement of MDD1 SI microbiota in GABA related metabolic pathways (Fig 10d, e).

4.4.4 Putative enteroglial remodelling in MDD1 mice

Enteroglial cells (EGCs), like astroglial cells in the CNS, are essential for ENS function and can take up a neuroprotective or neurodegenerative state depending on the extracellular environment (Cirillo C., et al. 2011). Studies have found that increased

expression of EGC marker S100B is accompanied by increased glial density and secretion of pro-inflammatory cytokines which in turn can sustain an aberrant pro-inflammatory gut environment in both mice and humans (Cirillo C., et al. 2011). Other studies found that pathogenic bacteria increase nitric oxide production from EGCs, along with S100B expression (Turco F., et al. 2013). MDD1 mice had increases in glial marker S100B in the colon (Table 1), implying increased proliferation of enteroglial cells (EGCs) in the ENS. Our SI gene expression analysis also found increases of neuronal NOS expression in MDD1 mice along with EGC marker P75, suggesting MDD1 mice gut compartments have proinflammatory EGC states in response to a dysbiotic microbiome. The consequences of altered glial density may be relevant to the changes in behaviour seen in MDD1 mice, as it is known glial density is associated with MDD in humans (Introduction 1.7.2). Serum S100B has been found to be increased in patients with MDD and changes in glial density have been found in patients with MDD and schizophrenia, however, this has been documented in the brain and not the gut (Arolt V., et al. 2003; Steiner J., et al. 2008; Rajkowska G., et al. 2013). EGCs have been directly implicated in the downregulation of occludin and concomitant decreases in intestinal barrier integrity and we have seen significant evidence of altered intestinal barrier function in MDD1 mice (Table1; Fig 3b), (Cirillo C., et al. 2011; Savidge TC., et al. 2007). MDD4 mice had decreased colonic expression of GDNF, another marker for glial cells, suggesting that MDD4 mice have a different EGC profile to MDD1 mice, and that MDD4 microbiota potentially also affects the proliferation of EGCs but through different pathways than MDD1 microbiota.

P75 is a neural growth factor receptor which can bind to BDNF; P75 has both cytoprotective and cytotoxic functions, and has been associated to MDD in studies associating gene variants with MDD treatment outcome (Fujii T., et al. 2011). P75 is also an important surface marker for EGCs and is essential for EGC proliferation (Goldstein A.M., et al. 2013). We saw alterations in P75 expression in MDD1 mice SI and hippocampus (Table 1) suggesting MDD1 mice have altered neuroproliferation along the gut-brain axis.

4.5 MDD microbiota alter murine brain physiology

4.5.1 Blood-brain-barrier integrity seems to be compromised in MDD1 mice

Occludin is a key component of the BBB along with other tight junction proteins; studies exploring BBB permeability have found that occludin downregulation and increased permeability of the BBB occur in response to inflammatory signalling (Huber J.D., et al. 2001). Some trends of gut gene expression were mirrored in the brain, namely occludin downregulation in the prefrontal cortex (Fig 5a). MDD1 mice likely have increased permeability of the BBB barrier in the prefrontal cortex. Loss of BBB integrity has been associated with depressive-like behaviour, with downregulation of occludin and claudin-5 being detrimental to mouse behaviour in the nucleus accumbens (Cheng Y., et al. 2018; Russo S., et al. 2018).

The prefrontal cortex is heavily implicated in MDD pathology and the effects of chronic stress in mice (Cotter D., et al. 2002; Hinwood M., et al. 2012). Alterations to

permeability in the prefrontal cortex of MDD1 mice may lead to changes in PFC function and mouse behaviour, similarly to how altered BBB permeability impacts nucleus accumbens functioning in chronic stress mice (Russo S., et al. 2018).

MDD4 mice had increased expression of occludin in the hippocampus compared to HC4 mice, which implies MDD4 mice have greater BBB integrity in certain limbic regions. MDD4 mice also showed normal behaviour overall (Table 1B; Fig 2e).

Gut microbiota have been implicated in the regulation of BBB integrity; germ free mice have decreased BBB integrity which can be rescued either by colonisation or administration of SCFAs which are derived from bacterial fermentation (Braniste V., et al. 2014). MDD1 SI microbiota had lower butyrate and propionate metabolism than HC1 mice (Fig 10c), which may have contributed to the lower BBB integrity seen in the PFC of MDD 1 mice.

LPS is another bacterial product which can alter barrier permeability in the gut and brain and influence depressive-like behaviour (Mass M., et al. 2008). We found that MDD1 mice had increased biosynthesis of LPS in the SI (Fig 10a), which was accompanied by decreased gut barrier integrity (Fig 3b), and would likely lead to the greater translocation of LPS into the circulatory system than in HC1 mice. Higher systemic LPS levels may contribute to the abnormal behaviour and altered gut-brain barrier function seen in MDD1 mice and implies MDD1 mice have an altered gut homeostasis. Antibiotic induced dysbiosis can alter BBB permeability in key limbic regions differentially, and this

is accompanied by altered cognitive behaviours, MDD1 mice show signs of having a dysbiotic microbiota (Fröhlich E. E., et al. 2016).

4.5.2 MDD1 mice have altered expression of hippocampal neuroproliferation markers

We saw a significant decrease of P75 in the hippocampus of MDD1 mice compared to HC1 mice (Table 1). This is the opposite to what we see in the gut (Table 1), and may imply the MDD1 mice have decreased neuroproliferation in the hippocampus. This is in concordance with the lower BDNF levels we measured in the dentate gyrus of MDD1 mice (Fig 11) (Bergamin M., et al. 2008).

P75 serves as a marker for neuronal differentiation and astroglial cells as it does in the gut for EGCs. We saw opposite P75 expression trends between the gut and brain, which merits further investigation into the glial profiles of MDD1 mice.

Astroglia are important sources of BDNF in the brain and lower astroglial density would likely affect BDNF levels in the brain (Rubio N., et al. 1997). Previous studies in the Bercik lab noted that *B. longum* can increase hippocampal BDNF via unknown mechanisms (Bercik P., et al. 2011). With this precedent we can theorise that the MDD1 microbiota is interfacing with the hippocampus, possibly via astroglia, to impact BDNF synthesis. Indeed when we analysed the taxonomical make up of MDD1 microbiota we found that MDD1 mice had significant alterations in *B. longum* abundance in the SI compared to HC1 mice. Reduced hippocampal BDNF has been associated with depression like

behaviour and MDD in humans and rodents (Tailiaz D., et al. 2010). It is also accompanied by decreased neurogenesis in the dentate gyrus (Ray MT et al. 2011). Taken together, our data indicates that MDD1 microbiota may be able to reduce hippocampal neurogenesis which might explain the abnormal behaviour of these mice.

4.5.3 Epigenetic alterations in the nucleus accumbens of MDD1 mice

We examined cerebral epigenetic markers, as literature has implied an important role for epigenetic mechanisms in MDD pathophysiology (Introduction 1.6.2). HDAC3 expression is increased in the nucleus accumbens of MDD1 mice compared to HC1 mice (Fig 5b). HDAC activity in the limbic system has been associated with depression like behaviour along with altered BDNF expression in chronic stress mice (Tsankova NM., et al. 2006; Covington 3rd H.E., et al. 2011b). Butyrate was used as a HDAC inhibitor in these studies, and was found to have potent antidepressive effects through HDAC inhibition (Covington 3rd H.E., et al. 2011b). Microbiota derived butyrate can likely alter cerebral epigenetics and may have influenced the emotional behaviour of the mice in this study. Lower levels of gut derived butyrate could increase of HDAC activity in certain brain regions, as lower butyrate levels would mean less inhibition of HDAC promoters and higher expression of these enzymes (Schroeder F.A., et al. 2007). We found that MDD1 mice had altered butyrate metabolism in the SI compared to HC1 mice, which may result in lower butyrate levels in the SI (Fig 10c). This was concurrent with increased cerebral HDAC expression in MDD1 mice (Fig 5b), which theoretically could have

occurred due to lower butyrate mediated inhibition. These results present a putative role for MDD1 microbiota in cerebral epigenetics.

4.5.4 MDD1 mice have altered GABA related gene expression in the nucleus accumbens

As we saw alterations to GABA related gene expression in the gut we also wanted to investigate if GABA related gene expression would be altered in the brain, as it is known that GABA related pathways are important in the pathophysiology of MDD (Gilabert-Juan J., et al. 2013; Tripp A., et al. 2011).

GABA related expression was found to be significantly altered in the nucleus accumbens of MDD1 mice, with GABAB1 and GAD67 expressed at higher levels than in HC1 mice (Fig 5a, b), implying an increased density of GABAergic neurons in MDD1 mice NAcc. Gut microbiota has been previously implicated with GABA related gene expression in the brain, and our gene expression results are in agreement with this (Bravo J.A., et al. 2011). In addition to this it was found the MDD1 SI microbiota participate in metabolic processes associated with the glutamatergic synapse and glutamate metabolism (Fig 10d, e). This implies that the MDD1 microbiota are involved in regulating the alterations in GABA related gene expression we saw in MDD1 mice SI (Fig 4). We predict that the changes in MDD1 mice cerebral GABA related gene expression is linked to microbiota mediated changes in the gut. Studies examining the effect of antibiotic microbiota depletion in mice found that antibiotic treated mice have altered responses to cocaine

in concurrence with altered gene expression in the nucleus accumbens (Kiraly D.D., et al. 2016). This complements our findings, and suggests that MDD1 microbiota mediated the alterations to gene expression in the nucleus accumbens that we saw.

Literature examining GABAergic neurons in chronic stress mice found that GABAergic interneurons are decreased in the hippocampus (Czéh B., et al. 2015). This is in line with what we see in the hippocampus of MDD4 mice (Table 1B); these mice have decreased $GABA_A$ expression, which suggests they have reduced GABAergic density in the hippocampus. MDD4 mice overall did not display abnormal behaviour, however the sub-group of mice used for gene expression analysis had significant components of depressive-like behaviour.

Studies so far have not examined the consequences of altered GABAergic gene expression in the nucleus accumbens on depressive behaviours, therefore, the consequences of increased GABAergic activity in this region is unknown. However, given the inhibitory activities of GABAergic neurons we can postulate that increased GABAergic innervation of the nucleus accumbens may result in more potent inhibitory signals in this region, and drive deficits in reward-motivation pathways (Pizzagalli D., et al. 2009). It is therefore possible to speculate that increased GABA related gene expression may be related to the anhedonic behaviour seen in MDD1 mice.

In the context of MDD, post mortem analysis of human patients suicide victims have been found to have increased GABA receptor expression in the prefrontal cortex, implying that alterations in GABAergic neurons affect limbic regions distinctly and are

important in the expression of MDD and its pathophysiology (Choudhary P.V., et al 2005).

5. Conclusions

We were able to induce depressive-like behaviour to gnotobiotic mice by colonising them with microbiota from an MDD patient. In addition to behavioural alterations, the microbiota of two MDD patients was able to alter gene expression related to both the immune and nervous system in the gut and brain. This implicates the microbiome in the expression of MDD in a subset of patients.

5.2 Future directions

We found that MDD1 microbiota were able to induce abnormal behaviour in gnotobiotic mice whereas microbiota from other MDD donors did not transfer abnormal behaviour. I suggest that we pool the microbiota of MDD1, 2, 3, and 4 and colonise a set of GF mice, in addition to mice colonised with pooled HC microbiota. I would expect that pooled MDD microbiota colonised mice will behave the same as pooled HC microbiota colonised mice. This experiment will strengthen our hypothesis that pooling microbiota would mask the effect of depressogenic microbiota such as MDD1 microbiota. In addition to these experiments it would be beneficial to explore the involvement of the vagus nerve in MDD1 microbiota colonised mice behaviour. I propose we use the same experimental set up I used for my project but use vagotomised mice to see what effect vagotomy would have on MDD1 mice behaviour and gene expression.

6. Limitations

Since this was a pilot study we encountered several limitations. Issues with mouse breeding hindered our ability to increase group numbers for the range of donors tested which may have masked behavioural trends that would have become significant with higher mouse numbers. Conversely, conducting large scale experiments with 30 vs 30 mice exceeds the resource capacity of our group meaning numbers had to be gradually boosted through three or four separate experiments, introducing more room for inter-experiment variability. The lack of a reliable staining protocol for microglia in fresh frozen tissue hindered our ability to assess glial density in the brains of MDD1 mice, which from looking at gut evidence, appears to be an important aspect in the pathogenesis of the abnormal behaviour in these mice. Initially, the pilot experiment was run with eight MDD1 colonised mice and six HC18 colonised mice. HC18 mice displayed abnormal behaviour compared to past HC colonised mice used in the lab, due to the donor having consumed excessive amounts of alcohol daily. For this reason HC18 was replaced with HC1 who was alcohol abstinent and had no additional confounding factors in his donor profile. This initial pilot experiment was useful as it allowed me to become accustomed to the behavioural protocols and taught me to scan patient profiles with more rigour to avoid unsuitable donors.

7. References

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