INVESTIGATING THE MECHANISMS OF DEPRESSION IN A MOUSE MODEL

INVESTIGATING THE MICROBIAL AND IMMUNE MECHANISMS OF DEPRESSIVE-LIKE BEHAVIOUR IN A HUMANIZED MOUSE MODEL OF MDD

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TITLE: Investigating the Microbial and Immune Mechanisms of Depressive-like Behaviour in a Humanized Mouse Model of MDD

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ABSTRACT

Major depressive disorder (MDD) is a highly heterogeneous disorder, with some patients displaying immune activation and altered intestinal microbiota composition when compared to healthy controls. In recent years, the transfer of fecal microbiota pooled from several MDD patients has been used to model depression in recipient rodents. However, we have previously observed the induction of donor-specific phenotypes in mice receiving microbiota from individual irritable bowel syndrome and generalized anxiety disorder patients. Therefore, we assessed the efficacy of fecal microbiota transplant (FMT) using individual versus pooled MDD patient microbiota to induce depressive-like behaviour in recipient rodents. We observed that pooling microbiota from several patients abrogated microbial features unique to individual donors. Mice that received pooled microbiota displayed different behavioural and immune phenotypes when compared to mice that received individual patient microbiota. Two individual MDD microbiota donors, patients MDD1 and MDD5, altered the behaviour of recipient mice when compared to controls. We identified several microbial species that may underlie the anxiety- and depressive-like behaviours observed in MDD1 and MDD5 mice. Additionally, altered expression of neural and immune genes was observed along the gut-brain axis of mice colonized with MDD1 microbiota. As microglia activation may play a role in our model, we developed a protocol for the isolation and phenotyping of adult mouse microglia that will facilitate future research efforts. Overall, our results demonstrate the heterogeneity of the microbial underpinnings of MDD and support the use of individual patient microbiota in future FMT experiments.

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LIST OF ABBREVIATIONS

ASVs	Amplicon sequence variants
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
DASS21	Depression Anxiety Stress Scale 21
EGC	Enteric glial cells
FMT	Fecal microbiota transplant
GAD	Generalized anxiety disorder
GF	Germ-free
GI	Gastrointestinal
GSRS	Gastrointestinal Symptom Rating Scale
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IECs	Intestinal epithelial cells
IL	Interleukin
iNKT	Invariant natural killer T
MAMPs	Microbial-associated molecular patters
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
NF-κB	Nuclear factor-κB
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns

PRRs	Pattern recognition receptors
SIgA	Secretory immunoglobin A
SPF	Specific pathogen free
TGFβ	Transforming growth factor β
TJ	Tight junction
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

DECLARATION OF ACADEMIC ACHIEVEMENT

All clinical data was collected by a team led by Dr. Rebecca Anglin. All experiments for colonization groups MDD1-4 and HC1-4 in Project 1 were completed by Marc Louis-Auguste. For groups MDD1 and HC1, hippocampal, small intestine, and colonic RNA was isolated by Marc. All cecal content samples from groups MDD1, MDD2, HC1, and HC2 were processed by Marc prior to 16S rRNA gene sequencing, along with a subset of samples from groups MDD3 and MDD4. Samples from groups HC3 and HC4 were processed by Jennifer Hanuschak, along with the remaining MDD3 and MDD4 samples. The experiments for colonization groups PMDD and PHC in Project 1 were completed by Jennifer. For groups PMDD and PHC, small intestine and colonic RNA was isolated by Jennifer. For Project 2, mice included in the representative experiment were colonized by Virginia Rabbia. All NanoString nCounter data were collected by the McMaster Genomics Facility. For Projects 1 and 3, 16S rRNA sequencing was completed by the Surette Lab at McMaster University. 16S rRNA sequencing data were analyzed by Giada De Palma using the QUIIME pipeline. All other analyses were completed by Jennifer and all figures were created by Jennifer.

Please note that similar methods were used in Projects 1 and 3. Full written descriptions of these methods will appear in Project 1 and all subsequent mention of these methods will be brief.

CHAPTER 1: INTRODUCTION

Hypotheses and Aims

In 2017, our group established a humanized mouse model of depression through the transfer of fecal microbiota from individual patients with major depressive disorder (MDD) to germ-free (GF) mice. Subsequently, three research projects were completed in order to address our overall aim which was **to further our understanding of the microbial and immune mechanisms underlying depressive-like behaviour in this mouse model.**

Project 1: MDD patient microbiota induces depressive-like behaviour in GF mice

Hypothesis 1: Individual MDD patient microbiota induces a donor-specific phenotype in recipient mice

Specific Aim 1: Investigate the effects of pooled microbiota from several MDD patients on mouse behaviour and gene expression

Project 2: High-yield isolation of adult mouse microglia for flow cytometric analysis

Hypothesis 2: Microbiota from individual MDD patients alters mouse behaviour through microglia activation

Specific Aim 2: Establish methods for measuring microglia activation in mice in our lab setting

Project 3: The ability of MDD patient microbiota to induce altered behaviour in mice is dependent on mouse age at colonization

Hypothesis 3: The early life period represents an important window for the programming of MDD risk through the intestinal microbiota and the immune system

Specific Aim 3: Investigate the effects of early life colonization with MDD patient microbiota on mouse behaviour and microglia activation

Background

Disease Overview

MDD is a significant global health concern. MDD affects nearly 3% of the global population and is the third leading cause of disability worldwide (Vos et al., 2016). MDD is characterized by episodes of debilitating symptoms, including persistent depressed mood, anhedonia, cognitive impairment, and suicide ideation (American Psychiatric Association, 2013). Furthermore, MDD is a highly heterogenous disorder with no established mechanisms (Belmaker & Agam, 2008). Depression onset most often occurs in young adulthood, followed by recurrent episodes throughout life (Fava & Kendler, 2000; Klein et al., 2013). However, symptomology, age at onset, and disease course can vary greatly among individuals (Belmaker & Agam, 2008; Klein et al., 2013). Treatment responses are also variable, with approximately 33% of depression patients responding to conventional treatments and up to 25% of patients experiencing no relief from symptoms (Belmaker & Agam, 2008; Fava & Kendler, 2000). Several risk factors have been identified for MDD, including gender, familial loading of depression, environmental adversities, and childhood trauma (Fava & Kendler, 2000; Klein et al., 2013). Women are at a greater risk of developing MDD, and the global prevalence rate among women is nearly 1.5 times that of men (Baxter et al., 2014; Fava & Kendler, 2000). Lastly, research suggests MDD has complex genetic underpinnings as no reliable genetic risk factors have been found (Belmaker & Agam, 2008). Twin studies have revealed that MDD has a relatively low heritability of 37%, although it is

believed that heritability may be higher in severe presentations of early-onset depression (Belmaker & Agam, 2008).

Intestinal Microbiota and MDD

Residing in the distal gastrointestinal (GI) tract, the intestinal microbiota is the largest community of microorganisms in the human body (Cerf-Bensussan & Gaboriau-Routhiau, 2010). The intestinal microbiota benefits host survival by facilitating nutrient absorption and immune system development (Eckburg et al., 2005; Gensollen, Iyer, Kasper, & Blumberg, 2016). The development of the microbiota occurs during early life; and although it was originally thought that the womb is sterile, there is evidence to indicate that exposure to microbes may begin in utero (Gensollen et al., 2016). To illustrate, microbes have been isolated from human meconium, cord blood, and amniotic fluid (Jiménez et al., 2005; Mändar et al., 2001; Moles et al., 2013). Nonetheless, birth marks the beginning of mass colonization from environmental sources, including the birth canal, maternal skin, and breast milk (Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015). Early colonizers of the infant gut include facultative anaerobes; these microbes create an anaerobic environment suitable for obligate anaerobes to later colonize the gut (Matamoros, Gras-Leguen, Le Vacon, Potel, & De La Cochetiere, 2013). During the first three years of life, the composition of the intestinal microbiota fluctuates and coverage gradually shifts towards that of an adult-like community (Gensollen et al., 2016; Palmer, Bik, DiGiulio, Relman, & Brown, 2007). Interestingly, gestation time, mode of delivery, mode of feeding, and prenatal maternal stress are all factors that have been shown to influence the composition of the infant microbiota (Matamoros et al., 2013). In addition, early life perturbations in the microbiota have been associated with behavioural abnormalities in mice and the development of psychiatric illness in humans (Köhler-Forsberg et al., 2019; Leclercq et al.,

2017). Mice exposed to penicillin during the perinatal period displayed decreased sociability in adulthood (Leclercq et al., 2017). Early life exposure to penicillin also induced anxiolytic behaviour in male but not female mice (Leclercq et al., 2017). In humans, the use of anti-infective agents was found to be associated with increased risk of treatment for mood disorders before the age of 18 (Köhler-Forsberg et al., 2019). Those who received anti-infective agents had an increased risk of 37% for hospitalizations and 18% for the use of psychotropic medication to treat mood disorders (Köhler-Forsberg et al., 2019). Risk estimates for all mental disorders were also found to increase with the number of anti-infective agents used from birth to late adolescence, and antibiotics were the anti-infective agents associated with the greatest risk increases (Köhler-Forsberg et al., 2019). Despite these associations, more clinical studies are needed to determine the effects of early life microbial perturbations on host health and disease in adulthood.

In adults, the intestinal microbiota is comprised of up to 10^{12} microbes per mL of luminal content (Palmer et al., 2007). In general, the adult microbiota is dominated by members of the Firmicutes and Bacteroidetes phyla (Eckburg et al., 2005). However, each individual harbours a distinct microbial community that is shaped by both host and environmental factors, including age, sex, diet, ethnicity, and geography (Deschasaux et al., 2018; Eckburg et al., 2005; He et al., 2018; Hopkins, Sharp, & Macfarlane, 2002; Markle et al., 2013). Stability and resilience are believed to be characteristics of a healthy adult microbiota, whereas perturbations in community composition have been associated with periods of antibiotic use, stress, and illness (Bercik, Collins, & Verdu, 2012; Matamoros et al., 2013).

The intestinal microbiota has been shown to influence both the gut and the brain, and it is believed to play a regulatory role along the gut-brain axis (Collins, Surette, & Bercik, 2012). The

gut-brain axis is the bidirectional communication between the GI tract and the brain, comprised of neural, immunologic, hormonal, and metabolic pathways (Collins et al., 2012). Recently, research on the gut-brain axis has provided greater insight into the pathophysiology of MDD. Altered microbiota composition has been reported in both rodent models of depression and MDD patients when compared to healthy controls (Aizawa et al., 2016; Y. C. E. Chung et al., 2019; Jiang et al., 2015; Jianguo, Xueyang, Cui, Changxin, & Xuemei, 2019; Kelly et al., 2016; Park et al., 2013; Tillmann, Abildgaard, Winther, & Wegener, 2019; Zheng et al., 2016). However, there is no consensus among groups regarding the microbial features associated with depression. Conflicting findings are likely a result of confounding factors, such as host sex, age, ethnicity, and geography, as each study investigates the intestinal microbiota in a different population. Indeed, both sex- and age-specific differences in microbiota composition have been reported between MDD patients and healthy controls (J. J. Chen et al., 2020, 2018). Lastly, two studies have found that the transfer of pooled fecal microbiota from several MDD patients to rodents induces anxiety- and depressive-like behaviours in the recipient animals (Kelly et al., 2016; Zheng et al., 2016).

Gut Mucosal Immune System

Along the GI tract, the mucosal immune system plays an important role in the maintenance of homeostasis by controlling the abundance and composition of the microbiota (Cario, 2010; Hansen, Gulati, & Sartor, 2010). As a first line of defence, a single layer of intestinal epithelial cells (IECs), joined by tight junction (TJ) proteins, forms a physical barrier between the intestinal microbiota and the host (Abreu, Fukata, & Arditi, 2005; Maynard, Elson, Hatton, & Weaver, 2012). The cells comprising this barrier constantly sense microbes through pattern recognition receptors (PRRs) (Maynard et al., 2012; Perez-Lopez, Behnsen, Nuccio, & Raffatellu, 2016).

PRRs recognize both microbial- (MAMPs) and pathogen-associated molecular patterns (PAMPs) and include toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Mills, 2011). Upon activation, both TLRs and NLRs can activate nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) singling pathways, leading to the production of cytokines (Mills, 2011). Specifically, NF- κB activation leads to the production of the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin (IL)-6, along with pro-IL-1β and pro-IL-18 (Mills, 2011). Pro-IL-1β and pro-IL-18 are converted to their active forms by caspase-1, which is produced through the activation of a subset of NLRs involved in inflammasome formation (Kim, Shin, & Nahm, 2016; Mills, 2011). Conversely, MAPK signalling results in the production of the cytokines IL-10 and IL-23 (Mills, 2011). Further, the production of antimicrobial peptides from IECs is also dependent on the activation of PRRs, such as TLR2, TLR4, and NOD1 (Abreu et al., 2005; Brandtzaeg, Kiyono, Pabst, & Russell, 2008). Antimicrobial peptides include α- and β-defensins which possess broad antimicrobial properties and help to regulate the composition of the intestinal microbiota (Abreu et al., 2005; Cario, 2010; Mackos, Maltz, & Bailey, 2017).

The gut mucosal immune system can be compartmentalized into inductive sites and effector sites (Brandtzaeg et al., 2008; McGhee & Fujihashi, 2012). Inductive sites house naïve lymphocytes and include gut-associated lymphoid tissues, such as Peyer's patches (Brandtzaeg et al., 2008). Differentiated lymphocytes migrate to effector sites where they initiate their immune responses (Brandtzaeg et al., 2008; McGhee & Fujihashi, 2012). Effector sites include the lamina propria and epithelial layer (Brandtzaeg et al., 2008). Signaling molecules released from IECs, such as cytokines produced through the activation of PRRs, play an important role in directing lymphocyte differentiation (Maynard et al., 2012; Mills, 2011). For instance, IL-6 and IL-23 are

involved in the induction of CD4 positive T helper 1 and 17 cells (Maynard et al., 2012). Signaling molecules, therefore, represent a key pathway of communication between the innate and adaptive immune systems of the gut.

Most fascinating is the ability of the mucosal immune system to differentiate commensals from pathogens, and the localization of PRRs facilitates this differentiation (Maynard et al., 2012). TLRs are transmembrane receptors embedded in either the apical or basolateral surfaces of IECs, whereas NLRs are cytosolic receptors (Kim et al., 2016; Maynard et al., 2012). Commensal microbes are more likely to activate TLRs on the apical surface of IECs, whereas pathogenic microbes are more likely to infiltrate IECs and activate NLRs (Maynard et al., 2012). Moreover, during instances of barrier dysfunction, it is possible for microbes to access TLRs expressed on the basolateral surface of IECs (Maynard et al., 2012). Following microbial sensing, the immune system elicits either a tolerogenic or pro-inflammatory response (Maynard et al., 2012; Perez-Lopez et al., 2016). Commensals stimulate the secretion of IL-25, IL-33, and transforming growth factor β (TGFβ), inducing the development of regulatory T cells (Maynard et al., 2012). In this homeostatic response, regulatory T cells and IECs release signals, such as TGFβ, that promote the development of plasma cells known to produce secretory immunoglobin A (SIgA) (Maynard et al., 2012; Perez-Lopez et al., 2016). The binding of SIgA to microbes limits their access to the epithelial layer and facilitates their excretion from the GI tract (Hansen et al., 2010). Therefore, SIgA plays an important role in controlling microbial abundance (Hansen et al., 2010). During pathogen exposure or the loss of homeostasis, the mucosal immune system elicits a pro-inflammatory response that involves the production of IL-6, IL-12, and IL-23; this is followed by the induction of T helper 1 and T helper 17 cells (Maynard et al., 2012).

Inflammatory Mechanism of Depression

There are several lines of evidence to suggest an immune mechanism underlying depression in a subset of MDD patients. MDD patients display elevated peripheral and central levels of pro-inflammatory cytokines and acute phase reactants, including IL-1β, IL-6, TNF, and C-reactive protein (Felger et al., 2018; Miller & Raison, 2016). There also exists a high co-morbidity between depression and chronic inflammatory conditions, such as cardiovascular disease, rheumatoid arthritis, and IBD (Chang, Szegedi, O'Connor, Dantzer, & Kelley, 2009; Graff, Walker, & Bernstein, 2009). Furthermore, several genetic and environmental risk factors for depression are associated with inflammation. For instance, polymorphisms for genes encoding pro-inflammatory cytokines have been implicated in depression (Bufalino, Hepgul, Aguglia, & Pariante, 2013).

Exposure to psychosocial stress is a risk factor for the development of depression in humans and has been widely used to induce depressive- and anxiety-like behaviours in laboratory animals (Fava & Kendler, 2000; Miller & Raison, 2016). Interestingly, exposure to psychosocial stress is known to elicit an inflammatory response through the activation of the sympathetic nervous system (Miller & Raison, 2016). Sympathetic nervous system activation results in the release of catecholamines which act on the bone marrow, leading to the mobilization of peripheral myeloid cells, such as monocytes and macrophages, into circulation (Miller & Raison, 2016). Furthermore, psychosocial stress has been shown to change the composition of the intestinal microbiota, as well as increase gut barrier permeability (Bailey et al., 2011; Bailey, Engler, & Sheridan, 2006). Increased permeability of the gut barrier can allow for the passage of bacteria and bacterial products, including MAMPs and PAMPs, from the intestinal lumen into the periphery where they can encounter myeloid cells (Miller & Raison, 2016). PRR ligands can

activate myeloid cells through NF-κB and NLRP3 inflammasome signalling, resulting in the release of the pro-inflammatory cytokines IL-1β, IL-6, and TNF (Miller & Raison, 2016).

Microglia

Microglia are resident brain immune cells of myeloid origin (Zhang, Zhang, & You, 2018). Microglia comprise up to 15% of the brain parenchyma (Orihuela, McPherson, & Harry, 2016). Microglia play an important role in the maintenance of homeostasis through their involvement in neuroprotection and normal brain development (Fung et al., 2017; Orihuela et al., 2016). Similar to peripheral myeloid cells, microglia can adopt different functional states in response to the demands of their local environment (Butovsky et al., 2014; Orihuela et al., 2016). The classic functional states of microglia include the M1 and M2 states (Boche, Perry, & Nicoll, 2013; Orihuela et al., 2016). M1 microglia are commonly referred to as activated microglia and express pro-inflammatory cytokines, including IL-1β, IL-6, and TNF (Orihuela et al., 2016). M1 microglia are also involved in antigen presentation and produce reactive oxygen species (Orihuela et al., 2016). Conversely, M2 microglia are known to be involved in tissue repair and express anti-inflammatory cytokines, including IL-4, IL-10, and TGFβ (Orihuela et al., 2016). Furthermore, phagocytosis has been described as a function of microglia (Brown & Neher, 2014). During normal brain development, it is believed that microglia phagocytize weak synapses (Cope et al., 2018). However, increased microglia phagocytosis of synapse components has been associated with chronic stress exposure and cognitive impairment (Cope et al., 2018; Milior et al., 2016). Lastly, although discrete functional states have been heavily described in the literature, it is now believed that microglia functions are diverse and that these functions are best represented on a spectrum (Fung et al., 2017).

In mice, the intestinal microbiota has been shown to influence microglia (Erny et al., 2015; Thion et al., 2018). Abnormal microglia morphology and decreased microglia densities have been reported in the brains of GF mouse embryos when compared to specific pathogen free (SPF) embryos (Thion et al., 2018). These findings suggest possible microbe exposure before birth or possible pre-pregnancy programming of gametes by the microbiota. In adulthood, the microglia of GF mice displayed abnormal morphology and decreased immune responses when stimulated with lipopolysaccharide (Erny et al., 2015). Lipopolysaccharide is a microbial product that has long been used to stimulate classic M1 microglia activation (Hanisch & Kettenmann, 2007). Notably, treatment with short-chain fatty acids, which are microbial fermentation products, was shown to reverse microglia abnormalities seen in adult GF mice, further demonstrating the potential of microbial products to influence microglia (Erny et al., 2015). Moreover, similar abnormalities were found in the microglia of tri-colonized mice which lack a complex microbiota; and colonization of these mice with a complex SPF microbiota was shown to normalize their microglia (Erny et al., 2015). In summary, animal studies have demonstrated that both colonization status and microbial products are capable of influencing microglia morphology and function.

Neuroinflammation and MDD

The direct passage of pro-inflammatory cytokines across the blood-brain barrier (BBB) is one mechanism through which peripheral inflammation can stimulate neuroinflammation, and it is through this mechanism that peripheral TNF is thought to activate microglia (D'Mello, Le, & Swain, 2009; Miller & Raison, 2016; Qin et al., 2007). An additional mechanism involves the transport of activated peripheral myeloid cells across the BBB (Miller & Raison, 2016). Activated peripheral myeloid cells can be recruited to the brain by microglia through the release

of the chemokine CCL2 (McKim et al., 2018). Elevated expression of *CCL2* by microglia has been shown in mice exposed to repeated social defeat stress, a form of psychosocial stress (McKim et al., 2018). Additionally, increased *CCL2* expression was reported in the dorsal anterior cingulate cortex of depressed suicide victims, suggesting increased microglia activation and peripheral myeloid cell recruitment (Torres-Platas, Cruceanu, Chen, Turecki, & Mechawar, 2014). This is supported by the finding of increased microglia activation in the prefrontal cortex and anterior cingulate cortex of MDD patients during a major depressive episode (Setiawan et al., 2015).

Neuroinflammation can lead to both structural and functional changes to the brain. Elevated levels of pro-inflammatory cytokines have been shown to increase the uptake of the neurotransmitter serotonin, reducing its synaptic availability (Zhu et al., 2010). Decreased availability of serotonin is well known to be associated with depressive-like behaviours, and selective serotonin reuptake inhibitors are widely used to treat depression in humans (Belmaker & Agam, 2008; Fava & Kendler, 2000). Furthermore, pro-inflammatory cytokines, such as TNF, are known to activate the tryptophan metabolizing enzyme indoleamine 2,3-dioxygenase (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Activation of this enzyme favours the production of kynurenine over serotonin in the tryptophan-kynurenine pathway (Sublette et al., 2011). Interestingly, plasma kynurenine levels were found to be significantly higher in MDD patients who have attempted suicide than patients who have not attempted suicide (Sublette et al., 2011).

In the brain, kynurenine can be metabolized to neurotoxic quinolinic acid by activated microglia and infiltrating peripheral myeloid cells (Miller & Raison, 2016). Quinolinic acid induces neuronal excitotoxicity which is the damage and death of neurons as a result of glutamate

overexposure (Dong, Wang, & Qin, 2009; Miller & Raison, 2016). Quinolinic acid does this by stimulating glutamate release through NMDA receptor activation and by inhibiting glutamate reuptake by astrocytes (Miller & Raison, 2016). In addition, the production of quinolinic acid is associated with the downregulation of brain-derived neurotrophic factor (BDNF) (Miller & Raison, 2016). This is notable given that BDNF deficiency has been associated with depression, and this deficiency forms the basis of the neurotrophic hypothesis of depression (Yu & Chen, 2011). To support this hypothesis, decreased BDNF protein levels have been found in the serum of MDD patients and in the hippocampus of suicide victims (Karege et al., 2002; Karege, Vaudan, Schwald, Perroud, & La Harpe, 2005). Lastly, findings of hippocampal BDNF deficiency could explain the decreased hippocampal volumes seen in depression patients when compared to healthy controls (Videbech & Ravnkilde, 2004).

CHAPTER 2: PROJECT 1

Introduction

To date, there have been no preclinical studies utilizing individual MDD patient microbiota to induce depressive-like behaviour in animals. However, the use of pooled fecal microbiota from several MDD patients has been shown to induce both anxiety- and depressive-like behaviours in recipient rodents (Kelly et al., 2016; Zheng et al., 2016). As previously mentioned, each individual has a distinct intestinal microbiota that is influenced by both host and environmental factors (Deschasaux et al., 2018; Eckburg et al., 2005; He et al., 2018; Hopkins et al., 2002; Markle et al., 2013). We have previously demonstrated the induction of donor-specific phenotypes in GF mice colonized with fecal microbiota from individual irritable bowel syndrome (IBS) and generalized anxiety disorder (GAD) patients (De Palma et al., 2017; Thrasher et al., 2019). Given the high interindividual variability in microbiota composition, we hypothesize that pooling microbiota from several patients conceals important microbial features unique to individual patients. In order to address this hypothesis, we compared the outcomes of colonization with individual versus pooled MDD microbiota. We then sought to identify the microbial and immune features underlying depressive-like behaviour in our model.

Methods

Animals

Adult (6-12 weeks old) GF NIH Swiss mice of both sexes were obtained from the Farncombe Family Axenic-Gnotobiotic Facility at McMaster University. Once colonized, all mice were housed in a Level 1 unit at McMaster University's Central Animal Facility. Mice were exposed to a 12-hour light/dark cycle and given access to food (Teklad 7904 diet; Envigo, USA) and water *ad libitum*. All animal experiments were performed in accordance with the McMaster

Animal Care Committee guidelines and were approved by McMaster's Animal Research Ethics Board.

Donor Selection

All human subjects were participants in a clinical study led by psychiatrist Dr. Rebecca Anglin. In the mouse experiments, four MDD patients (MDD1-4) and four age- and sex-matched healthy controls (HC1-4) were selected as microbiota donors (Table 2.1). MDD patients were chosen based on their depression scores and fecal β-defensin 2 levels. Fecal β-defensin 2 serves as a marker of immune activation occurring in response to a perturbation in the luminal environment of the GI tract, such as pathogen exposure (Langhorst et al., 2009; Perez-Lopez et al., 2016). Elevated fecal β-defensin 2 levels have been reported in conditions with altered microbiota composition, such as IBS (Langhorst et al., 2009). Two MDD patients (n=1 per sex) with high fecal β-defensin 2 levels (≥75 ng/g of feces) and two patients with low fecal β-defensin 2 levels (<75 ng/g of feces) were selected. Despite psychiatric comorbidities, all MDD patients were otherwise healthy. Patients were on no additional medications besides those used to treat their psychiatric illness.

Table 2.1: MDD and HC Donor Characteristics

Donor	Sex	Age	BMI	Depression Scores			Fecal β-Def 2 (High≥75ng/g feces)	Medications (1=antidepressants, 2=benzodiazephines, 3=atypical antipsychotics)	Comorbidities (GAD=generalized anxiety disorder, SAD=social anxiety disorder)
				DASS21	MADRS	HAM-D			
MDD1	M	25	17.0	42 (Extremely severe)	28 (Moderate)	17 (Moderate)	114.48	1,2,3	GAD, SAD
HC1	M	24	23.1	0 (None)	NA	NA	37.71	None	None
MDD2	F	42	21.2	12 (Mild)	29 (Moderate)	23 (Very severe)	155.48	1,3	GAD
HC2	F	42	23.5	2 (None)	NA	NA	39.66	None	None
MDD3	F	38	27.1	42 (Extremely severe)	26 (Moderate)	18 (Moderate)	53.19	1,2,3	GAD, SAD
HC3	F	35	NA	4 (None)	NA	NA	0.23	None	None
MDD4	M	19	18.4	36 (Extremely severe)	25 (Moderate)	11 (Mild)	34.08	None	None
HC4	M	22	21.2	2 (None)	NA	NA	48.92	None	None

Fecal Microbiota Transplant

Human fecal samples were diluted (10% w/v in saline) under anerobic conditions. Pooled samples contained equal parts fecal dilution from each donor. Fecal dilution was administered at a volume of 0.2 mL per mouse via oral gavage. GF mice were colonized with either fecal microbiota from a single MDD patient (MDD1-4), a single HC (HC1-4), or pooled fecal microbiota from MDD1-4 (PMDD) or HC1-4 (PHC; Fig. 2.1). Minimum n=10 per colonization group. Total n=110.

Behavioural Testing

Three weeks post-colonization, mouse behaviour was assessed using the open field test, three chamber sociability assay, tail suspension test, and sucrose preference test. Before each test, all mice were habituated to the testing room for a minimum of 30 minutes. Tests were performed in order of increasing stress with a minimum of 24 hours rest between each test. The sucrose preference test was performed last to control for any confounding effects sucrose may have on mouse behaviour. All equipment was cleaned between subject mice.

Open field test: The open field test is used to assess anxiety-like behaviour in rodents (Seibenhener & Wooten, 2015). The subject mouse was placed in the center of the open field and allowed to explore the field for 10 minutes. The movement of the mouse was tracked using the Activity Monitor software (Med Associates, USA). The center of the open field is viewed as a high-risk area for the mouse; and for this reason, less time spent in the center of the open field is indicative of anxiety-like behaviour (Seibenhener & Wooten, 2015).

Three chamber sociability assay: Social withdrawal is associated with depression (Girard et al., 2014). The three chamber sociability assay is used to assess sociability in rodents (Nadler et al., 2004). The test consisted of a 5-minute habituation phase, followed by a 10-minute testing phase, during which the mouse was recorded using a GoPro. In the habituation phase, an empty cage was placed in each side chamber. The subject mouse was placed in the center chamber of the testing apparatus and allowed to explore all three chambers for 5 minutes. During the testing phase, a novel mouse occupied one of the empty cages and a novel object occupied the other empty cage. The subject mouse was allowed to explore all three chambers for 10 minutes. From the video files, the time each subject mouse spent interacting with the novel mouse was measured. Interaction was defined as any movement occurring within 1-inch of the cage. In this test, decreased sociability was measured as decreased time spent interacting with the novel mouse.

Tail suspension test: The tail suspension test is used to assess behavioural despair in rodents (Castagné, Moser, Roux, & Porsolt, 2011; Steru, Chermat, Thierry, & Simon, 1985). Each mouse was suspended from its tail at a height of 30 cm for a testing period of 6 minutes. The experimenter measured time to immobility and total time spent immobile. Time immobile was

defined as time spent passively hanging. Lesser time to immobility and greater total time immobile is indicative of behavioural despair (Castagné et al., 2011; Steru et al., 1985).

Sucrose preference test: Anhedonia is a hallmark of depression (American Psychiatric Association, 2013). The sucrose preference test is used to assess anhedonia-like behaviour in rodents (Liu et al., 2018). The test consisted of a 48-hour habituation period, followed by a 72-hour testing period. During the habituation period, the subject mouse was placed in a cage with two bottles containing plain water. The bottles were weighed every 24 hours beginning at the start of the habituation period. During the testing period, one of the water bottles was replaced with a bottle containing a 2% sucrose solution. Again, the bottles were weighed every 24 hours. To control for side preference, the position of the bottles was switched every 24 hours. At the end of the test, overall percent sucrose preference was calculated. Decreased percent sucrose preference is indicative of anhedonia-like behaviour (Liu et al., 2018).

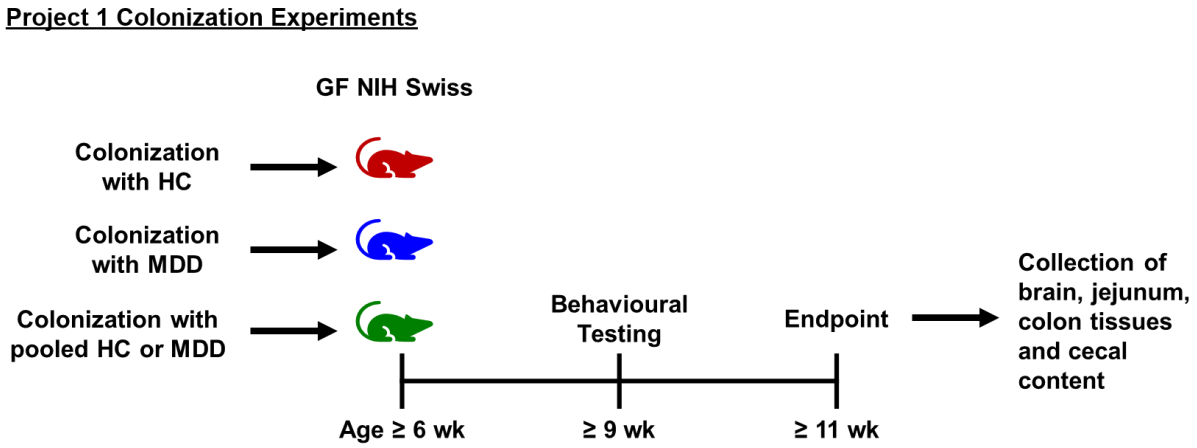

Figure 2.1: Experiment timeline for all colonization groups in Project 1.

Integrative Behavioural Z-Scoring

Integrative behavioural z-scoring was used to control for the intrinsic variability of single behavioural tests. This method combines individual behavioural parameters to create a single emotionality z-score for each mouse. Emotionality z-scores were calculated as described previously (Guilloux, Seney, Edgar, & Sibille, 2011). All healthy control data was used as the control group in the calculation.

RNA Isolation and nCounter Analysis

Mice were euthanized upon completion of behavioural testing. Tissues were collected and stored at -80°C for later processing. Brains were cryosectioned coronally at 200 μm. RNA was isolated from the ventral hippocampus, jejunum, and colon using the RNeasy Mini Kit (QIAGEN, Germany). RNA was normalized and pooled for nCounter analysis (NanoString Technologies, Inc., USA). A custom nCounter gene expression panel was used. Please see Appendix B for the complete gene list.

16S rRNA Gene Sequencing

Human fecal samples were collected by the clinical team. The cecal content of each mouse was collected at endpoint. All samples were stored at -80°C until sequencing. 16S rRNA gene sequencing of the V3 region was performed by the Surette Laboratory at McMaster University.

Statistical Analyses

Behavioural data were analyzed using GraphPad Prism 8 (GraphPad Software, Inc., USA). Outliers were removed using Grubb's test and normality was assessed using the Shapiro-Wilk test. Normally distributed data were analyzed using one-way ANOVA and Tukey's post hoc test

or two-way ANOVA and Sidak's multiple comparisons test. NanoString nCounter data were analyzed using nSolver 4.0 (NanoString Technologies, Inc., USA). Further, all microbiota data were analyzed using nonparametric tests. Coverage data were analyzed in GraphPad using either the Mann-Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparisons test. Microbial correlations and differences were determined using IBM SPSS Statistics 21 (IBM, USA). Microbial diversity metrics were generated and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline. For microbial differences, FDR adjusted p-values <0.05 were considered statistically significant. For all other analyses, p-values <0.05 were considered statistically significant. Figures were generated using GraphPad Prism, Microsoft Excel 2004, or Emperor.

Results

i. Select donor microbiota induced depressive-like behaviour in recipient mice

Mice colonized with microbiota from MDD1 displayed depressive-like behaviour in the sucrose preference test. Sucrose preference of MDD1 mice was significantly lower than that of HC1, PHC, and PMDD mice (Fig. 2.2A). No differences were seen between MDD1 and HC1 mice in the open field test, sociability assay, or tail suspension test (Fig. 2.2B-D). However, emotionality z-scores were significantly different between the HC1 and MDD1 groups (Fig. 2.2E). No differences in behaviour were found between the other donor pairs or the two pooled groups. However, both PHC and PMDD mice displayed anxiety-like behaviour in the open field test when compared to HC1 mice as seen by decreased time spent in the center of the open field (Fig. 2.2C).

We then employed sex-stratified analyses across all colonization groups. Mouse sex was found to be a significant source of variation in the open field and tail suspension tests, with females spending less time in the center of the open field and more time immobile when compared to male mice (Table 2.2). We also observed a significant donor x sex interaction in the tail suspension test (Table 2.2). In the sucrose preference test, the microbiota donor was found to be a significant source of data variation (Table 2.2). Neither mouse sex nor donor were found to be significant in the sociability assay; however, the donor x sex interaction nearly reached significance (*p=0.0521*).

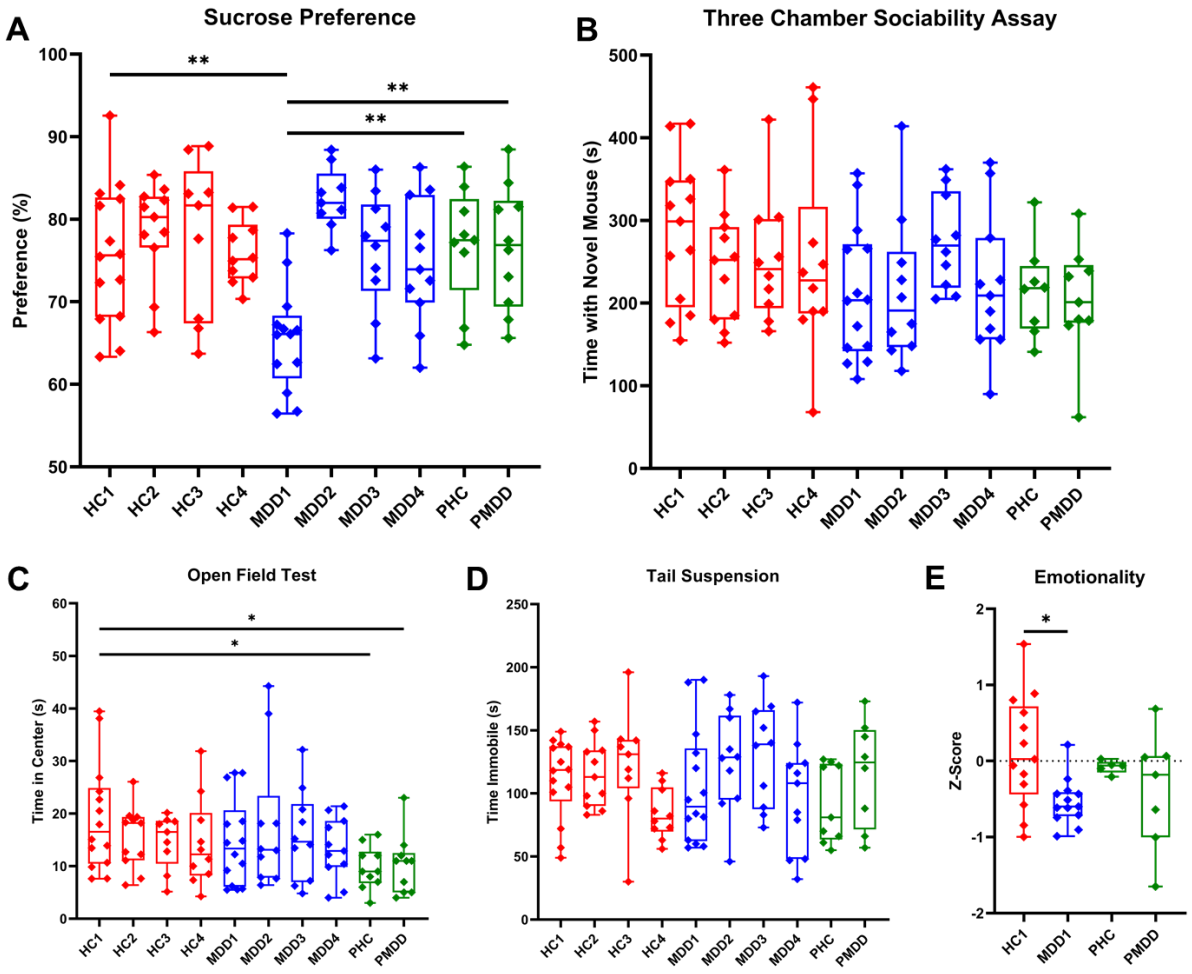

Figure 2.2: Effect of individual MDD patient microbiota on mouse behaviour. (A) Percent sucrose preference of mice colonized with MDD1 microbiota (n=13) was significantly lower when compared to HC1 (n=14), PHC (n=9), and PMDD (n=10) mice. (B) Time spent with the novel mouse did not differ between groups. (C) Mice in the pooled groups (n=10 per group) spent less time in the center of the open field when compared to HC1 (n=14) mice. (D) No differences were seen in the tail suspension test. (E) Emotionality z-scores differed significantly between the HC1 (n=13) and MDD1 (n=13) groups. Data presented as standard boxplots with individual values. One-way ANOVA and Tukey's post hoc test; *$p<0.05$, **$p<0.01$.

Table 2.2: Sex-Stratified Analyses (Two-way ANOVAs)

Time in Center of Open Field		
Source of Variation	**Percent of Variation**	**P-value**
Mouse Sex	5.895	0.0113
Time with Novel Mouse		
No significant sources of variation		
Time Immobile		
Mouse Sex	7.917	0.0014
Donor x Mouse Sex	18.65	0.0054
Percent Sucrose Preference		
Donor	23.58	0.0010

ii. Transfer of human microbiota to mice was dependent on individual donor

At phylum level, the microbiota profiles of recipient mice were different from those of the human donors (Fig. 2.3A). However, the human donor was a significant source of variation in the relative abundances of all phyla but Tenericutes in the microbiota of all recipient mice. Tenericutes was the only phylum that did not transfer from human to mouse.

To investigate the specific microbial features of mice displaying depressive-like behaviour, we compared MDD1 mouse microbiota to that of all other colonization groups. Increased Proteobacteria, Firmicutes, and Actinobacteria, but decreased Verrucomicrobia was found to be characteristic of MDD1 mouse microbiota (Fig. 2.3C). After sex-stratified analyses, differences in Firmicutes, Actinobacteria, and Verrucomicrobia remained in female but not male mice (Fig. 2.3C). In male mice, however, increased Verrucomicrobia abundance was found to be predictive of anhedonia like-behaviour (Fig. 2.3B). We also observed an inverse correlation between Firmicutes and anhedonia like-behaviour in males, although this correlation did not reach statistical significance ($p=0.068$).

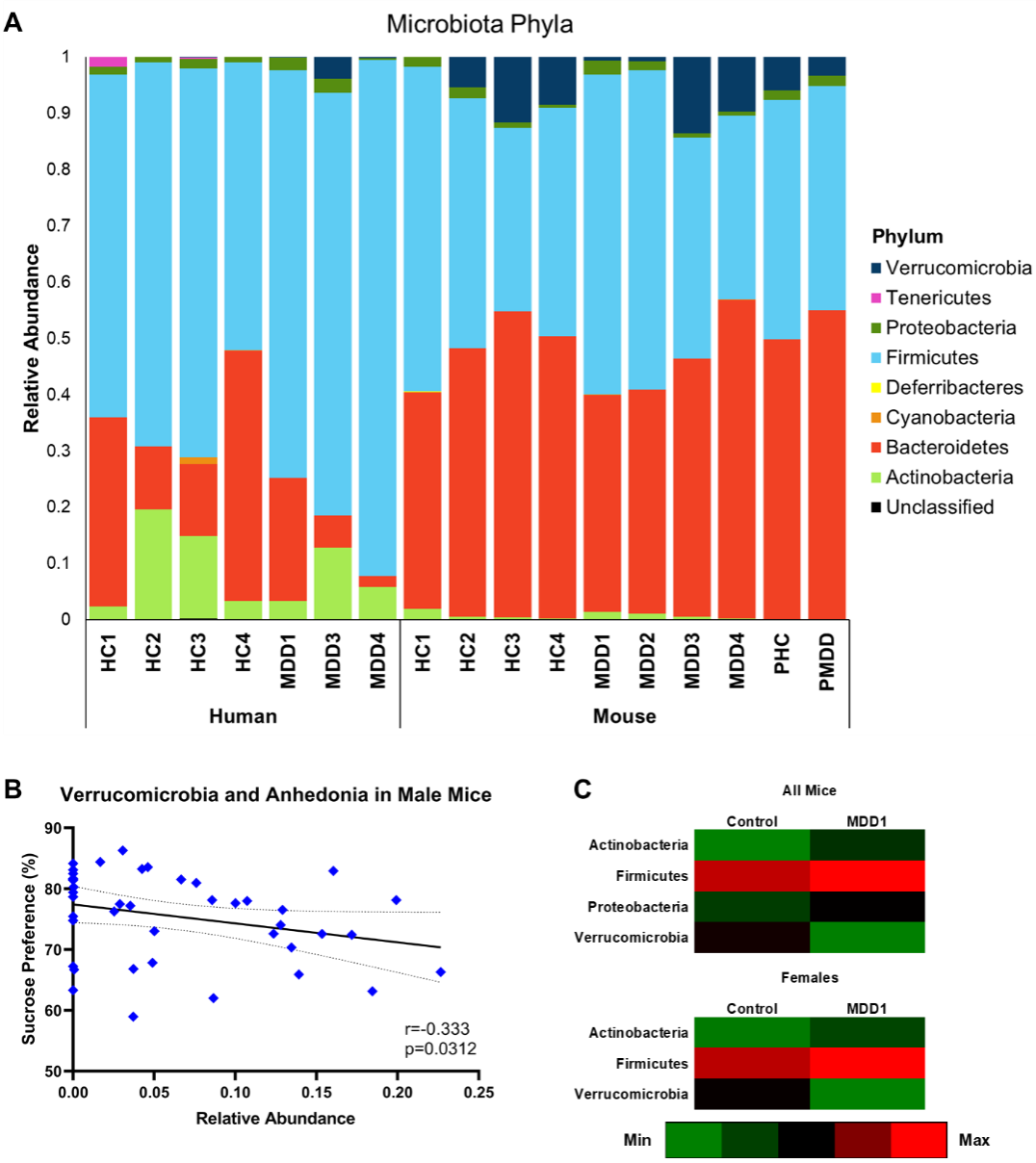

Figure 2.3: Phylum level differences among human donors and recipient mice. (A) Human donor (n=7) and recipient mouse (n=97) microbiota composition at phylum level. Data presented as mean relative abundance. (B) The relative abundance of Verrucomicrobia inversely correlated with percent sucrose preference in all male mice (n=42). (C) All significant phylum level features of MDD1 mouse microbiota. Data presented as mean relative abundance. Mann-Whitney test; $p<0.05$ FDR adjusted.

The transfer of microbiota from human to mouse was found to be dependent on the donor as seen by differences in percent amplicon sequence variants (ASVs) transferred (Fig. 2.4A). The

proportion of 100 most abundant (top 100) donor ASVs was significantly greater in the microbiota of MDD1-colonized mice when compared to the microbiota of mice colonized with HC1, HC2, or HC3 microbiota (Fig. 2.4B). MDD1 mouse microbiota contained fewer donor ASVs that were not in the top 100 (other donor ASVs) than the microbiota of HC1-, HC3-, and HC4-colonized mice (Fig. 2.4C). A higher proportion of ASVs not linked to the human donor was found in the microbiota of HC2 mice compared to that of HC4, MDD1, or MDD3 mice (Fig. 2.4D).

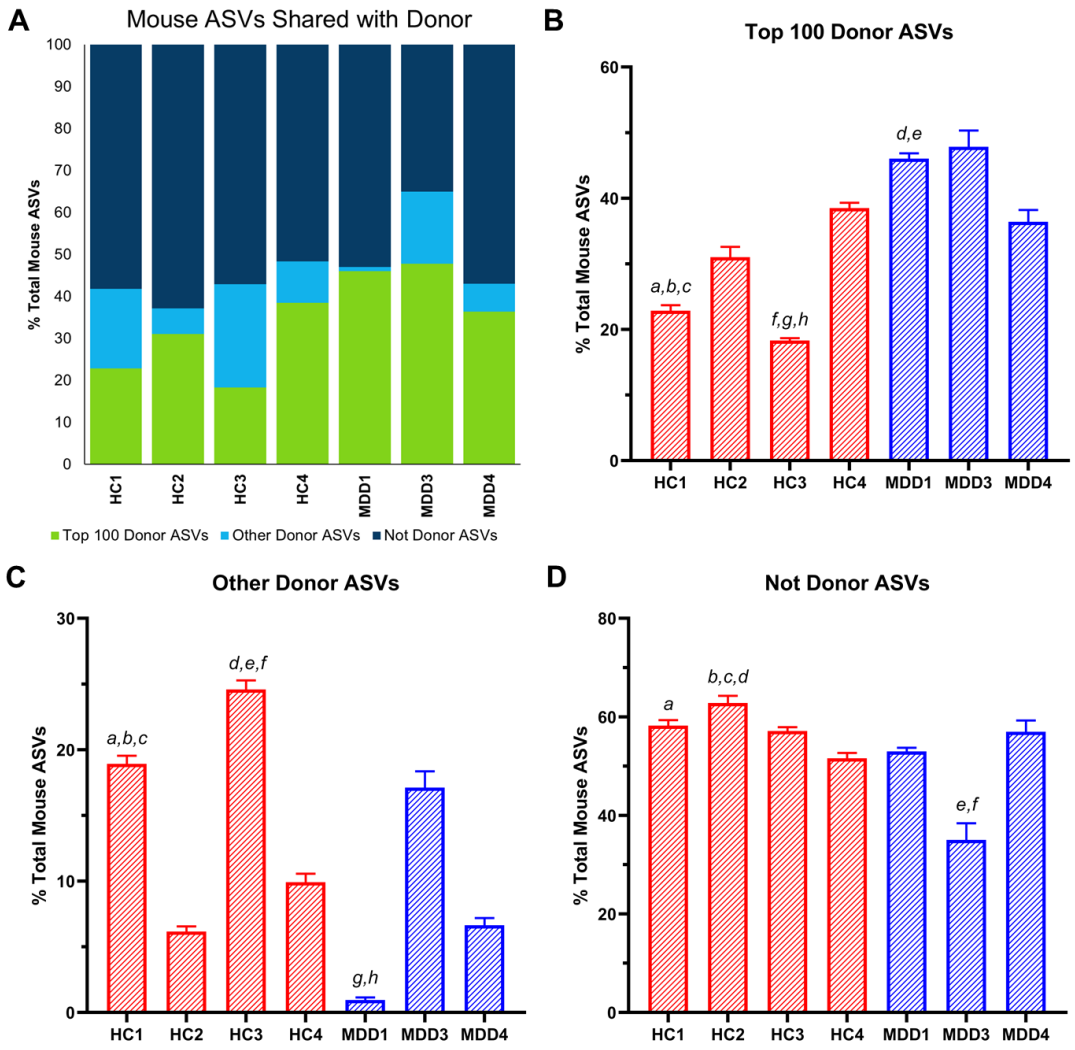

Figure 2.4: Success of transfer of donor microbiota to mouse. (A) Proportion of recipient mouse amplicon sequence variants (ASVs) shared with donor. (B) Significant differences in the percentage of total mouse ASVs that are the 100 most abundant (top 100) donor ASVs were found between HC1 and HC4 (*a*), HC1 and MDD1 (*b*), HC1 and MDD3 (*c*), HC2 and MDD1 (*d*), HC3 and MDD1 (*e*), HC3 and HC4 (*f*), HC3 and MDD3 (*g*), and HC3 and MDD4 (*h*). Data presented as mean + SEM. Kruskal-Wallis test and Dunn's multiple comparisons test; *b*, *c*, *e*, *g* $p<0.0001$; *f* $p<0.01$; *a*, *d*, *h* $p<0.05$. (C) Significant differences in the percentage of total mouse ASVs that are not 100 most abundant (other) donor ASVs were found between HC1 and HC2 (*a*), HC1 and MDD1 (*b*), HC1 and MDD4 (*c*), HC2 and HC3 (*d*), HC3 and MDD1 (*e*), HC3 and MDD4 (*f*), HC4 and MDD1 (*g*), and MDD1 and MDD3 (*h*). Data presented as mean + SEM. Kruskal-Wallis test and Dunn's multiple comparisons test; *b*, *e* $p<0.0001$; *d*, *f*, *h* $p<0.001$; *c* $p<0.01$; *a*, *g* $p<0.05$. (D) Significant differences in the percentage of total mouse ASVs that are not donor ASVs were found between HC1 and MDD3 (*a*), HC2 and HC4 (*b*), HC2 and MDD1 (*c*), HC2 and MDD3 (*d*), HC3 and MDD3 (*e*), and MDD3 and MDD4 (*f*). Data presented as mean + SEM. Kruskal-Wallis test and Dunn's multiple comparisons test; *d* $p<0.0001$; *a* $p<0.001$; *b*, *c*, *e*, *f* $p<0.01$.

In our model, HC1 and MDD1 were the donor pair that resulted in significantly different behavioural outcomes in recipient mice. Therefore, we compared ASV proportions between the HC1, MDD1, PHC, and PMDD colonization groups to determine the effect of pooling on the transfer of ASVs from donors HC1 and MDD1. Pooling the microbiota of several MDD donors resulted in the decreased transfer of top 100 MDD1 donor ASVs to recipient mice (Fig. 2.5A). PMDD mouse microbiota contained a greater proportion of ASVs not linked to donor MDD1 when compared to MDD1 mouse microbiota (Fig. 2.5C). We observed a similar difference in the percentage of ASVs not linked to donor HC1 between the microbiota of mice colonized with PHC and HC1 microbiota (Fig. 2.5C). Lastly, pooling the microbiota of several HC donors resulted in the decreased transfer of other less abundant ASVs found in donor HC1 (Fig. 2.5B).

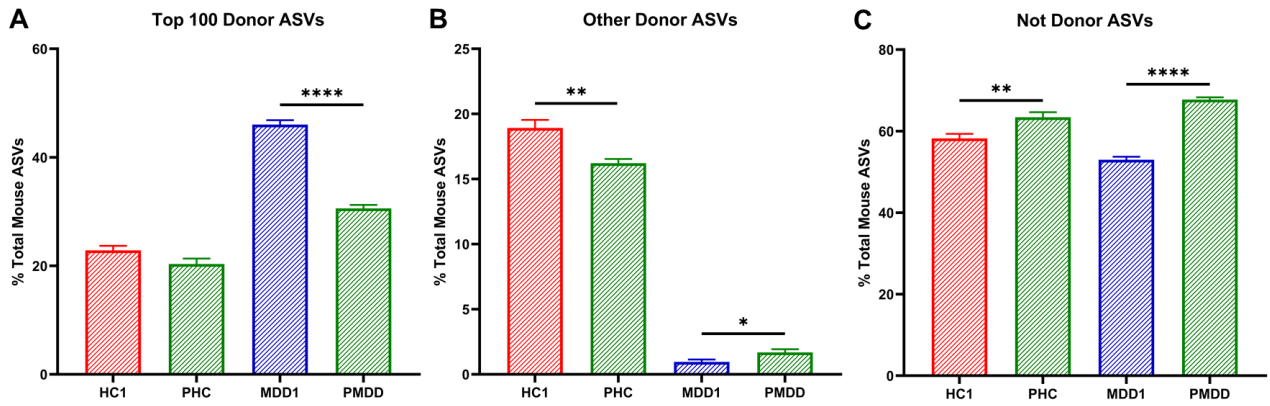

Figure 2.5: Effect of pooling microbiota on the transfer of individual donor ASVs. (A) The proportion of the 100 most abundant (top 100) MDD1 donor ASVs was significantly less in PMDD mouse microbiota when compared to MDD1 mouse microbiota. (B) The percentage of other less abundant ASVs from donor HC1 was significantly less in PHC mouse microbiota when compared to HC1 mouse microbiota. The percentage of other ASVs from donor MDD1 was significantly greater in PMDD mouse microbiota when compared to MDD1 mouse microbiota. (C) Microbiota of mice from the pooled groups contained a greater proportion of ASVs not linked to the individual donors HC1 and MDD1. Data presented as mean + SEM. Mann-Whitney test; *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

iii. Beta diversity of mouse microbiota clustered by individual donor

Jaccard beta diversity of mouse microbiota was significantly different between mouse colonization groups (PERMANOVA; *p=0.001*). In the PCoA plot, we observed the clustering of groups by individual donor (Fig. 2.6A), as well as by donor class (MDD vs HC; Fig. 2.6B). Conversely, no significant sex differences were observed in beta diversity.

We investigated the specific features of the microbiota of MDD1 mice which displayed depressive-like behaviour. MDD1 mouse microbiota had decreased phylogenetic diversity when compared to the microbiota of all other colonization groups; and no differences in alpha diversity were observed (Fig. 2.6C).

Several species were associated with decreased sucrose preference which indicates anhedonia-like behaviour in mice. The increased relative abundance of an unclassified species of *Phascolarcotbacterium* was found to be predictive of anhedonia like-behaviour in all mice, and further analyses revealed that this correlation was strong in female mice but absent in males (Fig. 2.6D). In female mice, the relative abundances of *Eggerthella lenta*, *Bacteroides ovatus*, and an unclassified species of *Bifidobacterium* positively correlated with anhedonia-like behaviour, whereas *Blautia obeum* inversely correlated with anhedonia-like behaviour (Fig. 2.6D).

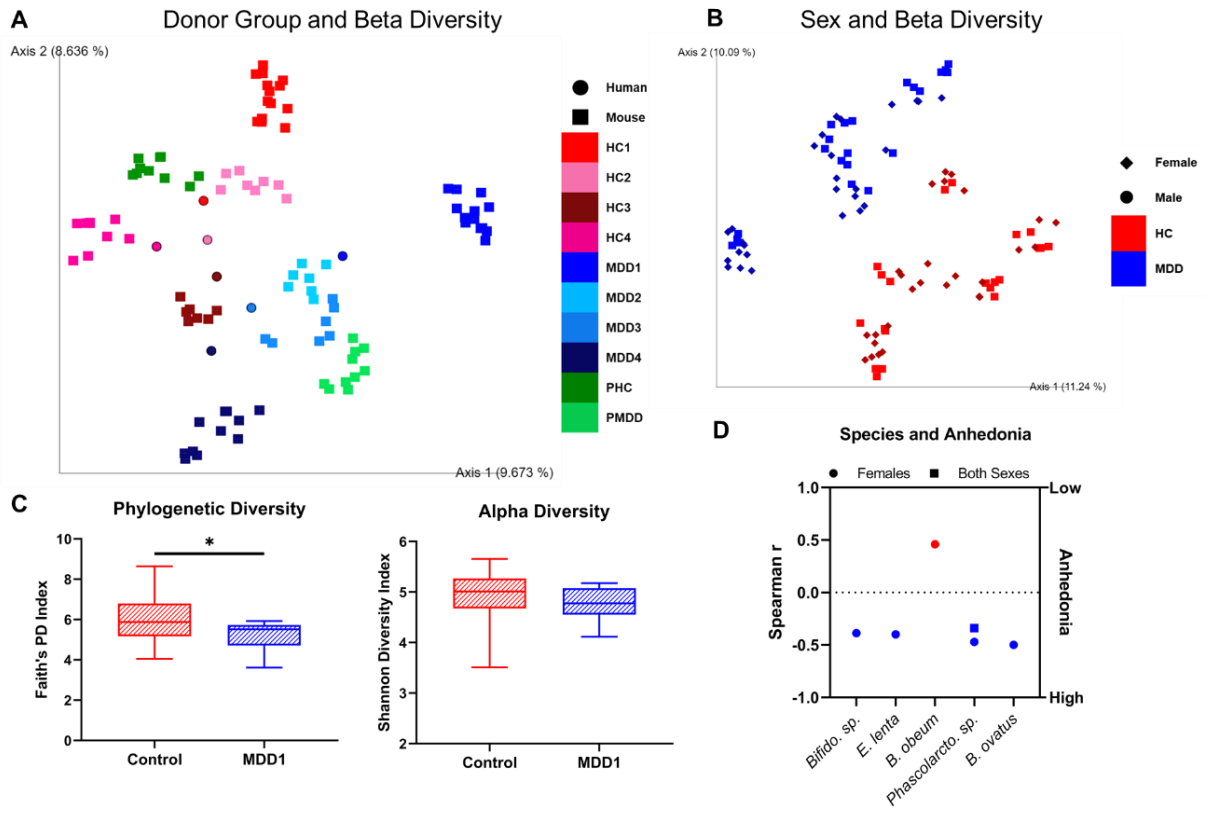

Figure 2.6: Beta diversity of mouse microbiota clustered by individual donor. (A) PCoA plot of Jaccard beta diversity for human donor (n=7) and recipient mouse microbiota (n=97). (B) PCoA plot of Jaccard beta diversity for recipient mouse microbiota depicting different sexes (females n=53; males n=42). (C) Phylogenetic diversity of MDD1 mouse microbiota (n=14) significantly decreased compared to microbiota of all other colonization groups (n=83). Alpha diversity of MDD1 mouse microbiota did not differ significantly when compared to microbiota of all other colonization groups. Data presented as standard boxplots. Mann-Whitney test; *$p<0.05$. (D) Significant species level correlations with percent sucrose preference in all mice (n=94) and females (n=52).

Several bacterial species were found to be characteristic of MDD1 mouse microbiota. Greater relative abundances of *E. lenta*, *Coprobacillus cateniformis*, as well as unclassified species of *Clostridiales*, *Pseudoramibacter Eubacterium*, and *Phascolarctobacterium* and decreased abundance of *Bacteroides acidifaciens* was found in the microbiota of MDD1 mice when compared to the microbiota of all other mice (Fig. 2.7A). Differences in these species were conserved after sex-stratified analyses (Fig. 2.7B, C). Furthermore, we observed significant

species level features that were unique to both sexes. Decreased relative abundance of *B. obeum* was characteristic of MDD1 female mice (Fig. 2.7B), whereas increased relative abundance of an unclassified species of *Allobaculum* was characteristic of MDD1 male mice (Fig. 2.7C).

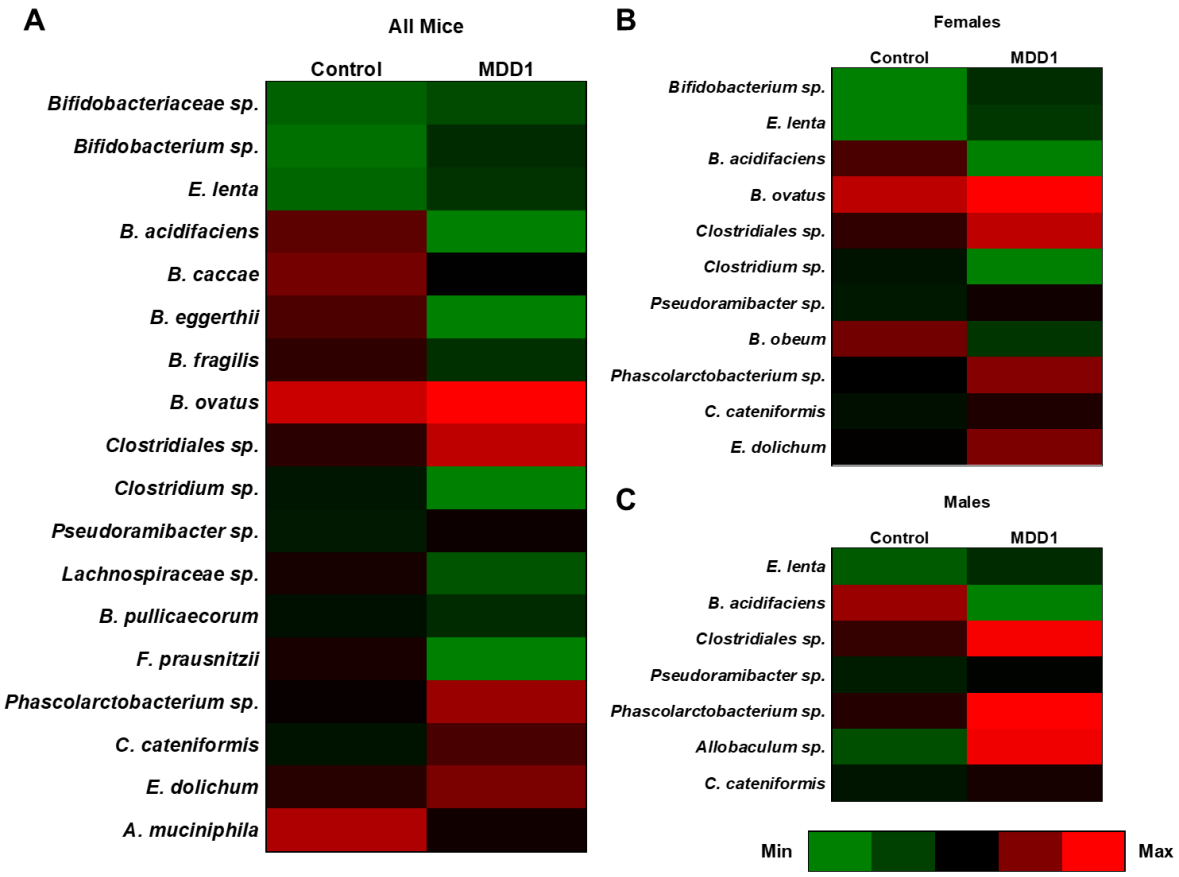

Figure 2.7: Significant species level features of MDD1 mouse microbiota for (A) all mice, (B) female mice, and (C) male mice. Data presented at log-transformed mean relative abundance. Kruskal-Wallis test; $p<0.05$ FDR adjusted.

iv. Pooling microbiota altered its effects on mouse gene expression

In the jejunum, the expression of several inflammatory genes, including *C3*, *CCR2*, *CCR6*, *NOD2*, *TLR2*, and *TLR5*, was increased in mice colonized with MDD1 microbiota when compared to HC1 mice (Fig. 2.8A, D). In PMDD mice, however, *CCR2* expression was found to

be decreased when compared to PHC mice (Fig. 2.8A). Moreover, elevated expression of the TJ protein-coding genes *CLDN1* and *CLDN2* was found in MDD1 mice when compared to HC1 mice (Fig. 2.8A, D). An opposing trend was seen for *CLDN2* between the pooled groups, with expression decreased in PMDD mice (Fig. 2.8A). Expression of *NOS1* was increased in MDD1 mice compared to HC1 mice (Fig. 2.8A, D). However, *NOS1* expression was significantly lower in PMDD mice when compared to PHC mice (Fig. 2.8A). In MDD1 mice, there was also increased expression of other genes associated with the nervous system, including the enteric glial cell (EGC) marker *GFAP* and the neurotrophin receptor-coding gene *P75* (Fig. 2.8A).

In the colon, MDD1 mice had reduced expression of the TJ protein-coding gene *OCLN* when compared to HC1 mice (Fig. 2.8B, D). Expression of the TLR and IL-1 adaptor-encoding gene *MYD88* was also decreased in mice colonized with MDD1 microbiota (Fig. 2.8B, D). Lastly, expression of the EGC marker *S100β* was increased in MDD1 mice when compared to HC1 mice (Fig. 2.8B).

In the hippocampus, the expression of *P75* and the capsaicin receptor *TRPV1* was decreased in MDD1 mice when compared to HC1 mice (Fig. 2.8C, D).

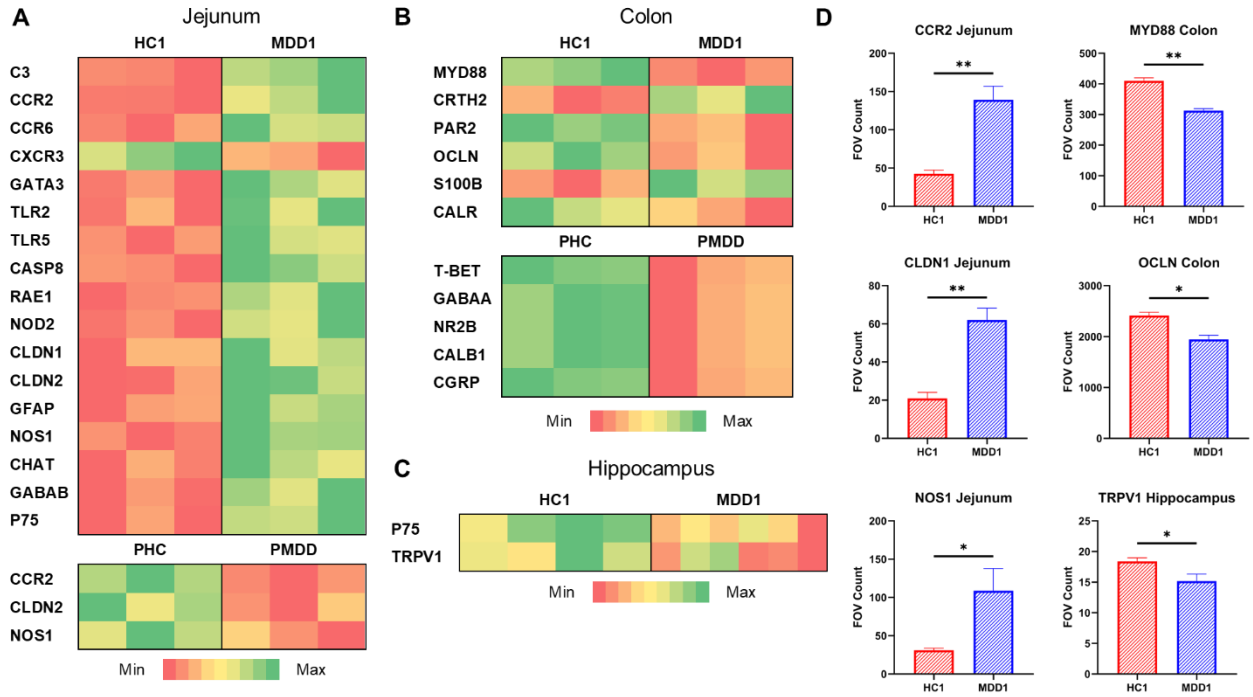

Figure 2.8: Heatmaps for genes with significantly different log2 ratios in mouse (A) jejunum, (B) colon, and (C) hippocampus. Data presented as normalized FOV counts. (D) Individual gene expression plots. Normalized FOV counts presented as mean + SEM. Unpaired, two-tailed t-test; *$p<0.05$, **$p<0.01$.

Discussion

MDD is a highly heterogeneous disorder, with only a subset of patients presenting with altered intestinal microbiota composition and elevated inflammatory biomarker levels (Aizawa et al., 2016; Belmaker & Agam, 2008; Z. Chen et al., 2018; Y. C. E. Chung et al., 2019; Felger et al., 2018; Jiang et al., 2015; Kelly et al., 2016; Lin et al., 2017; Zheng et al., 2016). Here, we demonstrate for the first time that individual MDD patient microbiota induces a donor-specific phenotype in recipient GF animals. Our behavioural results reveal that fecal microbiota from only one MDD donor (MDD1) induced depressive-like behaviour in GF mice. Additionally, we demonstrate that the unique composition of individual patient microbiota remains after fecal

microbiota transplant (FMT). Interestingly, MDD1 mice had altered intestinal microbiota when compared to all other colonization groups, and several of the unique microbial features of MDD1 mouse microbiota closely match what has been reported in the literature. For example, decreased phylogenetic diversity has been previously shown in rodents colonized with pooled MDD microbiota, as well as in patients with MDD (Kelly et al., 2016). The observed increases in Actinobacteria, Firmicutes, and Proteobacteria in MDD1 mice coincide with prior findings in MDD patients (Z. Chen et al., 2018; Y. C. E. Chung et al., 2019; Jiang et al., 2015; Lin et al., 2017; Zheng et al., 2016). Previous studies have also associated increases in *Eggerthella* and *Clostridiales* species with depression (J. J. Chen et al., 2020; Y. C. E. Chung et al., 2019; Kelly et al., 2016; Zheng et al., 2016). In the present study, decreased relative abundance of *Bacteroides caccae* was found to be a unique feature of MDD1 mouse microbiota. This is of particular interest as this species is a known producer of the neurotransmitter GABA (Strandwitz et al., 2019). Reductions in GABA have been reported in the brains of depression patients, supporting an additional mechanism for MDD pathophysiology (Duman, Sanacora, & Krystal, 2019). Lastly, MDD1 mice had decreased relative abundance of *Faecalibacterium prausitzii*, a species that has been found to exert protective effects against colitis in mice (Sokol et al., 2008).

Both immune and non-immune cells of the gut recognize commensal and pathogenic microbes via PRRs, such as TLRs and NLRs (Perez-Lopez et al., 2016). TLRs and NLRs can bind MAMPs and PAMPs, resulting in the production of pro-inflammatory cytokines and chemokines via the NF-κB and MAPK signaling pathways (Luster, 2002; Mills, 2011; Perez-Lopez et al., 2016). Aberrant PRR signaling has been implicated in inflammatory diseases associated with altered intestinal microbiota composition, such as IBD (Cario, 2010; Round & Mazmanian, 2009). Additionally, the intestinal microbiota may exert regulatory effects on TLR expression in

the gut. Both probiotic and pathogenic bacteria have been shown to increase the expression of TLRs by EGCs *in vitro* (Turco et al., 2014). In the present study, we observed elevated expression of the PRR-encoding genes *NOD2*, *TLR2*, and *TLR5* in the jejunum of MDD1 mice, suggesting possible increases in microbial sensing and its associated inflammatory responses. TLR5 is located on the basolateral surface of the gut epithelial barrier (Maynard et al., 2012). Therefore, increases in *TLR5* expression may indicate possible barrier dysfunction and increased microbial translocation. This is further supported by our findings of greater TJ protein-coding gene expression in the jejunum.

In the colon, we observed fewer genes that were differentially expressed between MDD1 and HC1 mice. This coincides with previous work demonstrating that the microbiota regulates fewer genes at the level of the colon compared to the small intestine (Larsson et al., 2012). MDD1 mice had decreased expression of the TLR adaptor protein-coding gene *MYD88* and protease-activated receptor-coding gene *PAR2*. During infection, PAR2 is activated by trypsin and trypsin-like proteases, leading to a pro-inflammatory response (Nhu et al., 2010; Velin et al., 2011). Furthermore, cooperative signalling between TLRs and PAR2 has been previously observed in response to immune challenge, and PAR2 deficiency in mice has been shown to decrease susceptibility to DSS-induced colitis (Hyun, Andrade-Gordon, Steinhoff, & Vergnolle, 2008; Nhu et al., 2010). Conversely, MYD88 knockout mice are more susceptible to DSS-induced colitis when compared to wild-type controls (Araki et al., 2005). Studies examining genetic MYD88 deficiency have also revealed associations with increased susceptibility to bacterial infections in mice and humans (Takeuchi, Hoshino, & Akira, 2000; Von Bernuth et al., 2008). Therefore, our findings of decreased *MYD88* and *PAR2* expression in MDD1 mice may indicate

impaired sensing of the microbiota by the innate immune system and altered activation of downstream pro-inflammatory signaling cascades.

Chemokines are chemotactic cytokines and interactions between chemokines and their receptors mediate adaptive immune cell trafficking to the gut (Perez-Lopez et al., 2016; Zimmerman, Vongsa, Wendt, & Dwinell, 2008). Chemokine receptors have been implicated in colitis. For instance, the inhibition of the chemokine receptors CCR2, CCR5, and CXCR3 has been shown to prevent DSS-induced colitis in mice (Tokuyama et al., 2005). Similarly, CCR6 knockout in mice decreased DSS-induced colitis severity when compared to wild-type controls (Varona, Cadenas, Flores, Martínez-A., & Márquez, 2003). In our model, the expression of the chemokine receptor-encoding genes *CCR2* and *CCR6* was increased in the jejunum of MDD1 mice, signaling increased adaptive immune cell trafficking to the small intestine.

The *CRTH2* gene encodes a receptor for the pro-inflammatory molecule prostaglandin D2. The CRTH2 receptor is expressed on the surface of some leukocytes, including T helper 2 cells; and similar to a chemokine receptor, activation of CRTH2 induces chemotaxis (Hata & Breyer, 2004). Increased CRTH2 expression has been associated with colitis, and it is postulated that prostaglandin D2-CRTH2 signaling mediates the trafficking of leukocytes to inflamed colon tissue (Sturm et al., 2014). In the present study, we observed increased expression of *CRTH2* in the colon of MDD1 mice, suggesting a greater adaptive immune response.

EGCs are a characteristic cell type of the enteric nervous system (Cirillo et al., 2011). EGCs are believed to play an important role in the maintenance of gut homeostasis through microbial sensing and regulating gut barrier permeability (Cirillo et al., 2011; Turco et al., 2014). Elevated GFAP and S100β expression has been repeatably linked to gut inflammation in both animals and humans (Cirillo et al., 2011; Costa et al., 2019; da Cunha Franceschi et al., 2017). Additionally,

S100β released from activated EGCs can perpetuate the inflammatory response by binding the receptor for advanced glycation end products which leads to the activation of NF-κB and MAPK signaling (Costa et al., 2019; Leclerc, Fritz, Vetter, & Heizmann, 2009; Xia, Braunstein, Toomey, Zhong, & Rao, 2018). Furthermore, EGCs have also been shown to regulate the expression of the TJ protein occludin in mice (Savidge et al., 2007). In the present study, we observed elevated expression of the EGC markers *GFAP* and *S100β* in the gut of mice colonized with MDD1 microbiota. We also observe decreased *OCLN* expression in the colon of MDD1 mice, possibly indicating increased gut barrier permeability.

P75 is a low affinity neurotrophin receptor that has been implicated in hippocampal neurogenesis (Catts, Al-Menhali, Burne, Colditz, & Coulson, 2008; Colditz et al., 2010; Peterson, Dickinson-Anson, Leppert, Lee, & Gage, 1999). Decreased neurogenesis in the dentate gyrus and impaired behaviour are characteristic of P75 knockout mice (Catts et al., 2008). Interestingly, we observed decreased expression of *P75* in the hippocampus of MDD1 mice which displayed depressive-like behaviour. This demonstrates a potential neurotrophic mechanism of depression and coincides with our previous finding of decreased BDNF protein expression in the dentate gyrus of MDD1 mice (Louis-Auguste, 2019). In the present study, *TRPV1* expression was also decreased in the hippocampus of MDD1 mice. Although well known for its roles in the periphery, the capsaicin receptor TRPV1 has been implicated in the regulation of synaptic plasticity and inflammation in the brain (Bassi et al., 2019; Chahl, 2011; Y. C. Chung et al., 2017). TRPV1 activation has been shown to prevent microglia activation in a mouse model of Parkinson's disease, as well as stress-induced long-term synaptic depression in rats (Y. C. Chung et al., 2017; Li et al., 2008). Antidepressant effects have also been linked to TRPV1 activation (Ghazizadeh-Hashemi et al., 2018; Hayase, 2011).

All together, our results demonstrate that colonization with MDD1 microbiota induced alterations along the mouse gut-brain axis. Specifically, the gene expression profiles of MDD1 mice indicate a possible decrease in hippocampal neurogenesis and synaptic plasticity, along with increased brain immune activation. Our findings indicate that MDD1 mice had altered gut immune activation and that immune responses differed along the length of the GI tract. At the level of the small intestine, our findings suggest increases in both innate and adaptive immune responses in MDD1 mice, characterized by increased PRRs signaling, adaptive immune cell trafficking, and EGC activation. In the colon, the immune response was characterized by increased adaptive immune cell trafficking. The results presented here also indicate possible gut barrier dysfunction as seen by altered expression of TJ protein-coding genes in both the small intestine and colon. Considering the altered intestinal microbiota composition found in MDD1 mice, we propose that the observed gut immune activation likely reflects a loss of homeostasis between the host and the intestinal microbiota.

The heterogeneity seen among donor groups in our behavioural, microbial, and gene expression data coincides with the known heterogeneity of MDD. The present results also reveal heterogeneity in the transfer of microbiota from humans to GF mice. This may be attributed to differences in host-microbiota compatibility. It is well known that both host and environmental factors shape the intestinal microbiota in humans (Deschasaux et al., 2018; He et al., 2018; Hopkins et al., 2002; Markle et al., 2013). In mice, both sex and genetics have been shown to influence the intestinal microbiota (Org et al., 2016). Indeed, we found that mouse sex influenced specific features of the microbiota in this study. Therefore, it is possible that interactions between the donor microbiota and the recipient mouse are also influenced by mouse sex, genetics, and diet. Given the heterogeneity observed in this study, we hypothesize that the intestinal

microbiota is likely involved in the inflammatory mechanism of depression in only a subset of MDD patients.

It is important to mention that the results presented here do not indicate that microbiota from MDD patients with signs of immune activation cannot induce a depressive-like phenotype in a host that is susceptible either due to genetic or environmental factors. In our model, all mice were of the same genetic background and depressive-like behaviour was induced in the absence of a psychosocial stressor. The investigation of the FMT model of depression using susceptible rodent hosts should be the focus of future experiments.

This study has several strengths. No previous study has utilized multiple individual MDD patients as microbiota donors. In the present study, we demonstrate that pooling microbiota from several MDD patients is disadvantageous in the FMT model of depression as pooled MDD microbiota did not induce a depressive-like phenotype in recipient mice. This study was also the first of its kind to include microbiota donors and recipient animals of both sexes. This is notable given that sex and gender are known to influence MDD risk, intestinal microbiota composition, and immune system function (Fava & Kendler, 2000; Markle et al., 2013; Rainville & Hodes, 2018). Our present results demonstrate sex differences in mouse behaviour and microbiota composition. Lastly, the influence of geography on the intestinal microbiota was controlled for in this study as all donors were recruited from a limited geographic region.

The main limitation of this study is that the majority of MDD donors were taking psychiatric medications at the time of sample collection. The effects of psychiatric medications on the composition of the intestinal microbiota are not fully understood. However, medication use did not appear to predict the ability of a donor sample to induce a depressive-like phenotype in this study as medication use was similar for both donor MDD1 and MDD3.

In summary, our study is the first to show that fecal microbiota from an individual MDD patient induces a donor-specific phenotype in GF mice, marked by behavioural, microbial, and immune changes. These findings lead us to propose that the intestinal microbiota is likely involved in the inflammatory mechanism of depression in some but not all MDD patients. Therefore, the use of individual psychiatric patient microbiota is warranted in future studies utilizing FMT.

CHAPTER 3: PROJECT 2

Introduction

The isolation of microglia from the mouse brain allows for functional phenotyping by means of gene expression or flow cytometric analysis. However, there are few published protocols that give reliable high cell yields. This presents challenges for the researcher and limits the study of microglia to research settings with previously established protocols. Here, we present a protocol for the isolation and flow cytometric analysis of adult mouse microglia. Our protocol gives high cell yields, averaging 1.28±0.34 million cells per adult mouse brain, whereas comparable protocols report typical yields of no more than 500,000 cells per brain (Grabert & McColl, 2018; Lee & Tansey, 2013). To allow for easy reproducibility, our protocol utilizes a commercially available enzyme kit.

Materials

Perfusion:

- Perfusion pump, tubing, 25G butterfly needle (blunted with pliers)
- Surgical tools: tweezers, hemostat, iris scissors, blunt scissors
- Metal basin
- 15 mL Falcon tubes
- Reagents: D-PBS, 1 x PBS

Microglia Isolation:

- Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, Germany; No. 130-092-628)
- Shaking water bath
- Bio-Rad TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc., USA)

- Round-edge scalpel
- 15 mL Falcon tubes
- 1.5 mL Eppendorf tubes
- P200, P1000 pipette tips
- Reagents: D-PBS, 1 x PBS, 1 x HBSS, 10 x HBSS, Percoll solutions (Note 12), FACS buffer (Note 11), Trypan blue dye, LIVE-DEAD Fixable Near-IR Stain (Thermo Fisher Scientific, USA; No. L10119), Mouse BD Fc Block (BD Biosciences, USA; No. 553141), CD11b BV605 (BD Biosciences, USA; No. 563015), CD45 BV786 (BD Biosciences, USA; No. 564225), CD86 BV421 (BD Biosciences, USA; No. 562432), 4% PFA, compensation beads

Methods

1. Transcardial Perfusion of Whole Mouse
    1. Anesthetize the mouse using isoflurane in chamber. Once unconscious, transfer the mouse to an ice-filled metal basin equipped with a nose cone. The mouse should be in anatomical position. Do not proceed until surgical plane is reached.
    2. Using scissors, make a sagittal cut through the skin of the abdomen. The cut should move upwards ending at the sternum (Note 3). Take special care to avoid cutting through the internal organs, particularly the liver.
    3. Make two transverse cuts at the base of the ribcage. Fold the skin of the abdomen to reveal the internal organs.
    4. Using the tip of the scissors, pierce the diaphragm. This creates a small opening where you can begin your cut through the diaphragm. Cut through the diaphragm to reveal the organs of the thoracic cavity.

5. Make two vertical cuts from the base of the ribcage to the collar. Clamp the sternum with a hemostat and lift the ribcage up to fully expose the organs of the thoracic cavity.
6. Using a pair of tweezers, lightly grasp the heart.
7. Pierce the apex of the heart with the blunted 25G butterfly needle. Position the needle in the left ventricle. The tip of the needle should be close to the ascending aorta.
8. Using a pair of iris scissors, cut the right auricle of the heart. Blood should immediately flood the thoracic cavity. Quickly turn on the perfusion pump.
9. Perfuse the mouse with ice-cold PBS at a rate of 4-10 mL/min until liver is bleached and perfusion twitches are observed (Notes 5 and 9).
10. Carefully remove the mouse brain and place it in a 15 mL Falcon tube containing 6 mL of D-PBS.

2. Microglia Isolation

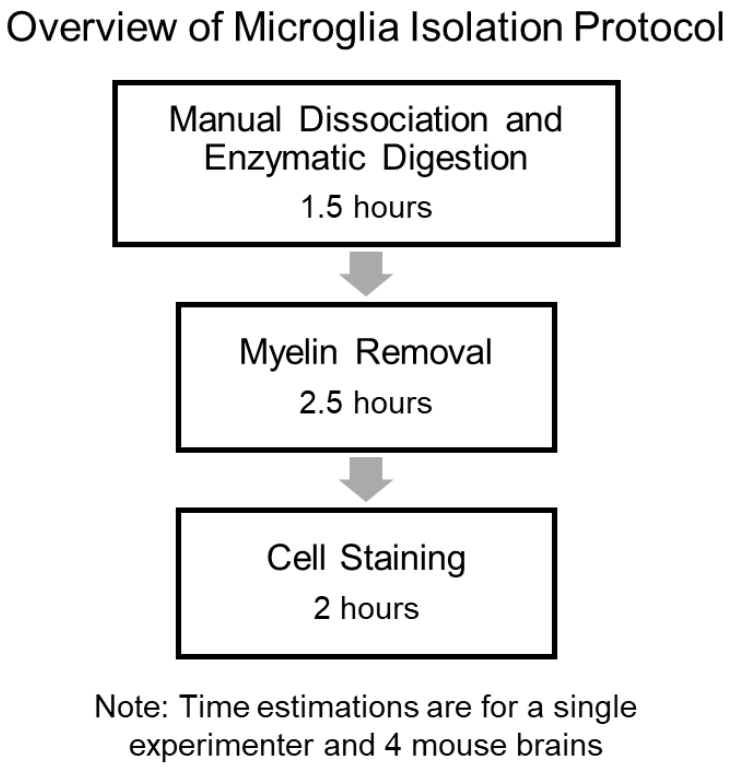

Figure 3.1: Microglia isolation protocol workflow and timeline.

2.1 Manual Dissociation of Brain Tissue and Enzymatic Digestion

1. Using a round-edge scalpel, cut the brain into fine pieces.
2. Remove the supernatant from the tube after tissue pieces have settled.
3. Add 1950 uL of enzyme mix 1 to the tube. Gently invert the tube to mix.
4. Incubate the tube for 15 min in a 37°C shaking water bath. Every 3-5 min, gently invert the tube to resuspend the pellet.
5. Add 30 uL of enzyme mix 2 to the tube.

6. Dissociate tissue pieces by passing the sample through a P1000 pipette tip several times. Stop once the tissue can easily pass through the pipette tip. Take special care to avoid forming air bubbles.
7. Incubate the tube for 10 min in a 37°C shaking water bath. Every 3-5 min, gently invert the tube to resuspend the pellet.
8. Dissociate tissue pieces by passing the sample through a P200 pipette tip several times. Stop once the tissue can easily pass through the pipette tip. Take special care to avoid forming air bubbles.
9. Centrifuge the sample at 300 x g for 10 min at RT. Carefully remove the supernatant.

2.2 Myelin Removal

1. Resuspend the pellet in 4 mL of 37% Percoll (RT). Make sure the pellet is fully resuspended before proceeding.
2. Using a glass pasture pipette, slowly underlay 4 mL of 70% Percoll (RT).
3. Using a plastic transfer pipette, slowly overlay 4 mL of 30% Percoll (RT), then slowly overlay 2 mL of 1 x HBSS.
4. Centrifuge the sample at 300 x g without break or acceleration for 40 min at RT.
5. Carefully collect the cell layer at the 70-37% Percoll interphase. This layer should amount to 2-2.5 mL.
6. Transfer the cell layer into a new 15 mL Falcon tube.
7. Wash the cells (500 x g for 7 min at 4°C) with 6 mL of 1 x HBSS. Resuspend the pellet in 500 uL of PBS.

*Optional: CD11b enrichment can be completed using the QuadroMACS system (Miltenyi Biotec, Germany). However, this step was found to be disadvantageous for flow cytometric analysis. Please see Note 13.

2.3 Cell Staining

1. Add 20 uL of the sample to 20 uL of Trypan blue dye. Transfer 10 uL of the mixture to a cell counter slide and count the cells using a Bio-Rad TC20 Automated Cell Counter.
2. Divide the sample into 1.5 mL Eppendorf tubes. Separate out cells for the dead cell and unstained controls. The remaining cells will be stained for cell surface markers.
3. Heat kill (heat at 70°C for 1 min) half of the cells separated out for the dead cell control. Place heat killed cells on ice for 1 min before mixing with the live cells of the dead cell control.
4. Add 1 uL of LIVE-DEAD dye to 1 mL of all samples (excluding the unstained control) and mix well.
5. Incubate the samples in the dark for 30 min on ice.
6. Wash the cells (1600 rpm for 5 min at 4°C) with 200uL of PBS. Resuspend in 100 uL of FACS buffer.
7. Incubate the samples with Fc Block (2uL/million cells) for 20 min on ice.
8. Incubate the samples with conjugated antibodies for CD11b (1:100), CD45 (1:100), and CD86 (3:100) for 20 min on ice.
9. Wash the cells (1600 rpm for 5 min at 4°C) with 200 uL of FACS buffer. Resuspend in 150 uL of FACS buffer and 50 uL of 4% PFA.

3. Flow Cytometry

3.1 Data Collection

Compensation beads should be used for single colour compensation controls in order to conserve cells. Run each cell sample on a LSRII Flow Cytometer (BD Biosciences, USA) until 100,000 live events have been collected.

3.2 Data Analysis

Analyze the flow cytometry data using FlowJo v10.6.2 (FlowJo, LCC, USA). The following gating strategy should be used. First, identify live singlet events from the leukocyte population (Fig. 3.2B-D). Then, identify microglia, macrophage, and lymphocyte events using a quadrant gate (Fig. 3.2A). Microglia events are defined as CD11b positive, CD45 low. Macrophage events are defined as CD11b positive, CD45 high. Lymphocyte events are defined as CD11b low, CD45 high. The activated microglia population is the subset of microglia events that are CD86 positive (Fig. 3.3). Calculate the microglia, macrophage, and lymphocyte cell population percentages as a percentage of live singlet events. Calculate the percentage of activated microglia as a percentage of all microglia events. Plot the data using GraphPad Prism 8 (GraphPad Software, Inc., USA) or a similar software of your choice.

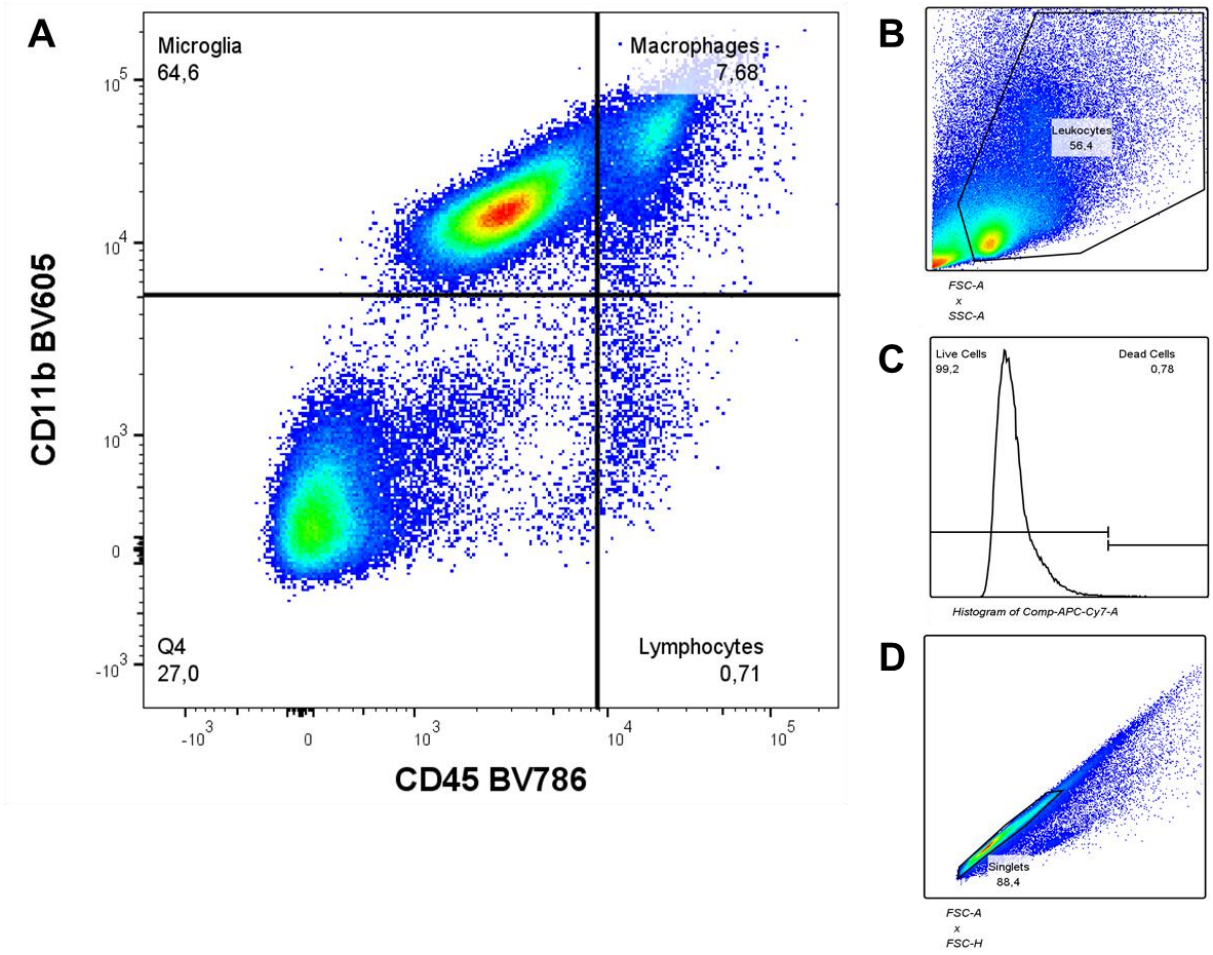

Figure 3.2: Flow cytometry gating strategy. (A) Quadrant gate for the identification of microglia, macrophages, and lymphocytes. (B) Leukocyte gate. (C) Gating for live leukocytes. (D) Gating for live singlet leukocytes.

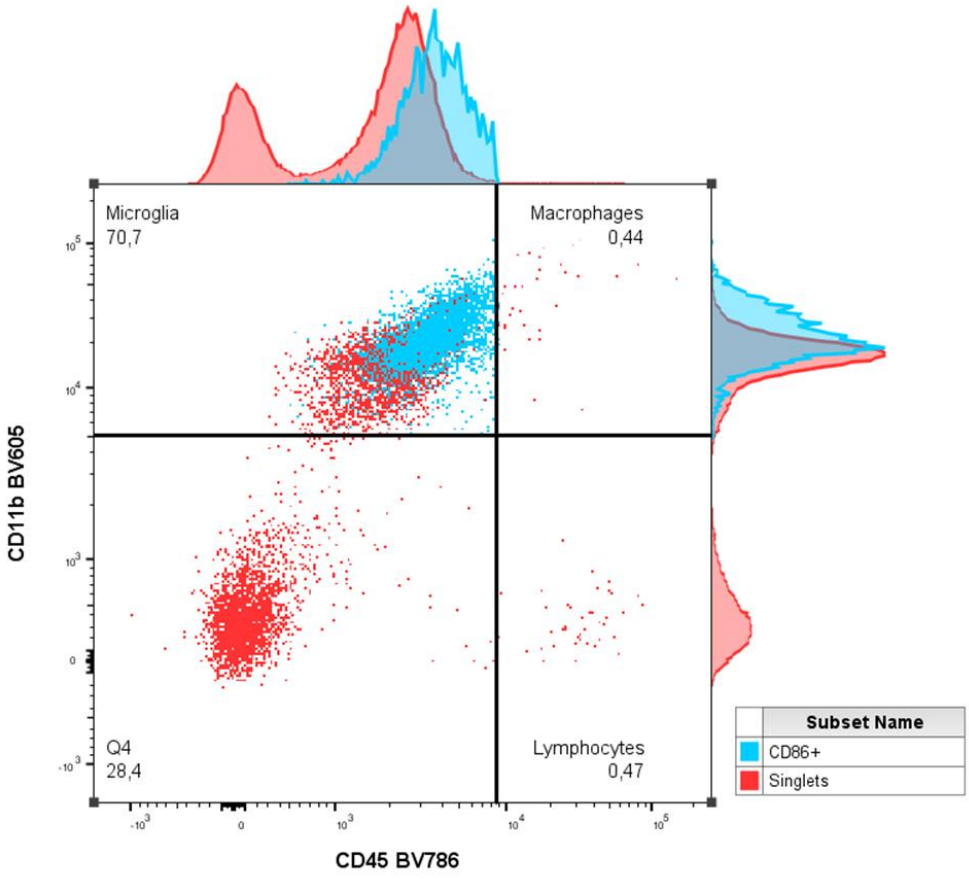

Figure 3.3: Activated CD86 positive microglia population.

Representative Experiment

Design:

Male GF mice (n=2 per group) were colonized with fecal microbiota from either a GAD patient (GAD32) or an age- and sex-matched healthy control (HC40). Mice were euthanized at 6 months old and their microglia were isolated for flow cytometric analysis.

Results:

Mice colonized with GAD32 microbiota displayed increases in percent microglia and infiltrating macrophages when compared to the control mice (Fig. 3.4A, C). However, decreases were seen

in percent activated microglia and percent infiltrating lymphocytes (Fig. 3.4B, C). No differences reached statistical significance.

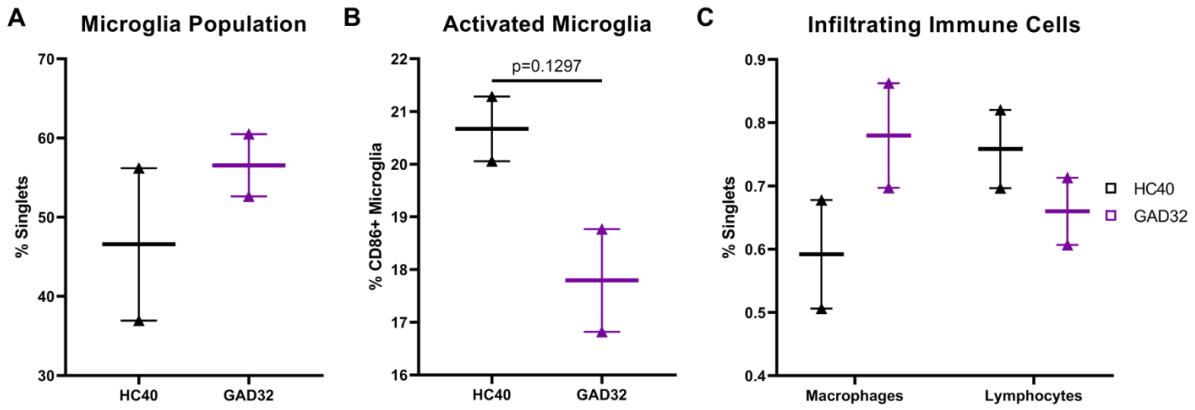

Figure 3.4: Brain immune cell profiles of mice colonized with GAD microbiota. (A) Trend for increased percent microglia seen in mice colonized with GAD32 microbiota compared to control mice. (B) GAD32-colonized mice had decreased percent CD86 positive microglia when compared to control mice. (C) Larger macrophage populations were seen in GAD32-colonized mice. Larger lymphocyte populations were seen in mice colonized with microbiota from HC40. Unpaired, two-tailed t-test. Data are standard boxplots with individual values.

Notes

General:

1. Work in a sterile environment.
2. All reagents should be ice-cold unless otherwise specified.
3. All buffers should not contain calcium, magnesium, or Phenol red.
4. Mouse age significantly affects isolation yield (Fig. 3.5). More microglia can be isolated from younger mice.

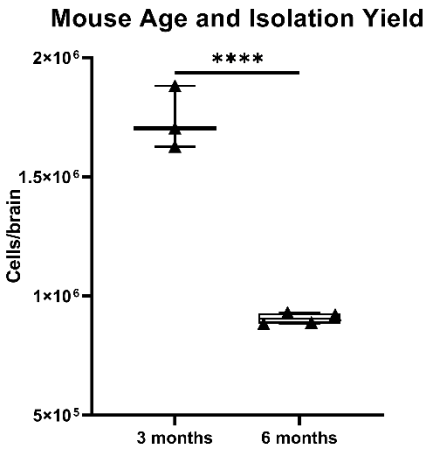

Figure 3.5: Mouse age and isolation yield. Significant decrease in cells isolated from mice at 6 months of age (n=4) compared to mice at 3 months of age (n=3). Data presented as standard boxplots with individual values. Unpaired, two-tailed t-test; ****$p<0.0001$.

Perfusion:

3. See Fig. 3.6 below for surgical plan.

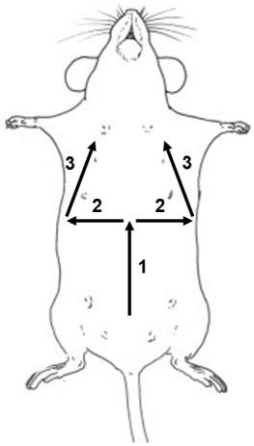

Figure 3.6: Perfusion surgical plan. Incisions are numbered in the order that they occur.

4. Isoflurane can be turned off once significant blood loss has occurred.

5. Test your perfusion pump to determine the flow rate.

6. To avoid swelling of the head and incomplete perfusion of the brain, you should not observe fluid coming out of the nose and the lungs should not inflate with fluid during the procedure.
7. If fluid is coming out of the nose of the mouse, retract the needle slightly and then point the tip of the needle towards the ascending aorta. Do not remove the needle completely from the heart.
8. If the lungs of the moue inflate, reposition the needle and cut the lungs to allow fluid to escape.
9. If liver bleaching is not observed within a few minutes, reposition the needle.

Microglia Isolation:

10. Keep all samples on ice in between steps.
11. To prepare FACS buffer, add 0.1 g BSA to 100 mL PBS. Filter sterilize and store at 4°C.
12. Percoll solutions should be made fresh. For 4 mouse brains, prepare a standard isotonic Percoll (SIP) solution (25.5 mL Percoll, 2.8 mL 10 x HBSS). Then, prepare 30% (6 mL SIP, 12 mL 1 x HBSS), 37% (7.4 mL SIP, 10.6 mL 1 x HBSS), and 70% (14 mL SIP, 4 mL 1 x HBSS) Percoll solutions.
13. CD11b enrichment results in samples with high purity (Fig. 3.7B). However, macrophages and lymphocytes are no longer present in the samples. Additionally, enrichment increases the isolation protocol length by approximately 2 hours and significantly decreases total cell yields (Fig. 3.7A). Low cell yields result in increases in flow cytometry data collection time. CD11b enrichment is suggested if isolated cells are to be used for gene expression analyses.

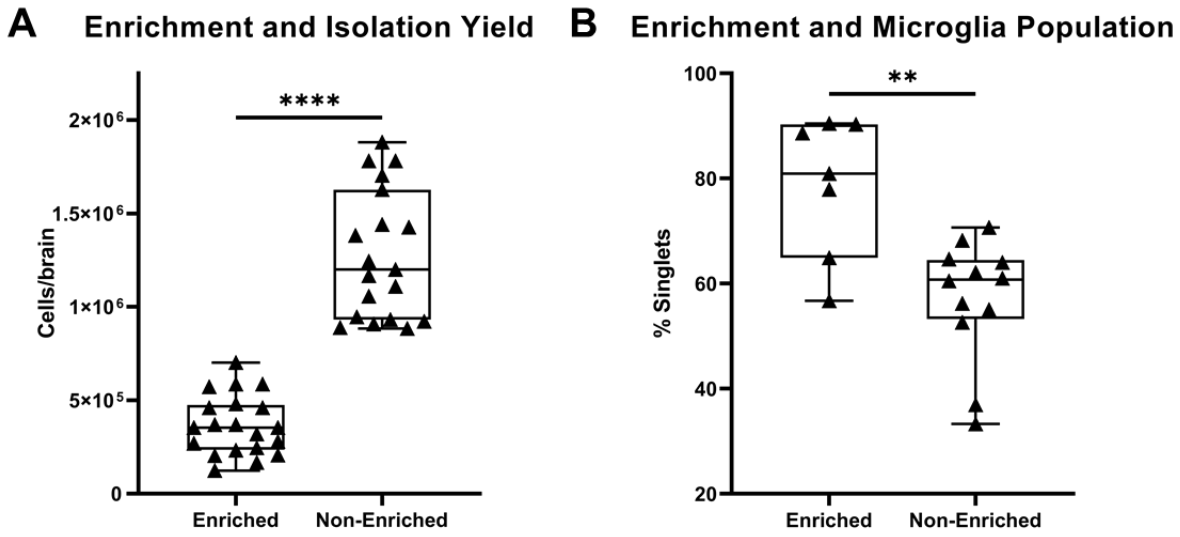

Figure 3.7: Enrichment, isolation yield, and sample purity. (A) Enrichment decreases cell isolation yield by nearly 3-fold. Enriched n=20. Non-enriched n=19. (B) Enrichment significantly increases sample purity. Enriched n=7. Non-enriched n=12. Data presented as standard boxplots with individual values. Unpaired, two-tailed t-test; ***p<0.01*, ****p<0.0001*. Data presented as standard boxplots with individual values.

CHAPTER 4: PROJECT 3

Introduction

The early life period represents an important window for the programming of health and disease in adulthood (Catalano & Ehrenberg, 2006; Edlow, 2017; Pantham, Aye, & Powell, 2015; Rivera, Christiansen, & Sullivan, 2015). There is evidence to suggest that early life programming may contribute to the development of depression and anxiety in adulthood. Clinical studies have revealed that adverse events during childhood, including sexual and physical abuse, are associated with increased risk of MDD and GAD later in life (Fava & Kendler, 2000; Safren, Gershuny, Marzol, Otto, & Pollack, 2002). Additionally, early life maternal separation is widely used to induce anxiety- and depressive-like behaviours in adult mice (De Palma et al., 2015). Interestingly, mice exposed to maternal separation display altered intestinal microbiota in adulthood, suggesting that early life exposure to stress can shape the composition of the microbiota (De Palma et al., 2015). Furthermore, our group was the first to report that the maternal separation model of depression is dependent on colonization (De Palma et al., 2015). GF mice exposed to maternal separation did not display anxiety- or depressive-like behaviours in adulthood (De Palma et al., 2015). However, anxiety- and depressive-like behaviours could be induced in GF mice exposed to maternal separation with colonization with SPF microbiota in adulthood (De Palma et al., 2015).

The early life period also marks the time point of colonization (Gensollen et al., 2016). Preclinical studies have demonstrated that colonization shapes both host physiology and behaviour (Abrams & Bishop, 1967; Bauer, Horowitz, Levenson, & Popper, 1963; Erny et al., 2015; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011; Olszak et al., 2012; Thion et al., 2018) . In addition, the influence of colonization on the development of the immune system is of particular interest and has been extensively reviewed (Gensollen et al., 2016;

Macpherson & Harris, 2004; Romano-Keeler & Weitkamp, 2015). To summarize, GF mice display numerous immune abnormalities when compared to their SPF counterparts; although many of these immune abnormalities can be normalized with colonization at any age, others appear to have limited plasticity (Gensollen et al., 2016; Macpherson & Harris, 2004). For example, early life colonization is important for the proper programming of colonic invariant natural killer T (iNKT) cells (Olszak et al., 2012). In GF mice, iNKT cells accumulate in the lamina propria of the colon, resulting in increased susceptibility to oxazolone-induce colitis (Olszak et al., 2012). Colonization during the neonatal period but not adulthood can normalize colonic iNKT cells and reduce susceptibility to oxazolone-induce colitis (Olszak et al., 2012).

In conclusion, the early life period is an important time point for the development of MDD risk, the intestinal microbiota, and the immune system. Given the suggested roles of both the intestinal microbiota and immune system in MDD, we hypothesize that the early life period represents an important window for the programming of MDD risk through the intestinal microbiota and the immune system. We sought to investigate this hypothesis by comparing early life and adulthood colonization in our humanized mouse model of depression.

Methods

Animals

Adult (7-16 weeks old) GF NIH Swiss mice of both sexes were obtained from the Farncombe Family Axenic-Gnotobiotic Facility. Once colonized, all mice were housed in a Level 1 unit at the Central Animal Facility. Mice were exposed to a 12-hour light/dark cycle and given access to food (Teklad 7904 diet; Envigo, USA) and water *ad libitum*. All animal experiments were

performed in accordance with the McMaster Animal Care Committee guidelines and were approved by McMaster's Animal Research Ethics Board.

Human Subjects

All human microbiota donors were participants in a clinical study led by Dr. Rebecca Anglin. One MDD patient (MDD5) and one age- and sex-matched healthy control (HC5) were selected as microbiota donors. MDD5 was chosen based on their Depression Anxiety Stress Scale 21 (DASS21) scores, Gastrointestinal Symptom Rating Scale (GSRS) score, and fecal β-defensin 2 levels (Table 4.1). Fecal β-defensin 2 is an overt marker of immune activation (Langhorst et al., 2009). MDD5 has a high fecal β-defensin 2 level (≥75 ng/g of feces). MDD5 has the psychiatric co-morbidities GAD and obsessive-compulsive disorder. MDD5 also suffers from obesity. MDD5 was taking antidepressants at the time of the study.

Table 4.1: MDD5 and HC5 Donor Characteristics

Donor	Sex	Age	BMI	DASS21 Depression Score	GSRS Sum Score	Fecal β-Def 2 (High≥75ng/g feces)	Medications (1=antidepressants)	Comorbidities (GAD= generalized anxiety disorder, OCD=obsessive compulsive disorder)
MDD5	M	41	39.2	40 (Extremely severe)	27	132.81	1	GAD, OCD
HC5	M	39	21.2	0 (None)	0	12.28	None	None

Study Design

To investigate the effects of long-term colonization in adulthood on mouse behaviour, adult GF mice (n=7 per group) were colonized with 0.2 mL of fecal dilution (10% w/v in anerobic saline) from either MDD5 or HC5 via oral gavage (Fig. 4.1). Mouse behaviour was assessed at two time points during adulthood: T1 at 3 weeks post colonization and T2 at 8 weeks post-colonization. Fecal samples were collected at T1 for 16S rRNA gene sequencing.

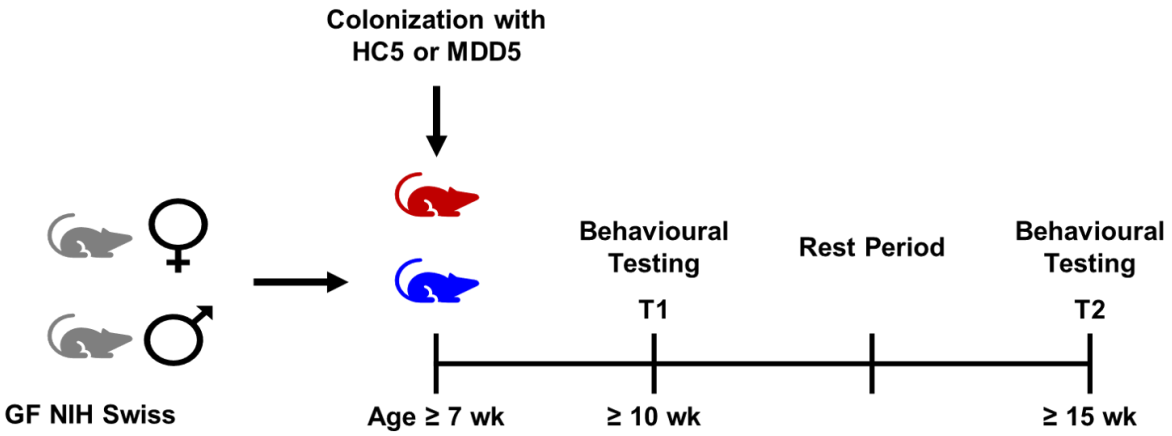

Figure 4.1: Experiment timeline for all mice colonized in adulthood (HC5 and MDD5).

To investigate the effects of early life colonization on behaviour, a GF breeding pair was colonized with 0.2 mL of MDD5 fecal dilution (Fig. 4.2). Offspring born to the colonized breeders were weaned at 3 weeks (T1). At weaning, offspring were gavage with 0.1 mL of MDD5 fecal dilution. Offspring underwent behavioural testing at 9 weeks of age (T3). The microglia of offspring were isolated at 12 weeks of age (T4) to investigate the effects of early life colonization on neuroinflammation. Offspring microglia were compared to those of age-matched GF control mice. Lastly, offspring fecal samples were collected at T1-T3 and cecal content was collected at T4 for 16S sequencing. Fecal samples were also collected from MDD5-colonized breeders at T1 and T2.

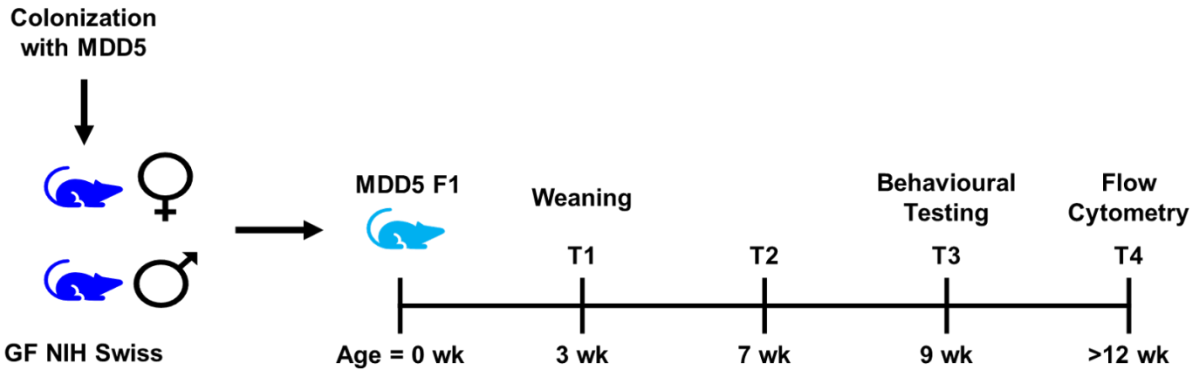

Figure 4.2: Experiment timeline for all MDD5 offspring (MDD5 F1).

Behavioural Testing

Mouse behaviour was assessed using the open field test, three chamber sociability assay, tail suspension test, and sucrose preference test. Tests were performed as described in Project 1.

Integrative Behavioural Z-Scoring

Emotionality z-scores were calculated as described previously (Guilloux et al., 2011).

Microglia Isolation and Flow Cytometry

Mice were anesthetized with isoflurane and transcardially perfused with PBS. Perfused brains were carefully removed. Brain tissue was digested using the Neural Tissue Dissociation Kit (Miltenyi Biotec, Germany; No. 130-092-628). Following this, myelin was removed using a three-phase Percoll gradient. Dead microglia were stained using LIVE/DEAD Near-IR Stain (Thermo Fisher Scientific, USA; No. L10119). To control for unspecific Fc receptor binding, microglia were incubated with Mouse BD Fc Block (BD Biosciences, USA; No. 553141). Microglia were stained with 1:100 CD11b BV605 (BD Biosciences, USA; No. 563015), 1:100 CD45 BV786 (BD Biosciences, USA; No. 564225), and 1:300 CD86 BV421 (BD Biosciences,

USA; No. 562432). For each sample, 100,000 live events were acquired using an LSR II Flow Cytometer (BD Biosciences, USA). Flow cytometry data were analyzed using FlowJo v10.6.2 (FlowJo, LCC, USA).

16S rRNA Gene Sequencing

As mentioned in Project 1, 16S rRNA gene sequencing of the V3 region was performed by the Surette Laboratory at McMaster University.

Statistical Analyses

Statistical analyses of behavioural and flow cytometry data were completed using GraphPad Prism 8 (GraphPad Software, Inc., USA). Outliers were removed using Grubb's test and normality was assessed using the Shapiro-Wilk test. Normally distributed data were analyzed using a Student's t-test or one-way ANOVA or three-way ANOVA followed by Tukey's post hoc test. Paired statistical analyses were completed for measures repeated over time. Further, all microbiota data were analyzed using nonparametric tests as described previously in Project 1. For microbial differences, FDR adjusted p-values <0.05 were considered statistically significant. For all other analyses, p-values <0.05 were considered statistically significant. Figures were generated using GraphPad Prism, FlowJo, Microsoft Excel 2004, or Emperor.

Results

i. Colonization with MDD5 microbiota in adulthood induced anxiety-like behaviour in recipient mice at the early time point

At T1, microbiota from donor MDD5 induced anxiety-like behaviour, with MDD5 mice spending less time in the center of the open field compared to HC5 mice (Fig. 4.3A). MDD5 microbiota did not induce depressive-like behaviour in the recipient mice as no differences

between the MDD5 and HC5 groups were seen in the sucrose preference test, sociability assay, or tail suspension test (Fig. 4.3B-D). We also observed no differences in emotionality z-scores between the two groups at T1 (Fig. 4.3E).

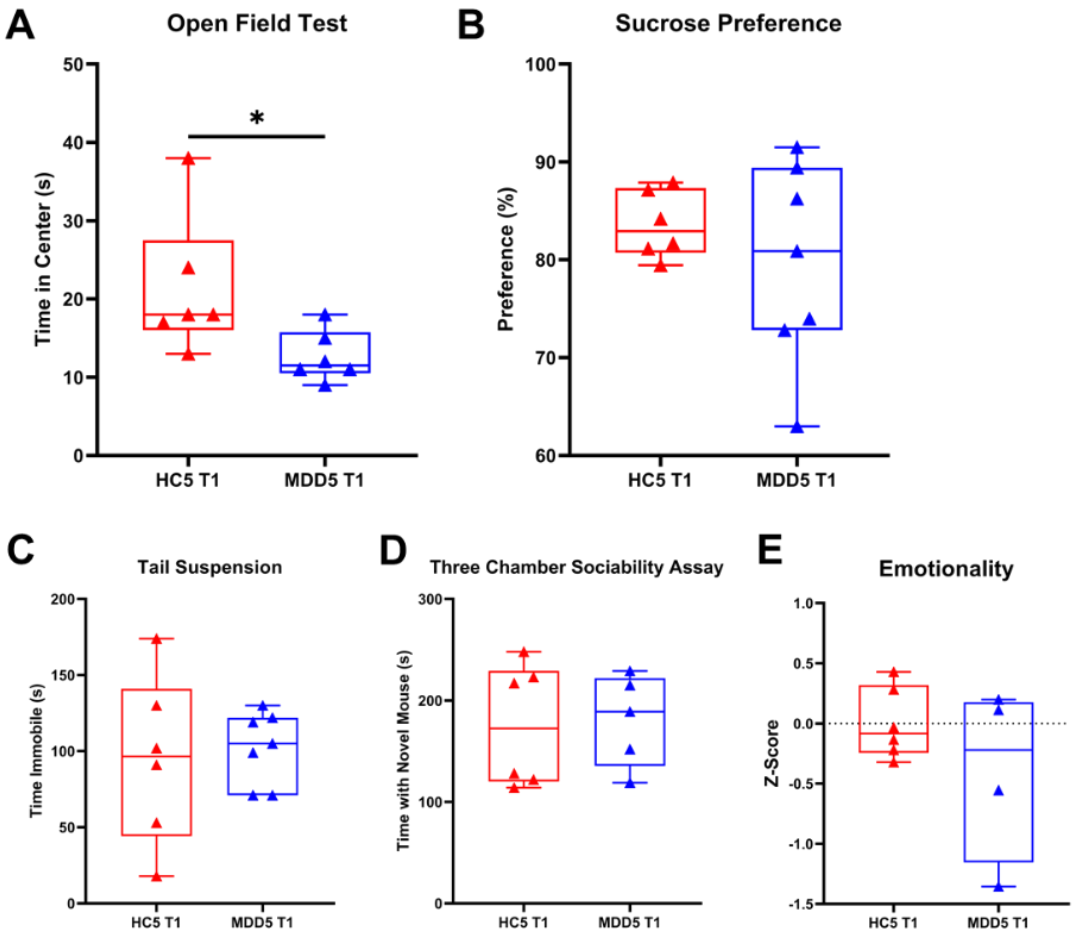

Figure 4.3: Effect of MDD5 microbiota at on mouse behaviour at T1. (A) Mice colonized with MDD5 microbiota spent less time in the center of the open field when compared to HC5-colonized mice. Behaviour of the groups did not differ in (B) the sucrose preference test, (C) tail suspension test, or (D) three chamber sociability assay. (E) MDD5-colonized mice exhibited a trend for decreased emotionality z-scores when compared to HC5 mice. Data presented as standard box plots with individual values. Unpaired, two-tailed t-test; *$p<0.05$.

ii. Behavioural outcomes were dependent on time point and mouse sex

In mice colonized in adulthood, the time point of behavioural testing and the microbiota donor were significant sources of variation in the open field test data (Table 4.2). In the open field test, significant interactions between time point and mouse sex, as well as microbiota donor, time point, and mouse sex were also observed (Table 4.2). Furthermore, an increase in the time spent in the centre of the open field was observed across time for HC5 females but not males (Fig. 4.4A). At T2, HC5 males and MDD5 females spent less time in the center of the open field when compared to HC5 female mice (Fig. 4.4A). In the sucrose preference test, time point was found to be the greatest source of variation in outcomes, with the sucrose preference of all mice decreasing over time (Table 4.2; Fig. 4.4B). In the tail suspension test, we observed no differences across time (Fig. 4.4C). Mouse sex was the greatest source of variation in this test, and male mice spent less time immobile when compared to female mice (Table 4.2; Fig. 4.4C). Moreover, there were no differences between any of the variables in the three chamber sociability assay (Fig. 4.4D). Nearly 60% of the variation in time spent with the novel mouse was attributed to the individual subject mouse. Lastly, emotionality z-scores for all mice significantly decreased across time (Table 4.2; Fig. 4.4E).

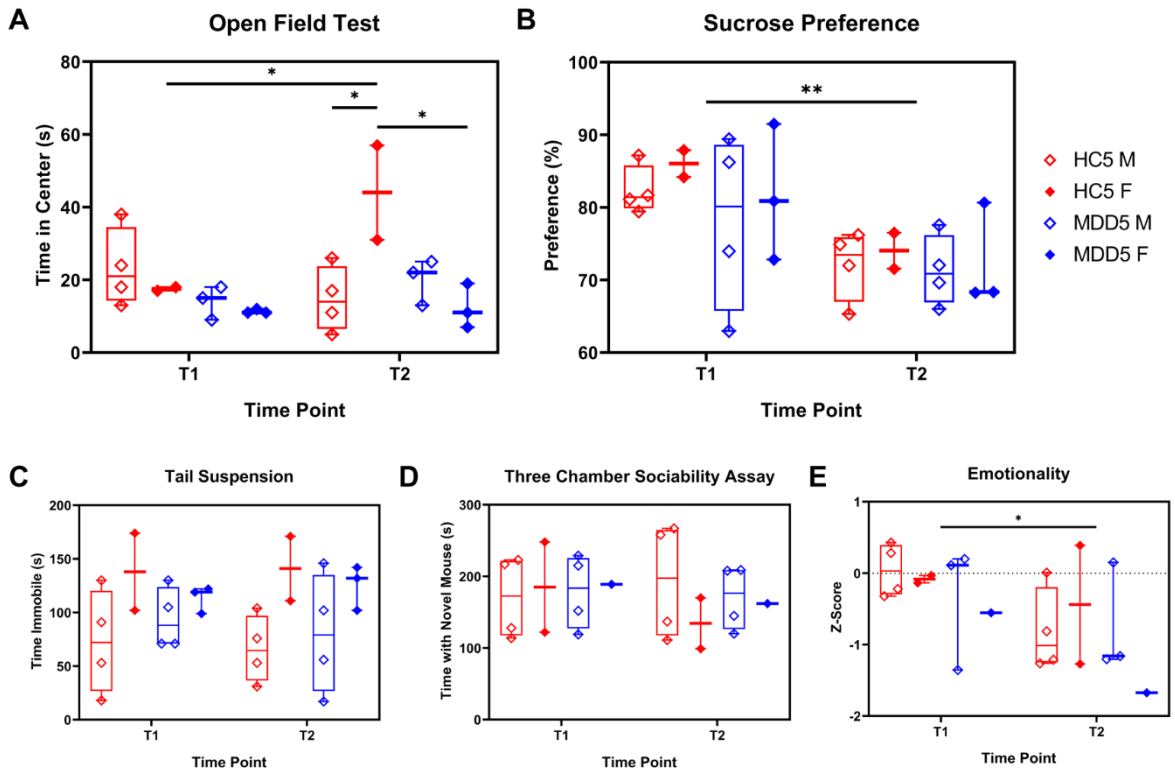

Figure 4.4: Sex-specific effects of MDD5 microbiota on mouse behaviour across time. (A) HC5 females spent significantly less time in the center of the open field across time. At T2, HC5 males and MDD5 females displayed increased anxiety-like behaviour when compared to HC5 females. Three-way ANOVA and Tukey's post hoc test; *$p<0.05$. (B) A significant decrease in percent sucrose preference was observed across time. Three-way ANOVA; **$p<0.01$. (C) Male mice spent significantly more time immobile than female mice at both time points. Three-way ANOVA; $p=0.0158$. (D) No differences were seen between the donor groups, sexes, or time points in the sociability assay. (E) Emotionality z-scores of all mice significantly decreased across time. Three-way ANOVA; *$p<0.05$. All data presented as standard box plots with individual values.

Table 4.2: MDD5 Time- and Sex-Stratified Analyses (Three-way ANOVAs)

Source of Variation	Percent of Variation	P-value
Time in Center of Open Field		
Time	7.582	0.0231
Donor	21.23	0.0472
Time x Mouse Sex	10.92	0.0099
Time x Donor x Mouse Sex	19.41	0.0020
Time with Novel Mouse		
No significant sources of variation		
Time Immobile		
Mouse Sex	34.65	0.0158
Percent Sucrose Preference		
Time	35.28	0.0026
Emotionality		
Time	19.64	0.0454

iii. MDD5 offspring displayed more severe anxiety-like behaviour

Compared to HC5 mice, mice colonized in early life with MDD5 microbiota (MDD5 F1) displayed more severe anxiety-like behaviour than mice colonized in adulthood (MDD5). MDD5 F1 mice spent less time in the center of the open field when compared to mice colonized with microbiota from HC5 (Fig. 4.5A). There was no significant difference between the MDD5 and HC5 groups; however, there was a decreasing trend (*p=0.0662*; Fig. 4.5A). Neither MDD5 F1 nor MDD5 mice displayed depressive-like behaviour as we observed no differences between any of the groups in the sucrose preference test, tail suspension test, or sociability assay (Fig. 4.5B, C). Lastly, no differences were observed in emotionality (Fig. 4.5D).

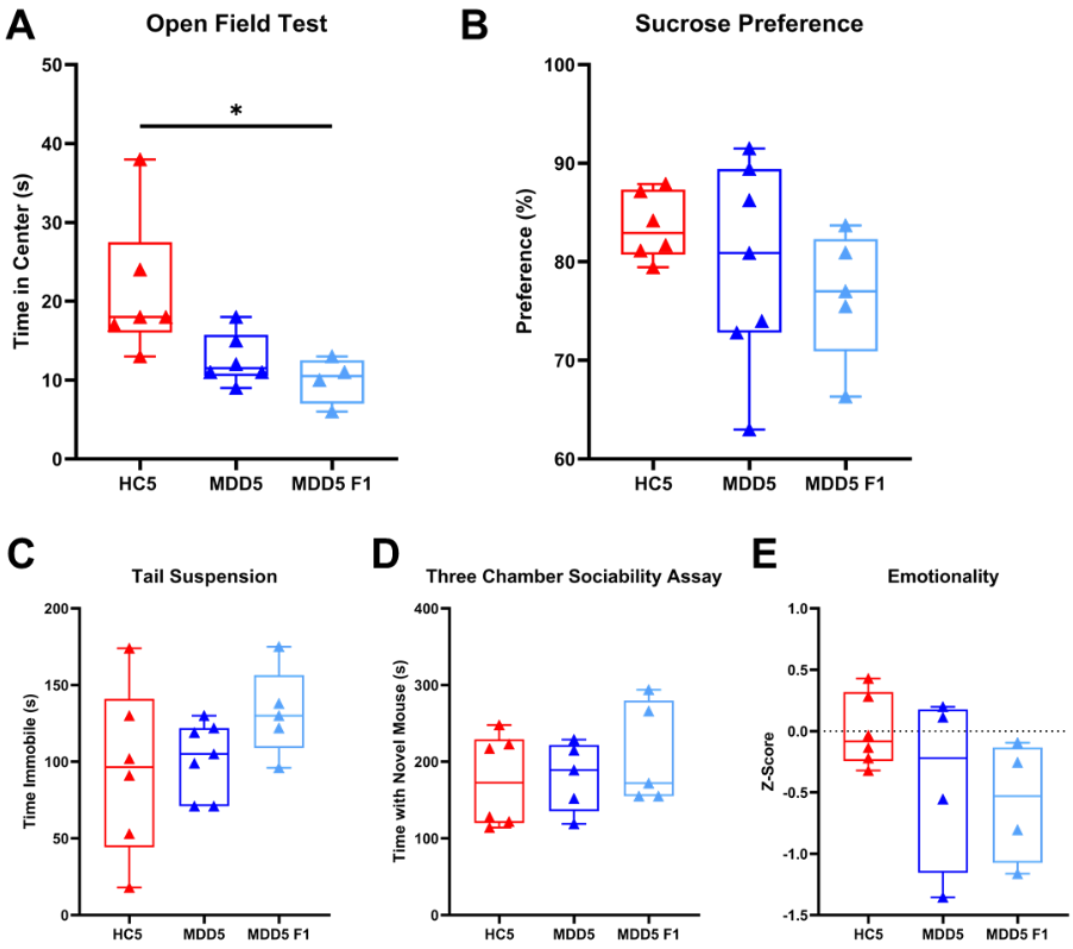

Figure 4.5: Behavioural outcomes in adult mice colonized with MDD5 microbiota in early life verses adulthood. (A) Offspring born to MDD5-colonized parents (MDD5 F1) spent less time in the center of the open field when compared to HC5 mice. A trend was found among the two MDD5 groups, with early life colonization associated with less time spent in the center. (B) Percent sucrose preference followed a decreasing trend across the three colonization groups, with the MDD5 F1 group displaying the lowest preference. (C) An increasing trend was observed among the three colonization groups in the tail suspension test. MDD5 F1 mice spent more time immobile when compared to the HC5 and MDD5 groups. (D) No differences were seen among the three groups in the sociability assay. (E) A decreasing trend in emotionality z-scores was seen across the three colonization groups. HC5 mice had the highest z-scores when compared to MDD5 and MDD5 F1 mice. Data presented as standard box plots with individual values. One-way ANOVA and Tukey's post hoc test; *$p<0.05$.

iv. Increased Cyanobacteria was predictive of decreased anxiety-like behaviour in mice

With microbiota transfer to mice, we observed expansion of the phyla Bacteroidetes and Verrucomicrobia (Fig. 4.6A). In MDD5 offspring, the relative abundances of all phyla except Bacteroidetes fluctuated over time (Fig. 4.7). The greatest fluctuations appeared over the course of the behavioural testing period, which was between T3 and T4 (Fig. 4.7). For this reason, all microbiota analyses between the HC5, MDD5, and MDD5 F1 groups were completed using data from the time of the open field test on the first day of behavioural testing.

The phylum Cyanobacteria was present in the microbiota of donor HC5 but not MDD5. Accordingly, the relative abundance of Cyanobacteria was significantly greater in the microbiota of HC5 mice when compared to the microbiota of MDD5 and MDD5 F1 mice (Fig. 4.6C). Furthermore, the relative abundance of Cyanobacteria was also found to be predictive of increased time in the center of the open field which represents anxiolytic behaviour in mice (Fig. 4.6B). The relative abundance of Actinobacteria was greater in the microbiota of donor MDD5 when compared to that of donor HC5; and in recipient mice, we observed a significant increase in Actinobacteria in MDD5 mice when compared to HC5 and MDD5 F1 mice (Fig. 4.6C). Although colonized with microbiota from donor MDD5, we did not observe increased Actinobacteria in MDD5 F1 mice (Fig. 4.6C). Lastly, no significant sex differences in phyla were found between the three colonization groups.

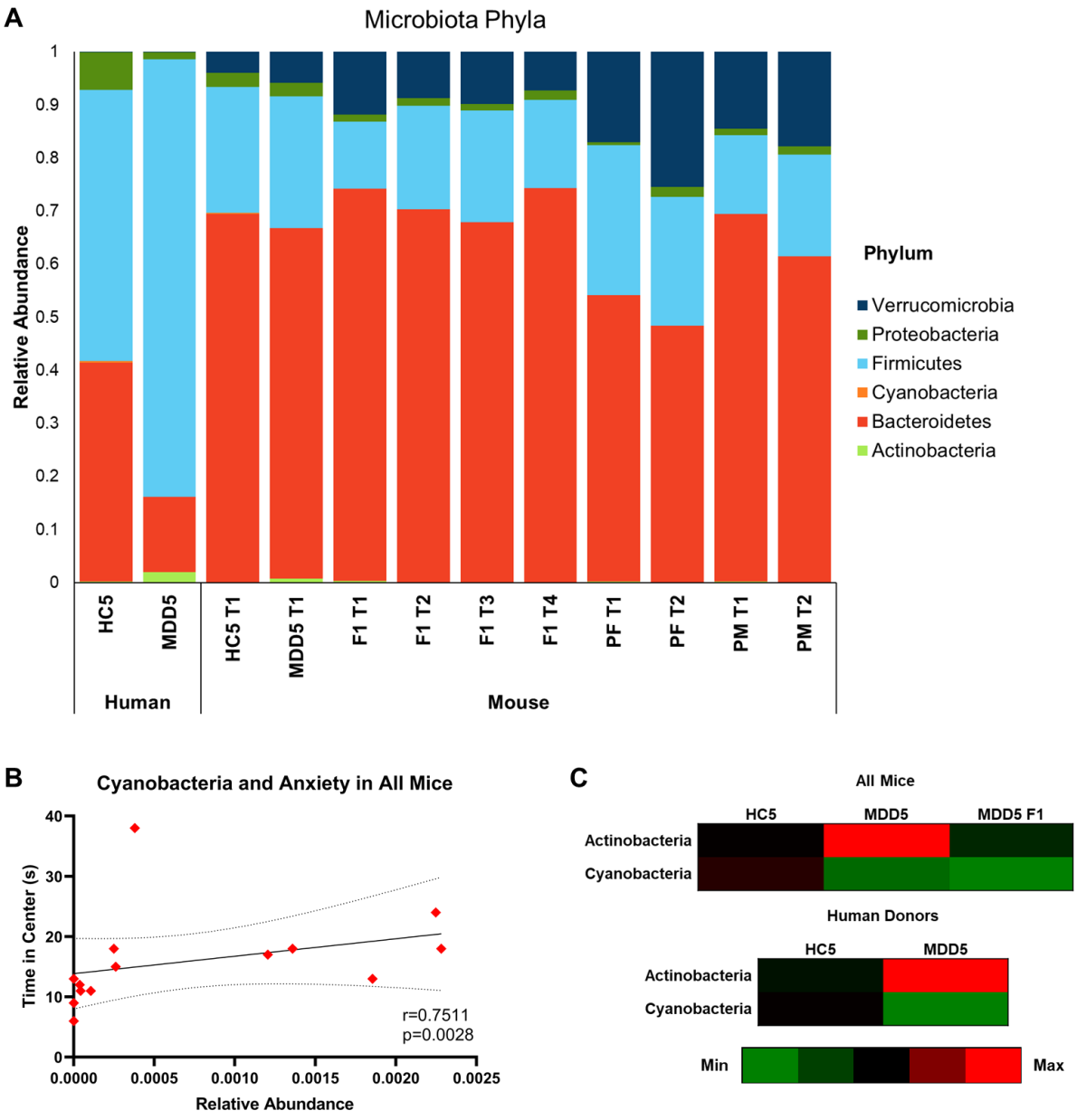

Figure 4.6: Phylum level differences among human donors and recipient mice. (A) Human donor and recipient mouse microbiota composition at phylum level. Single time point shown for mice colonized with HC5 (n=6) or MDD5 (n=7) in adulthood. Four time points shown for early life colonization study. MDD5 PF and MDD5 PM represent MDD5-colonized female parent mouse and male parent mouse, respectively. MDD5 F1 represents MDD5 offspring (n=3). (B) The relative abundance of Cyanobacteria positively correlated with time in center of the open field in all mice (n=14). (C) Significant phylum level differences in microbiota of all mice and corresponding trends in species of donor microbiota. Kruskal-Wallis test; $p<0.05$ FDR adjusted.

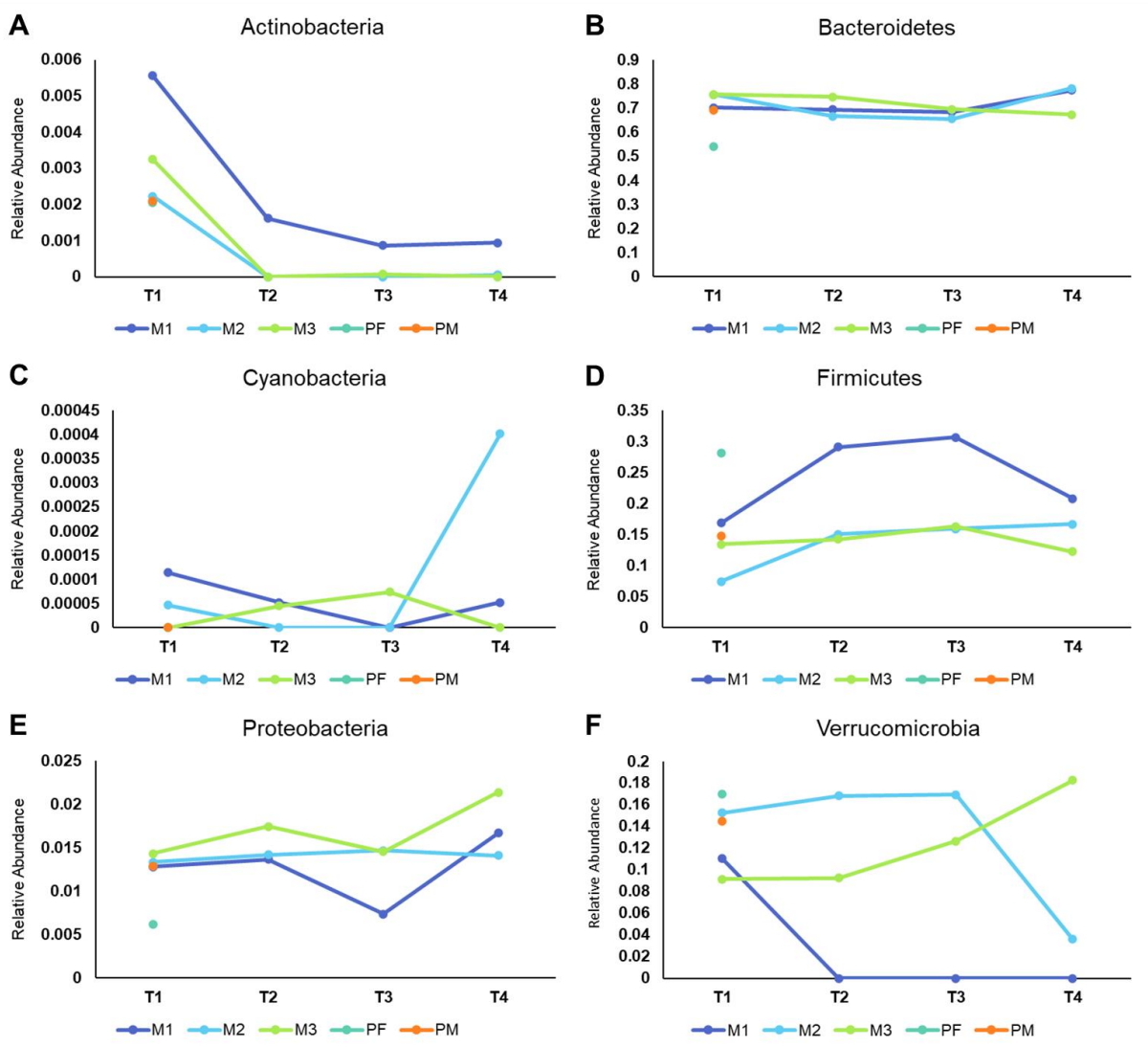

Figure 4.7: Relative abundances of (A) Actinobacteria, (B) Bacteroidetes, (C) Cyanobacteria, (D) Firmicutes, (E) Proteobacteria, and (F) Verrucomicrobia over time in MDD5 P and F1 mice. PF and PM represent female parent mouse and male parent mouse, respectively. M1-3 represent MDD5 offspring. M2 and M3 are both females and were housed together after weaning. Weaning was at T1, the open field test was at T3, and endpoint was at T4.

v. MDD5 offspring had distinct microbiota composition

In the PCoA plot of Jaccard beta diversity, we observed distinct clustering of the microbiota of MDD5 breeders (MDD5 P) and offspring (MDD5 F1) when compared to the microbiota of the

HC5 and MDD5 groups (Fig. 4.8A). The HC5 and MDD5 groups clustered close to their respective donors (Fig. 4.8A). Significant differences in Jaccard beta diversity were found between all groups (Fig. 4.8B). Similar differences were observed for the Bray-Curtis beta diversity metric; however, the Bray-Curtis beta diversity of MDD5 F1 and MDD5 mouse microbiota did not differ significantly (Fig. 4.8C). Furthermore, we observed a significant difference in alpha diversity between HC5 and MDD5 F1 mice (Fig. 4.9B). No differences in phylogenetic diversity were found in this study (Fig. 4.9A).

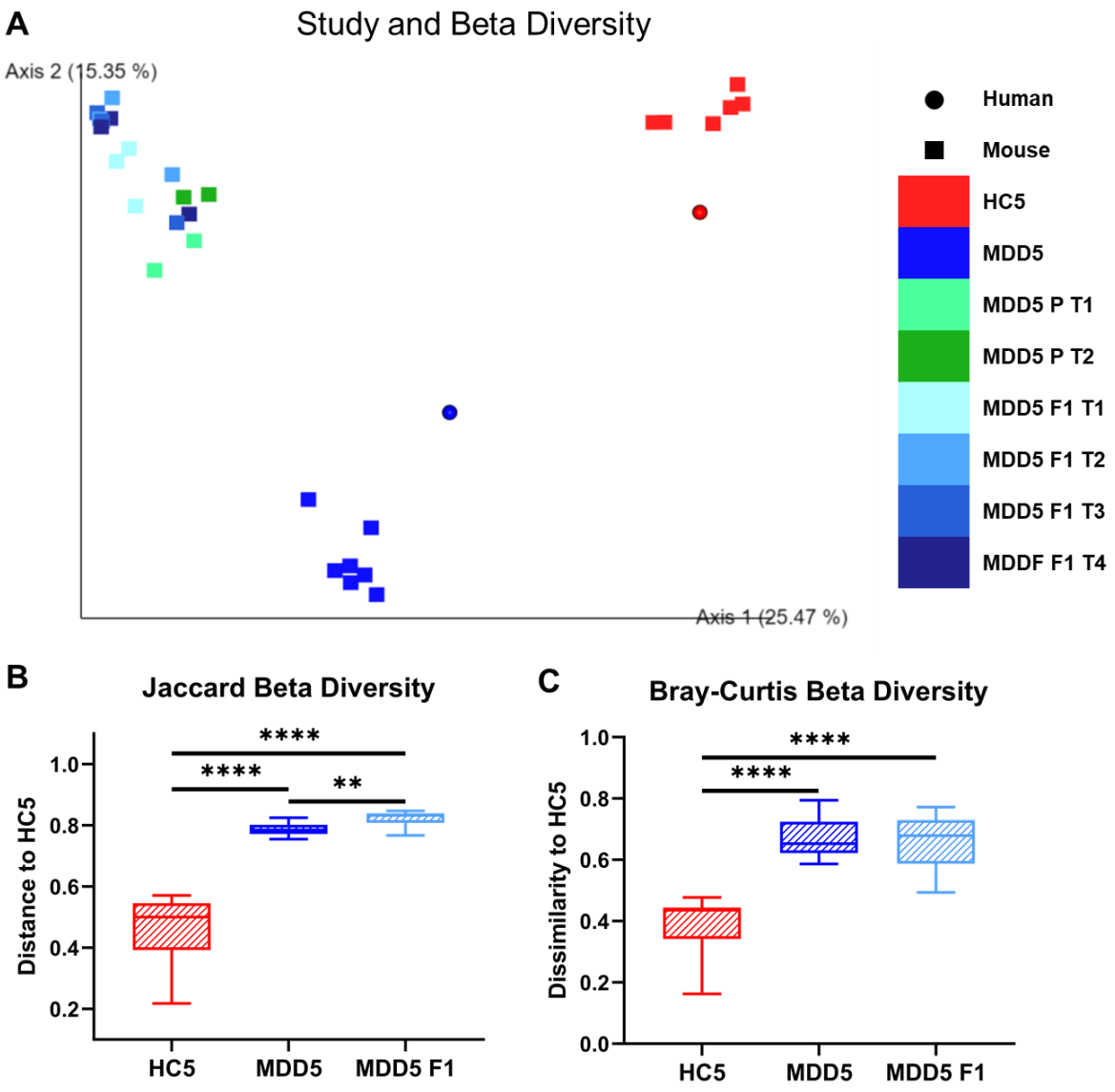

Figure 4.8: Beta diversity of mouse microbiota clustered by study group. (A) PCoA plot of Jaccard beta diversity for human donor and recipient mouse microbiota. Four time points shown for early life colonization study. MDD5 P represents parent mouse colonized with MDD5 microbiota (n=2). MDD5 F1 represents MDD5 offspring (n=3). (B) Jaccard distances at time of open field test. Data presented as standard boxplots. Kruskal-Wallis test and Dunn's multiple comparisons test; ***p<0.01*, *****p<0.0001*. (C) Bray-Curtis dissimilarities at time of open field test. Data presented as standard boxplots. Kruskal-Wallis test and Dunn's multiple comparisons test; ******p<0.0001*.

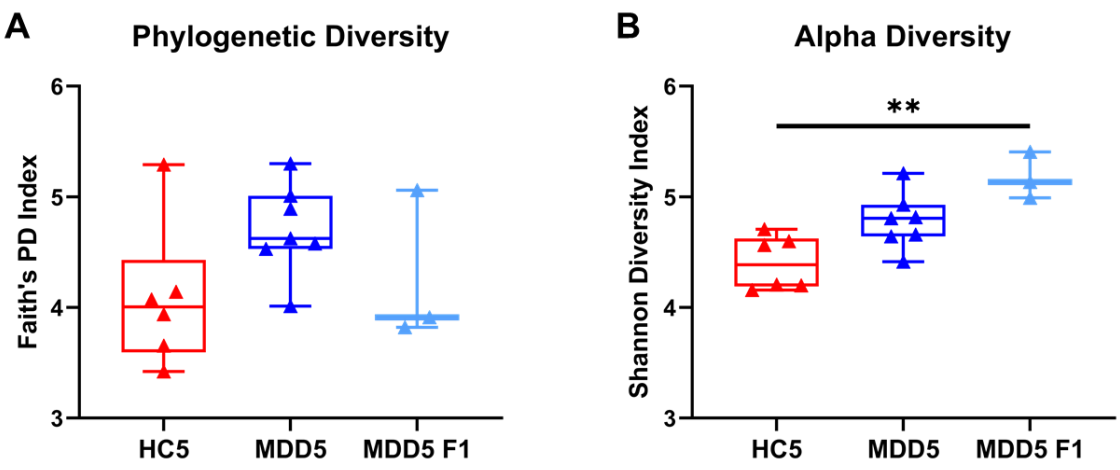

Figure 4.9: Alpha diversity of MDD5 F1 mouse microbiota was significantly increased when compared to that of HC5 mice. (A) Phylogenetic diversity and (B) alpha diversity at time of open field test. Data presented at standard boxplots with individual values. Kruskal-Wallis test and Dunn's multiple comparisons test; ***p<0.01*.

vi. Microbial drivers of anxiety-like behaviour in mice were sex-specific

The relative abundance of 16 species, including unclassified species of *Blautia* and *Paraprevotella*, were found to be different between the HC5 and MDD5 groups (Fig. 4.10). Fewer species level differences were found between the HC5 and MDD5 F1 groups and between the MDD5 and MDD5 F1 groups (Table C.4 in Appendix C). MDD5 F1 mouse microbiota was characterized by increases in unclassified *Holdemania* and *Turicibacter* species and lower relative abundance of an unclassified species of *Barnesiellaceae* (Fig. 4.10). Lastly, not all microbial features in recipient mice were detected in the human donors. For example, increased relative abundance of *Bacteroides acidifaciens* was found in MDD5 mouse microbiota. However, this species was not detected in either of the donor samples (Fig. 4.10).

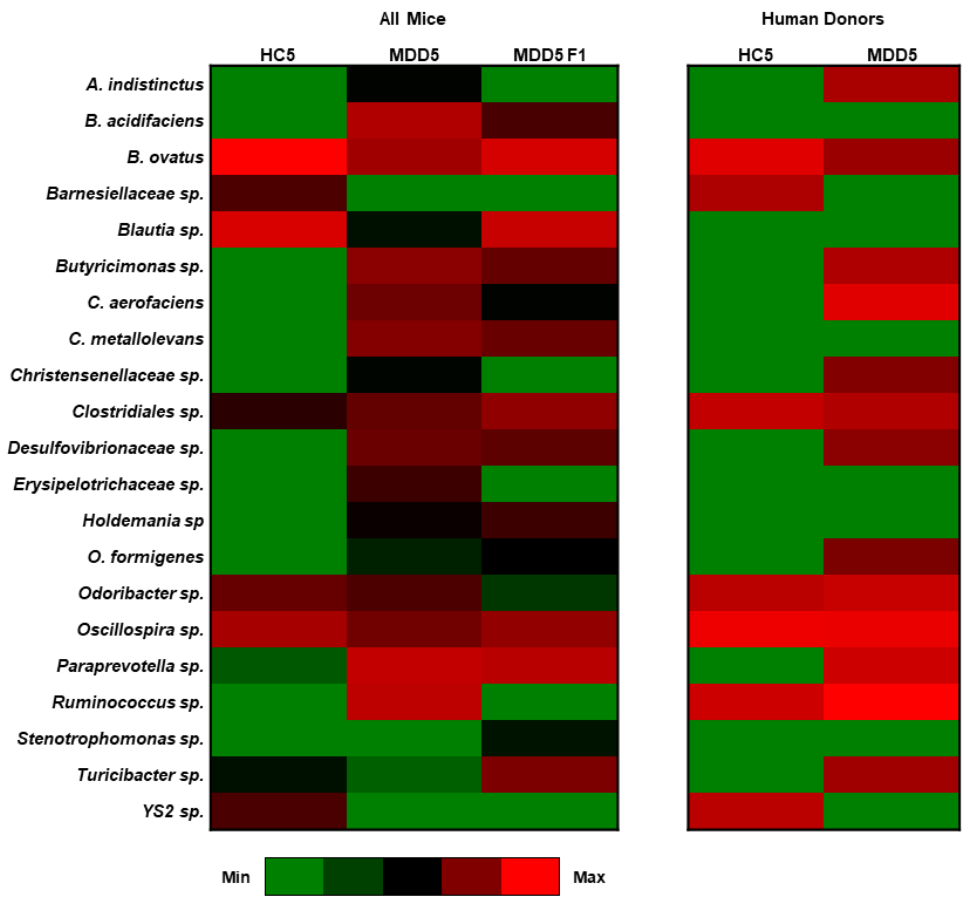

Figure 4.10: Significant species level differences in microbiota of all mice and corresponding trends in species of donor microbiota. Data presented at log-transformed mean relative abundance. Kruskal-Wallis test; $p<0.05$ FDR adjusted.

In all mice, several species correlated with anxiety-like behaviour in the open field test. Increased relative abundance of unclassified *Blautia* and *Ruminococcus* species were found to be predictive of decreased anxiety-like behaviour in mice (Fig. 4.11A). After sex-stratified analyses, these correlations remained significant in male but not female mice (Fig. 4.11B). Additionally, the relative abundances of *Oxalobacter formigenes* and an unclassified species of *Holdemania* positively correlated with anxiety-like behaviour in all mice (Fig. 4.11A). Further analyses revealed that the correlation with *O. formigenes* was driven by female mice (Fig. 4.11B).

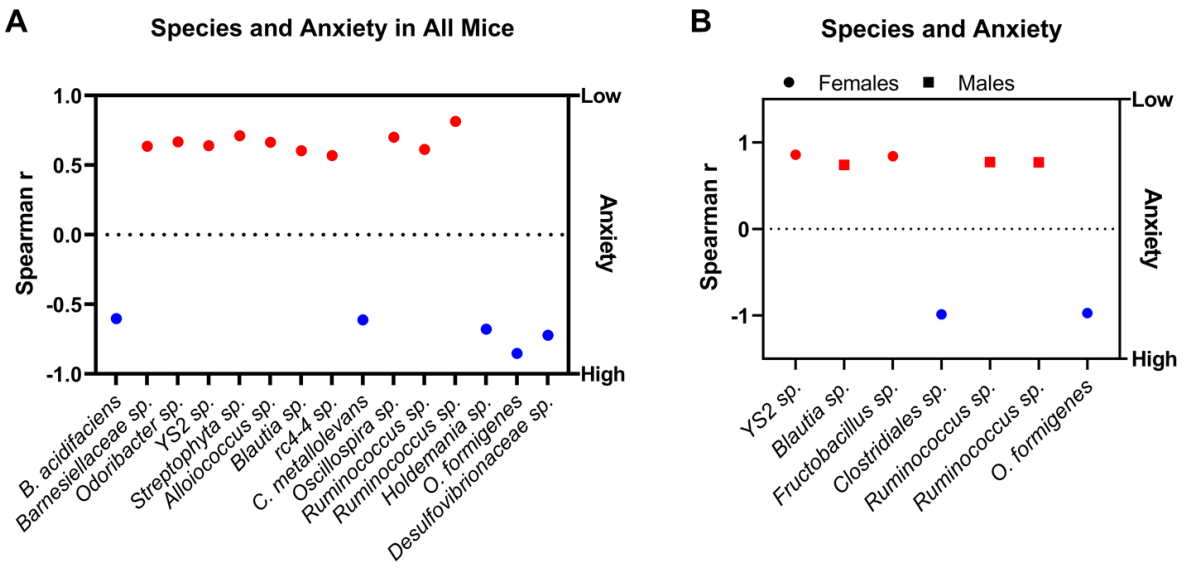

Figure 4.11: Significant species level correlations with time in the center of the open field in (A) all mice (n=14) and (B) females (n=6) and males (n=8).

vii. More data is needed to determine the effects of MDD5 microbiota on neuroinflammation

No significant differences were observed in the percentages of microglia, macrophages, lymphocytes, or CD86 positive microglia in the brains of MDD5 F1 mice when compared to those of age-matched GF controls (Fig. 4.12A-C).

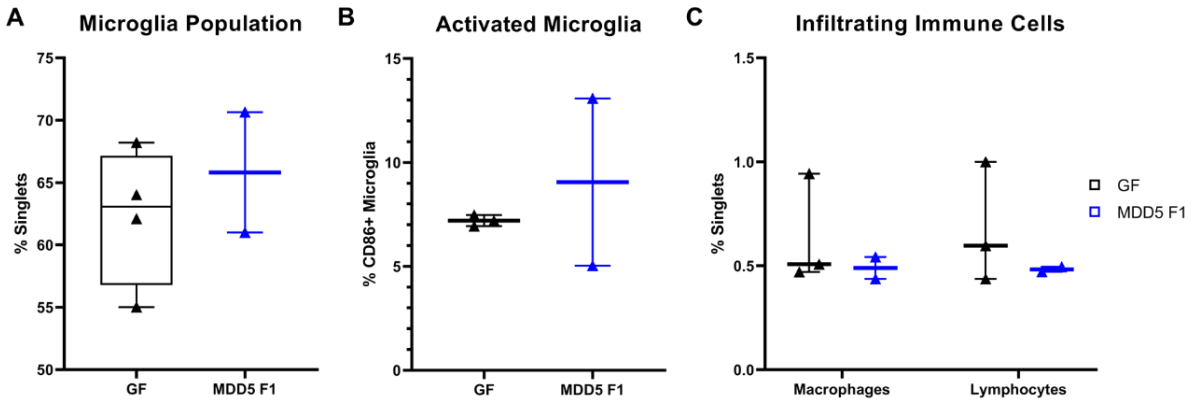

Figure 4.12: Brain immune cell profiles of MDD5 offspring (MDD5 F1) when compared to age-matched GF control mice. (A) All microglia as a percentage of live singlet events. (B)

Activated (CD86 positive) microglia as a percentage of all live microglia events. (C) Infiltrating macrophages and lymphocytes as a percentage of live singlet events. All data presented as standard box plots with individual values.

Discussion

Despite having a principal diagnosis of MDD, the microbiota of donor MDD5 induced anxiety-like behaviour in the recipient animals. Patient MDD5 has comorbid GAD and signs of gut level immune activation as seen by high fecal β-defensin 2 levels. Furthermore, depression and anxiety are related disorders with similar putative mechanisms, and both disorders have been associated with altered intestinal microbiota composition (Aizawa et al., 2016; Y. C. E. Chung et al., 2019; Jiang et al., 2015, 2018; Kelly et al., 2016; Lin et al., 2017; Miller & Raison, 2016; Zheng et al., 2016). We have previously shown that individual GAD patient microbiota can induce anxiety-like behaviour in recipient adult GF mice (Thrasher et al., 2019). The present study demonstrates that fecal microbiota from an individual MDD patient with comorbid GAD can induce anxiety-like behaviour in mice colonized in early life.

Taken alone, the behavioural data suggest that both the time point of behavioural testing and age at colonization affect behavioural outcomes in mice. However, it is possible that altered microbiota composition influenced mouse behaviour as the microbiota composition of both MDD5 breeders and offspring differed when compared to that of mice colonized with MDD5 microbiota in adulthood. The source of this difference in composition is unknown as all mice were colonized using the same MDD5 donor sample. Moreover, sex hormones are known to influence the intestinal microbiota of mice; and mouse microbiota composition has been shown to change during pregnancy, with shifts occurring as early as embryonic day 0.5 (Gohir et al.,

2015; Org et al., 2016). Therefore, it is possible that sex hormones influenced how the donor microbiota established in the GF breeding pair and the subsequent colonization of the offspring.

Features of both MDD5 F1 and MDD5 mouse microbiota correspond with what has been previously reported in the literature. The observed increase in Actinobacteria in MDD5 mice agrees with prior findings of increased Actinobacteria abundance in the microbiota of MDD patients (Y. C. E. Chung et al., 2019; Zheng et al., 2016). Additionally, we previously reported increased Actinobacteria in the microbiota of mice displaying depressive-like behaviour (Project 1). Prior research has also associated increases in *Turicibacter*, *Paraprevotella*, and *Holdemania* species with MDD (Kelly et al., 2016). Moreover, in MDD patients, increased *Holdemania* relative abundance was previously found to be predictive of high Beck Anxiety Index scores (Y. C. E. Chung et al., 2019). In the present study, we also observed a positive correlation between an unclassified species of *Holdemania* and anxiety scores in mice. Furthermore, both our present results and previous findings suggest *Blautia* species are protective against anxiety- and depressive-like behaviours (Project 1). Interestingly, *Blautia* are known producers of the short-chain fatty acid butyrate (Vacca et al., 2020). In mice, butyrate treatment has been found to enhance gut barrier and BBB integrity (Braniste et al., 2014; Stilling et al., 2016). Butyrate has also been shown to have anti-inflammatory effects in microglia, inhibiting the activation of the NF-κB pathway (Huuskonen, Suuronen, Nuutinen, Kyrylenko, & Salminen, 2004).

This study is not without limitations. We were unable to find evidence of increased neuroinflammation in our MDD5 offspring. It is possible that greater numbers and additional controls, including HC5 offspring, will reveal an inflammatory mechanism in our model. Secondly, this study only utilized one MDD microbiota donor. The use of multiple MDD donors will allow us to identify additional donor samples that can induce depressive- or anxiety-like

behaviours in this early life model. Alternative donors that induce depressive-like behaviour in mice will allow us to investigate the early life programming of MDD risk through the intestinal microbiota and the immune system. Moreover, we were unable to trace all significant microbial features in recipient mice back to the human donor samples. It is unlikely that this represents contamination as the majority of these species are obligate anaerobes. We postulate that these species are present in the donor samples at levels below the limit of detection. Further, maternal behaviour is known to affect the programming of offspring behaviour (Sullivan, Nousen, & Chamlou, 2014). With only one breeding pair, we cannot rule out the influence of maternal behaviour on the behaviour of the MDD5 offspring. Additionally, performing multiple rounds of behavioural testing may influence behavioural outcomes. Although we provided mice with three weeks rest between the two rounds of testing, it is possible that mice can remember the initial testing period and that this memory affects their behaviour in the subsequent testing period. Lastly, we observed changes to the microbiota of MDD5 offspring over the course of one behavioural testing period. It is possible that the initial testing period altered the microbiota composition of HC5 and MDD5 mice, leading to the differences in behaviour observed at the second time point.

Interactions between the host and microbiota are complex, and these interactions likely influence how the microbiota establishes in a new host. The present study is the first to demonstrate a humanized mouse model of GAD using early life colonization. Our data suggest that the use of a colonized breeding paradigm changes microbiota composition. We also present evidence to suggest that the composition of adult mouse microbiota changes over the course of a behavioural testing period. Thus, further experimentation is needed to optimize methods of early life

colonization. We recommend that future colonization studies include the careful monitoring of mouse microbiota composition over time.

CHAPTER 5: CONCLUSION

With the development of a novel mouse model of MDD, our group aimed to further our understanding of the microbial and immune mechanisms underlying depressive-like behaviour in this model. This aim was addressed with the three research projects presented here. In Projects 1 and 3, we identified two individual MDD microbiota donors, patients MDD1 and MDD5, that altered the behaviour of recipient mice. In both instances, the microbiota of the patient induced a donor-specific phenotype in recipient GF mice. We also evaluated multiple individual donor samples and pooled donor samples; these samples did not significantly alter mouse behaviour. These findings led us to conclude that the intestinal microbiota is likely involved in the inflammatory mechanism of depression in only a subset of MDD patients. Therefore, we recommend the use of individual, rather than pooled, donor samples in future studies utilizing FMT.

Altered microbiota was observed in mice colonized with microbiota from donor MDD1, as well as mice colonized with MDD5 microbiota. We identified several microbial targets associated with depressive- and anxiety-like behaviours in recipient mice. Some notable targets include *E. lenta*, *Holdemania* species, and *Blautia* species, given that these findings coincide with what has been previously reported in the literature. These data will contribute to the formation of new hypotheses linking individual microbial species to effects in the host. Future experiments will be needed to test these hypotheses.

Our studies are the first of their kind to use mice of both sexes and we observed clear sex differences in mouse behaviour. In Project 1, female mice displayed more anxiety-like behaviour in the open field test and more depressive-like behaviour in the tail suspension test. Female mice also spent more time immobile in Project 3. These findings are important given the known gender bias in the prevalence of mood disorders, with depression and anxiety being more

prevalent in women than men (Palanza, 2001; Rainville & Hodes, 2018). Sex differences in microbiota were also observed in our studies; and this further supports the use of animals of both sexes, along with sex-stratified statistical analyses.

Neuroinflammation may be involved in the behavioural outcomes observed in these colonization studies. Interestingly, in the hippocampus of mice displaying depressive-like behaviour, we observed decreased *TRPV1* expression. Activation of this receptor has previously been shown to have anti-inflammatory effects in microglia (Bassi et al., 2019; Y. C. Chung et al., 2017). Therefore, this finding suggests possible brain immune activation in MDD1 mice. Furthermore, Project 2 offers our group new methods to study neuroinflammation in future experiments. This will allow our group to better understand the immune mechanisms involved in the humanized mouse model of MDD.

In conclusion, our findings suggest that the intestinal microbiota plays a key role in the pathogenesis of MDD for some but not all patients. Through the completion of three research projects, we were able to gain additional insight into the microbial and immune mechanisms of a novel mouse model of MDD. Lastly, the data presented here will help to generate new hypotheses and design future experiments, using the best available methods.

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APPENDIX A

Table A.1: Identification of Microbiota Donors

Donor ID	Alternative ID
HC1	HC79
HC2	HC74
HC3	HC27
HC4	HC71
HC5	HC86
MDD1	MDD24
MDD2	MDD17
MDD3	MDD7
MDD4	MDD25
MDD5	MDD21

APPENDIX B

Table B.1: nCounter Custom Panel Gene List

C3	TOLLIP	NOD2	CALR
T-BET	CCR6	HIF1A	GFAP
MAPK1	CCR2	TGR5	S100B
CXCR3	DEFCR1	MYLK3	I-FABP
CXCR4	LYZ1	MMP9	TAAR1
CD11C	CNLP	TRPV1	TAAR4
CD11B	REG3G	GDNF	SEMAPHORIN
GPR44	MUC2	BDNF	SOX10
CD86	CDH1	NOS1	GABAA
MYD88	CLDN2	VIP	GABAB
NFKB1	CLDN1	NPY	NR2B
GATA-3	TJP1	SUBP	NR2D
TLR2	OCLN	CHAT	HMGB1
TLR4	CASP8	NFAT	PGP9.5
TLR5	F2RL1	P75	GAPDH
IL17A	TFF3	CALB1	PGK1
IL23R	RAE1	CGRP	GUSB
IL22RA2	PPARG	5HT	

APPENDIX C

Table C.1: Project 1 Phylum Level Correlation Analyses

Males Only			
Phylum	**Spearman's r**	**P-value**	**Rel. Abundance in Depression**
Verrucomicrobia	-0.333	0.0312	↑
Both Sexes			
No significant correlations			
Females Only			
No significant correlations			

Table C.2: Project 1 Species Level Correlation Analyses

Both Sexes			
Species	**Spearman's r**	**P-value**	**Rel. Abundance in Depression**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Phascolarctobacterium;s__	-0.3386	0.0008	↑
Females Only			
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium;s__	-0.387	0.0046	↑
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Eggerthella;s__lenta	-0.3989	0.0034	↑

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;s__obeum	0.4599	0.0006	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Phascolarctobacterium;s__	-0.4704	0.0004	↑
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__ovatus	-0.4984	0.0002	↑
Males Only			
No significant correlations			

Table C.3: Project 3 Phylum Level Analyses (Kruskal-Wallis and Dunn-Bonferroni)

Both Sexes			
Phylum	**HC5 vs MDD5**	**HC5 vs MDD5 F1**	**MDD5 vs MDD5 F1**
Actinobacteria	0.024	ns	0.015
Cyanobacteria	0.015	0.013	ns
Females Only			
No significant differences			
Males Only			
No significant differences			

Table C.4: Project 3 Species Level Analyses (Kruskal-Wallis and Dunn-Bonferroni)

Both Sexes			
Species	**HC5 vs MDD5**	**HC5 vs MDD5 F1**	**MDD5 vs MDD5 F1**
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;s__	ns	0.018	0.014
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella;s__aerofaciens	0.007	ns	ns
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter;s__	ns	0.014	0.008
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Oxalobacter;s__formigenes	ns	0.005	ns
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;s__	0.004	ns	ns
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;NA	0.003	ns	ns
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;NA;NA	0.009	ns	0.049
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;NA;NA	0.009	ns	0.049
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes;s__indistinctus	0.009	ns	0.049
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Odoribacter;s__	ns	0.004	ns
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;NA;NA;NA	0.032	ns	0.009

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__;s__	0.006	0.038	ns
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__ovatus	0.002	ns	ns
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__Paraprevotella;s__	0.002	ns	ns
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Holdemania;s__	ns	0.002	ns
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Clostridium;s__metallolevans	0.001	ns	ns
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__acidifaciens	0.001	ns	ns
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Butyricimonas;s__	0.001	ns	ns
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus];s__	0.003	ns	0.022
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__;s__	0.002	0.019	ns
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];NA;NA	0.002	0.019	ns
Females Only			
No significant differences			
Males Only			
No significant differences			

Table C.5: Project 3 Phylum Level Correlation Analyses

Both Sexes			
Phylum	**Spearman's r**	**P-value**	**Rel. Abundance in Anxiety**
Cyanobacteria	0.7511	0.0028	↓

Females Only
No significant correlations
Males Only
No significant correlations

Table C.6: Project 3 Species Level Correlation Analyses

Both Sexes			
Species	**Spearman's r**	**P-value**	**Rel. Abundance in Anxiety**
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__acidifaciens	-0.6022	0.0254	↑
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];NA;NA	0.6358	0.0171	↓
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Odoribacter;s__	0.6678	0.0108	↓
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__;s__	0.6407	0.0161	↓
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__;s__	0.7111	0.0058	↓
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Alloiococcus;s__	0.6639	0.012	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;NA	0.6045	0.0248	↓

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__rc4-4;s__	0.5695	0.0345	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Clostridium;s__metallolevans	-0.6114	0.0229	↑
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;s__	0.7013	0.0066	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;NA	0.6135	0.011	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;s__	0.8142	0.0007	↓
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Holdemania;s__	-0.6782	0.0098	↑
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Oxalobacter;s__formigenes	-0.8529	0.0003	↑
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__;s__	-0.7219	0.0051	↑
Females Only			
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__;s__	0.8575	0.0333	↓

k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Fructobacillus;s__	0.8407	0.0444	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;NA;NA;NA	-0.9856	0.0056	↑
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Oxalobacter;s__formigenes	-0.9706	0.0056	↑
Males Only			
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;NA	0.7408	0.042	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;NA	0.773	0.0179	↓
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;s__	0.7711	0.0329	↓