FUNCTIONAL RELATIONSHIP BETWEEN GUT MICROBIOTA AND ANXIETY

THE FUNCTIONAL RELATIONSHIP BETWEEN THE GUT MICROBIOTA AND GENERALIZED ANXIETY DISORDER IN THE MURINE MODEL

By

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ABSTRACT

Depression and anxiety are etiologically heterogeneous disorders and their pathophysiology remains largely unknown. Gut microbiota has been shown to modulate brain function, behavior, and immune responses, and it has also been proposed to play a role in the pathophysiology of depression and anxiety. Our study aimed to investigate whether microbiota from patients with Generalized Anxiety Disorder (GAD) can induce anxiety and depressive-like behaviour in germ-free mice and whether this is accompanied by changes in immune markers and brain activity.

Germ-free NIH Swiss mice (n=27) were colonized with microbiota from either a GAD patient (n=13) with severe anxiety and comorbid depression or an age and sex-matched healthy control (HC) (n=14). Six mice from each group were treated with infliximab for three weeks (5mg/kg/week) starting at week 1 post-colonization. Microbiota profiles were assessed via 16S rRNA based Illumina. Three weeks post-colonization, all mice underwent six standard psychometric tests, including the open field, digging, marble-burying, and tail-suspension test. Cecal β-defensin-3 and serum kynurenine/tryptophan were measured via ELISA. BDNF expression was assessed by immunofluorescence, and gene expression by using Nanostring gene assay.

Fecal β-defensin levels were higher in GAD patients than in healthy controls. Similarly, β-defensin levels were higher in GAD-colonized mice than in HC-colonized mice. GAD and HC-colonized mice had a unique and distinct microbiota, similar to that of their respective human donors. GAD-colonized mice exhibited anxiety and depressive-like behavior compared to HC-

colonized mice, as assessed by the open field, digging, marble burying and tail suspension tests. BDNF expression was decreased in the hippocampus but increased in the amygdala of GAD-colonized mice. GAD-colonized mice also had a greater kynurenine/tryptophan ratio than HC-colonized mice. GAD and HC infliximab-treated mice showed no differences in behavior, central BDNF expression or kynurenine/tryptophan levels.

Our results suggest that GAD microbiota has the ability to induce anxiety and depressive-like behavior and alter brain BDNF expression in a murine host. These changes are accompanied by the activation of the innate immune system and seem to be TNF-α dependent.

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LIST OF ABBREVIATIONS

BDNF	Brain-derived Neurotrophic Factor
CRP	C-reactive Protein
CMS	Chronic Mild Stress
DASS	Depression, Anxiety and Stress Scale
DSMV	Diagnostic and Statistical Manual, Fifth Edition
ELISA	Enzyme-linked Immunosorbent Assay
ENS	Enteric Nervous System
GAD	Generalized Anxiety Disorder
GFAP	Glial Fibrillary Acidic Protein
GRIN2D	Glutamate Receptor, Ionotropic, N-methyl-o-aspartate 2D
GF	Germ Free
GI	Gastrointestinal
HBD2	Human β-defensin-2
HC	Healthy Control
HPA	Hypothalamic-pituitary-adrenal
Reg3g	Regenerating Islet-derived protein 3 Gamma
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-A	Irritable Bowel Syndrome with comorbid Anxiety
IL17a	Interleukin 17a
IDO	Indoleamine-2-3-deoxygenase

MDD	Major Depressive Disorder
OTU	Operational Taxonomic Unit
SSRIs	Selective Serotonin-Reuptake Inhibitors
TDO	Tryptophan-2-3-deoxygenase
TNF-α	Tumour necrosis factor-alpha

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis was a subsection of larger project initiated by Dr. Bercik, Dr. Anglin, Dr. Bailey, Dr. Surette, and Dr. Collins. All experiments for this thesis were conducted by Elizabeth Perez Guzman with assistance from Jun Lu and Dr. De Palma. Data analysis and its interpretation was performed by Elizabeth Perez Guzman under the guidance and supervision of Dr. Bercik, except for Figure 2 and 7, which was produced by Ryan Potts and Dr. Surette. Insightful direction of this project was provided by Dr. Collins, Dr. Surette, and Dr. Mishra, who were members of Elizabeth's advisory committee.

INTRODUCTION

1.1 Intestinal Microbiota

Humans host nearly 100 trillion microbes in their digestive tract, most of which are bacterial species (Turnbaugh & Gordon, 2009). Moreover, the number of bacterial genes in the digestive tract outnumbers human genes by 150-fold (Backhed *et al*., 2011). This bacterial community and their genes are collectively termed as the gut microbiota, which share a mutualistic relationship with its host (Backhed *et al*., 2005). Although the gut microbiome is represented by over 1,800 bacteria genera, it is primarily composed of *Prevotella*, *Ruminococcus*, and *Bacteroides* (Arumugam *et al*., 2011). Development of the gut microbiome begins at birth, as in utero the intestinal tract is sterile. After 3 years of age, the gut microbiome remains relatively stable but can be altered by drug intake, changes in environment, diet, and gene expression (Palmer *et al*., 2007).

The microbiome lives in symbiosis with its host; the host provides the microbiota a protected and energy-rich environment and the microbiota provides numerous benefits, aiding the development of the innate and adaptive immune responses (Hooper & Macpherson, 2010; Hooper *et al*. 2012), modulating gut motility (Husebye *et al.,* 2001), regulating absorption of nutrients (Jumpertz *et al.* 2011), and contributing to neural communication, especially as some bacterial strains can produce neurotransmitters (Lyte, 2011; Barrett *et al,* 2012; Stephenson *et al*, 1947). These physiological effects of the microbiota aid the host in achieving optimal health and well being. Perturbations of the intestinal microbiota, or dysbiosis, can therefore be disruptive to the host's health in diverse ways, including changes in behavior and brain function.

1.2 Gut-Brain Axis

The relationship between the gut and brain has long been observed in medicine. However, only recently, studies have demonstrated bi-directional communication between the brain and gut. This bidirectional communication has been termed as the gut-brain axis, in which the intestinal microbiota acts as a key signalling component (Heijtz *et al.,* 2011; Neufeld *et al.,* 2011; Clarke *et al.,* 2013; Sudo *et al.,* 2004). Furthermore, monoaminergic pathways and the hypothalamic-pituitary-adrenal (HPA) axis mediate cross-talk between the gut and the brain (Mayer, 2011). The outputs from the brain to the gut are generated by the hypothalamus and amygdala, which receive signals from the medial prefrontal cortex and anterior cingulate cortex (Ongur *et* al., 2000). These brain structures comprise the network that integrates and transmits homeostatic information from the intestinal tract, including visceral pain and food intake (Mayer, 2011). Homeostatic changes in the gut are communicated to the brain through the enteric nervous system (ENS), a system comprised of 200-600 million neurons (Furness, 2008). Information of the gut can be encoded via several mechanisms, including primary afferent neurons, enteroendocrine cells, and immune cells (Furness *et al*., 2004).

The directionality of the gut-brain axis allows both "bottom-up" and "top-down" communication and studies have provided evidence for both direction pathways. For example, studies have shown that administration of certain bacterial strains into a murine host alters neural activity and behavior, which confirm "bottom-up" communication. In one such study, mice challenged with *Campylobacter jejuni* exhibited increased anxiety-like behaviour and altered brain activation (Goehler *et al.,* 2008). Studies have also shown "top-down" communication by primarily studying how psychological stress can influence the microbiota. Early life stress in a murine

model, via maternal separation, has been shown to alter immune and microbiota profiles (O' Mahony *et al.,* 2009) increase intestinal permeability of mice (Varghese *et al.,* 2006) and alter behavioural profiles (De Palma *et al*., 2015). Although associations within the gut-brain axis have been established, further mechanistic and translational studies are needed to render plausible applications to psychiatric conditions in humans.

1.3 Depression and Anxiety Disorders

Depression is the leading cause of long-term disability, affecting approximately 350 million people worldwide (WHO, 2012). The fifth edition of the Diagnostic and Statistical Manual (DSMV) characterizes Major Depressive Disorder (MDD) by consistent depressed mood, diminished pleasure in activities, changes in sleep, energy, and weight, feelings of worthlessness or guilt, and suicidal ideation. These psychological and physical symptoms of depression can cause significant occupational impairment, as the greatest prevalence of depression occurs between 15-45 years of age. This occupational impairment and the treatment-related costs of MDD has led to an economic burden of over $83 billion in the United States alone (Kessler *et al.,* 2001)

Likewise, anxiety disorders have a tremendous economic burden, costing the United States approximately $46 billion (Dupont *et al*., 1996). Anxiety disorders are the most prevalent psychiatric conditions that affect up to 29% of people at least once in their lifetime (Swinson *et al*., 2006). Even though there are six primary types of anxiety disorders, this project has focused on Generalized Anxiety Disorder (GAD), which affects 3% of the population in a given year

(Kessler *et al*., 2005). GAD is characterized by uncontrollable worrying from everyday activities, with no specific stimuli that provoke the out of proportion stress response (Tyrer & Baldwin, 2006). GAD is also accompanied by physiological symptoms, including fatigue, difficulty concentrating, muscle tension, and sleep disturbances that are not a result of a medical condition (American Psychiatric Association, 1995). Untreated or severe GAD is often accompanied by depression and patients afflicted with both conditions have considerably more impairment than with either condition alone (Kessler *et al*., 2001).

The pathophysiology of both depression and anxiety remains poorly understood, and their etiology is likely heterogeneous (Hasler, 2010). Moreover, the current treatment for depression and anxiety, mainly selective serotonin re-uptake inhibitors (SSRIs) and benzodiazepines, have limited efficacy and can produce dire side effects (Anderson *et al*., 2008; Nutt, 2000). Thus finding a novel avenue in understanding depression and anxiety would be extremely beneficial in reducing the economic burden of these psychiatric conditions and in the development of alternative treatments.

1.4 Microbiota and Behaviour

Gut microbiota plays a central role in gut-brain axis communication and influences brain function and behaviour. One example of how gut microbiota influences gut-brain axis "bottom up" communication is the demonstration that germ-free (GF) mice colonized with gut microbiota from mice of different strains adopted the behavioral tendencies of the donor mice. More specifically, this was confirmed by colonization of GF Balb/c mice, who are naturally timid and

anxious, with microbiota from NIH Swiss mice, who are more exploratory and less anxious. The recipient Balb/c mice consequently adopted the behaviour of its NIH Swiss donor, as the Balb/c mice exhibited decreased anxiety and greater exploratory-like behaviour. Conversely, NIH Swiss mice colonized with Balb/c microbiota exhibited increased anxiety-like behaviour and decreased hippocampal brain-derived neurotrophic factor (BDNF) (Bercik *et al.,* 2011). Conversely, one example of "top-down" gut-brain axis communication is the report of how mice raised in a stressful environment showed altered microbiota composition and an increase in depressive-like behaviour (Bendsten *et al*., 2012).

There is high prevalence of psychiatric comorbidities, namely anxiety and depression, in patients with chronic bowel disease (Walker *et al.,* 2008; Folks, 2004). Patients with an Inflammatory Bowel Disease (IBD) were found to be three times more likely to experience depression than the general population, with up to 17% of IBD patients reporting symptoms of depression and suicidal thoughts (Fuller, Thomson & Sulman, 2006). Patients with IBD or with Irritable Bowel Syndrome (IBS), often present abnormal intestinal microbiota profiles (Maukonen *et al.,* 2006; Matto *et al.,* 2005), which suggest that microbiota may play an important role within the gut-brain axis. A recent study used germ-free mice colonized with microbiota from IBS patients with comorbid anxiety (IBS-A) to investigate the functional role of microbiota in the expression of psychiatric comorbidities. The study found that mice that were colonized with IBS-A microbiota displayed higher anxiety-like behaviour and altered expression of colonic innate immune genes, as compared to mice colonized with microbiota from IBS patients without anxiety or microbiota from healthy controls (De Palma *et al.,* 2017). Given all this evidence, it is plausible to hypothesize the advantage of gut microbiota directed therapies at modulating brain function and mood.

1.4.1 Probiotics and mood amelioration

Probiotics were defined in 1965 and are currently known as live microorganisms, that when ingested, provide non-specific health benefits to its host (Lily & Stillwell, 1965; Caselli *et al*., 2013). The idea of probiotics ameliorating psychiatric symptoms is not a new one; in 1910, a whey formula with living lactic acid bacteria was found to improve depressive symptoms in melancholic adults (Philips, 1910). Since then, several clinical studies have investigated the effects of probiotic intake on mood. For example, daily ingestion of *Lactobacillus casei* for two months resulted in an improvement of anxiety symptoms in patients with chronic fatigue syndrome (Rao *et al*., 2009). Likewise, daily intake of *Lactobacillus helveticus* and *Bifidobacterium longum* for two weeks alleviated symptoms of depression and anxiety in healthy volunteers (Messaoudi *et al*., 2011). Probiotic intake has not only been shown to affect mood but also brain activity. Four-week consumption of fermented milk product with probiotic was found to affect brain regions of emotional processing in healthy women (Tillisch *et al*., 2013). However, clinical studies on probiotics have not included patients with diagnosed depression or anxiety when testing for mood amelioration or brain activity. The aforementioned results thus have limited applicability to people afflicted with GAD or MDD and further studies are needed to establish the efficacy of probiotics on mood amelioration. What these studies have demonstrated, however, is that changes in microbiota profiles (in these cases via probiotics) can lead to changes in mood.

1.4.2 Microbiota and mood disorders

To date, only three published studies have directly explored the relationship between microbiota profiles and mood disorders in humans. The first study analyzed the fecal microbiota of depressed patients and healthy controls and found that depressed subjects had a significant association with both *Oscillibacter* and *Alistipes* operational taxonomic units (OTU's) and exhibited an overrepresentation of the order Bacteroidales but an underrepresentation of the family Lachnospiraceae (Naseribafrouei *et al.,* 2014). This study was important in establishing correlations between depression and specific OTUs, which provided support to the idea that microbiota may play a role in psychiatric disorders. Similarly, the second study analyzed gut microbiota composition of depressed and healthy subjects and found that depressed subjects had increased microbiota α-diversity and altered levels of bacterial species, as compared to healthy controls (Jiang *et al.,* 2015). Moreover, there was a negative correlation between serum BDNF and *Clostridium* XIVb as well as a negative correlation between depression severity and *Faecalibacterium*. These two studies were important in establishing a link between altered gut microbiota and mood disorders in humans. Only one study has explored the functional implications of microbiota in patients with clinical depression. A study published in 2016 found that rats colonized with microbiota from patients with MDD displayed significantly greater depressive-like behaviour, TNF-α concentration, and kynurenine/tryptophan ratio than rats colonized with healthy control microbiota (Kelly *et al.,* 2016). However, in this paper, the colonized rats were not germ-free but simply treated with antibiotics prior to colonization, and the fecal microbiota used for colonization was a pool of several donors samples, thus the OTUs

of individual patients, or the differences between them, could not be delineated. To date, no study has investigated the functional relationship between gut microbiota and clinical anxiety.

1.5 Inflammation as a Cause of Anxiety and Depression

The immune system modulates communication within the gut-brain axis, which is imprinted and regulated by the intestinal microbiota (Bengmark, 2013; Macpherson & Uhr 2004). The largest immune organ of the human body is comprised of the gut and gut-associated lymphoid tissues (GALT) that provide the alimentary canal a defensive barrier from external pathogens (Vighi *et al*., 2008). A fine balance between the microbiome and the mucosal immune system is found in homeostatic conditions; however, perturbations of the microbiota can result in immune activation, such as activation of toll-like receptors and consequent release of pro-inflammatory cytokines (Dinan *et* al., 2013). This cascade of events leads to inflammation and can often be accompanied with altered behaviour. For example, rodents that received peripheral pro-inflammatory cytokines exhibited symptoms of depression, disrupted circadian rhythm, and appetite loss (Bilbo and Schwarz, 2012; Dantzer, 2009). Furthermore, animal models of intestinal inflammation often present with concurrent anxiety. Mice infected with *Trichuris muris,* a parasite that elicits a robust immune response, exhibited intestinal inflammation and increased anxiety-like behaviour (Bercik *et al*., 2010). Mouse models of experimental colitis display intestinal inflammation and anxious behaviour with changes in central BDNF, but mice with colitis treated with probiotic *Bifidobacterium longum*, exhibited normalized anxiety-like behaviour and BDNF levels (Bercik *et al*., 2011).

Depression has been associated with elevated biomarkers of inflammation, including interleukin-6, tumor necrosis factor (TNF)-α, and C-reactive protein (CRP) (O'Brien *et al*., 2004). Likewise, somatic symptoms of anxiety have been associated with greater levels of interleukin-6, TNF-α, and CRP (Duivis *et* al., 2013). Currently, it is not known whether elevated inflammation is essential for the expression of anxiety and depression or whether it is just epiphenomenal.

1.5.1 C-Reactive Protein

C-reactive protein (CRP) is an acute phase reactant that increases in response to inflammation. Thus, CRP has been historically used as blood test marker for systemic inflammation and is often utilized to predict risk of coronary heart disease, stroke, and mortality (Koenig *et al*., 1999; Emerging Risk Factors Collaboration; 2010). CRP concentrations that exceed 10mg/L are indicative of an inflammatory disease (Pepys *et al.,* 2003; Nordestgaard *et* al., 2009). However, lower CRP concentrations, approximately 1-3mg/L, or low-grade inflammation has been implicated to contribute to the pathophysiology of depression (Liukkonen *et* al., 2006; Howren *et al*., 2009; Valkanova *et al*., 2013). A recent study examined 73 men and 131 women and found that increased CRP levels are associated with an increased risk of depression and psychological distress, even when adjusted for confounding variables such as BMI, age, sex, alcohol intake, smoking, and chronic disease (Wium-Andersen, 2013). Similarly, higher CRP concentrations have been found to be associated with anxiety. For example, one study found that anxiety symptoms increased the risk of elevated CRP-levels by 2-fold in males (Liukkonen *et al*., 2011). Studies investigating the link between low grade inflammation and anxiety, however, are sparse.

Further research is needed to establish if the association between CRP and anxiety is as consistent as has been shown in depression.

1.5.2 TNF-α and Infliximab

Tumor-necrosis factor (TNF)-α is a pro-inflammatory cytokine primarily produced by activated macrophages. TNF-α, like other pro-inflammatory cytokines, responds to microbial pathogens and mediates systemic inflammation, but can also act on the brain and induce behavioral changes (Dantzer *et al*., 2008). The large size of TNF-α restricts its flow past the blood-brain-barrier but it can affect the brain via the circumventricular organs (parts of the blood-brain-barrier which is more permeable) or through afferent fibers of the vagus nerve (Konsman *et* al., 2002; Capuron and Miller, 2004). It is possible that depression may result from exacerbated inflammation, as mediated by maladaptive or increased cytokine activation. Indeed, studies have found that MDD patients have greater blood concentrations of TNF-α than healthy controls (Sutcigil *et al*., 2007; Yang *et al*., 2007). Animal studies have demonstrated that elevation of pro-inflammatory cytokines can induce depressive-like behaviour. For example, mice treated with LPS (which triggers production of pro-inflammatory cytokines) showed increased depressive-like behaviour even after sickness behaviour and motor activity had returned to normal (Frenois *et al*., 2007). Based on these studies, it has been speculated that decreasing TNF-α could alleviate inflammation and symptoms of depression. Infliximab, a chimeric mouse-human antibody targeting TNF-α, has been used to treat inflammatory bowel disease (IBD) for over a decade with accepted efficacy and safety (Fidder *et al*., 2009). Interestingly, patients with IBD and IBS often present with comorbid depression and anxiety, with increased psychiatric symptoms during

severe periods of active disease (Walker *et* al., 1992; Walker *et al*., 2008; Graff *et al*., 2009). A study in 2014 found that anti-TNF therapy, including infliximab, decreased depressive symptoms and increased quality of life in IBD patients (Horst *et al*., 2014). Moreover, infliximab treatment has been found to decrease depressive symptoms in MDD patients with high baseline inflammatory biomarkers (Raison *et al*., 2013). Animal studies have also shown that infliximab has the potential to alleviate psychiatric symptoms even outside of inflammatory bowel disease. For example, infliximab-treated rats that underwent chronic mild stress (CMS) exhibited a reduction in depressive and anxiety-like behaviour, as compared to placebo-treated rats (Karson *et al*., 2013). Both clinical and animal studies provide support that inflammation, mediated via TNF-α, may contribute to the expression of depression and anxiety. However, the majority of research has primarily been focused on depression and further research is needed to delineate the relationship between infliximab treatment and anxiety.

1.5.3 β-defensins and Calprotectin

β-defensins are antimicrobial peptides that limit microbial colonization in epithelial tissues and at high concentrations generate pro-inflammatory signals (Ganz, 2003). In this project, human β-defensin-2 (HBD2) was the specific defensin under investigation. HBD2 is produced by epithelial cells, in response to cytokine stimulation, such as TNF-α and IL-1β, to act against Gram-negative bacteria (Schroder & Harder, 1999). In recent years, increased HBD2 has been used as an indicator of intestinal innate immune activation (Langhorst *et al*., 2009; Aldhous *et al*., 2009; Zilbauer *et al*., 2010) likely due to dysbiosis. Because inflammation has been proposed to play a role in the expression of depression and anxiety, it is possible that innate immune

activation may also be involved. Interstingly, a recent study found that mice colonized with microbiota from patients with IBS and co-morbid anxiety had higher HBD2 concentrations than mice colonized with microbiota from patients with IBS but no comorbid anxiety or from healthy controls (De Palma *et al*., 2017). No studies to date, however, have investigated whether patients with clinical depression or anxiety have elevated levels of HBD2 or other β-defensins.

Calprotectin is a protein found in the cytosol of inflammatory cells (Røseth, 1999). Fecal calprotectin has been widely accepted as a biomarker for overt intestinal inflammation and differentiates IBD, such as Crohn's or colitis, from IBS (Konikoff *et al*., 2006, Von Roon *et al*., 2007; Van Rheenen *et al*., 2010; D'haens *et al*., 2012). In this study, fecal calprotectin was measured to ensure that any increase in other inflammatory markers is not attributed to an active GI disease such as IBD.

1.6 Tryptophan Metabolism in Anxiety and Depression

Serotonin availability in the brain is limited by the concentration of its precursor tryptophan. Tryptophan is an essential amino acid, which can either be converted into serotonin or kynurenine (Borre *et al*., 2014). Cytokines, like TNF-α, increase indoleamine-2-3-deoxygenase (IDO) and corticosteroids increase tryptophan-2-3-deoxygenase (TDO), both of which catalyze the synthesis of kynurenine from tryptophan (Slyepchenko *et al*., 2014). Therefore, an increase in IDO or TDO results in a decrease in tryptophan available for serotonin production. For this reason, the metabolism of tryptophan is indicative of serotonin availability and has been used in

research studying depression and anxiety (Capuron *et* al., 2003; Muira *et* al., 2008; Myint *et al*., 2013). More specifically, however, psychiatric research can use the kynurenine/tryptophan ratio as a measure of IDO activation, tryptophan metabolism, and relative serotonin production (O' connor *et al*., 2009) and thus as a useful tool in assessing inflammation-driven depression. For example, rats that underwent 8 weeks of Chronic Mild Stress (CMS) had a greater kynurenine/tryptophan ratio than rats that did not undergo the CMS regimen. Moreover, in this same experiment decreasing TNF-α, via infliximab treatment, decreased the kynurenine/tryptophan ratio and depressive and anxiety-like behaviour even in rats that received CMS (Karson *et al*., 2013). Moreover, rats that were colonized with microbiota from MDD patients showed an elevated kynurenine/tryptophan ratio compared to rats colonized with microbiota from healthy controls (Kelly *et al*., 2016).

1.7 BDNF in Anxiety and Depression

Brain-derived neurotrophic factor (BDNF) is a ubiquitous growth factor in the central nervous system (CNS) that promotes synapse formation and neuronal growth (Lewin and Barde, 1996; Huang and Reichardt, 2001). More recently, BDNF has also emerged as a prominent regulator of synaptic plasticity and behaviour (Poo, 2001; Lu, 2003; Martinowhich *et al*, 2007). BDNF has also been associated with depression. For example, studies have observed that stress decreases expression of BDNF mRNA in the hippocampus (Smith *et* al., 1995; Duman and Monteggia, 2006). Conversely, electroconvulsive shock therapy and antidepressant treatment increases BDNF mRNA expression in the hippocampus (Nibuya *et al*., 1995; Russo-Neustadt *et al*., 2000).

These observations have led to the development of the neurotrophin hypothesis of depression, which proposes depression is correlated with decreased BDNF and that antidepressant treatment alleviates depressive symptoms via increasing BDNF and consequent synaptic plasticity (Duman, 2002; Castren *et al*., 2007). Indeed, infusions of BDNF protein in to the hippocampus can have antidepressant effects in a murine model (Siuciak *et al*., 1997; Shirayama *et al*., 2002). Conversely, antidepressant treatment is not effective in BDNF knockout mice, which indicates that BDNF is involved in the therapeutic effects of antidepressants (Saarelainen *et al*., 2003). BDNF has also been implicated in the expression of anxiety. For example, BDNF deficient mice show increased anxiety-like behaviour and do not respond to treatment with the antidepressant fluoxetine (Chen *et al*., 2006). Moreover, GF Balb/c mice (naturally more anxious and timid) that received microbiota from NIH Swiss mice (naturally less anxious) exhibited lower anxiety-like behaviour and greater BDNF levels in the hippocampus (Bercik *et al*., 2011). Human studies have also provided support for the neurotrophin hypothesis, in that postpartum BDNF levels were lower in untreated depressed patients than in patients that had taken antidepressants (Chen *et al*., 2001). Because depression and anxiety disorders have been postulated to share common etiology, such as monoamine disturbances (Morilak and Frazer, 2004), it is possible that depression and anxiety also share associations with central BDNF. However, research investigating the relationship between BDNF and anxiety is sparse and further studies are needed.

1.8 Objectives and Hypotheses

The overall aim of this project was to explore the functional role of microbiota in the expression of anxiety using a gnotobiotic mouse model colonized with microbiota from a patient with Generalized Anxiety Disorder (GAD).

We investigated these specific hypotheses:

1) Colonization of gnotobiotic mice with microbiota from GAD patients induces anxiety-like behavior compared to mice colonized with microbiota from healthy controls.

2) The abnormal behavior in mice with GAD microbiota is accompanied by changes in immune activation and brain chemistry.

3) Treatment with TNF-α antibody infliximab attenuates abnormal behavior and brain chemistry in GAD-colonized mice by affecting kynurenin/tryptophan metabolism.

These aims were attained by conducting two separate experiments. The first experiment was conducted by colonizing gnotobiotic mice with either GAD or healthy control (HC) microbiota then measuring anxiety and depressive-like behaviour via psychometric testing, innate immune activation via cecum β-defensin concentrations, tryptophan metabolism via sera kynurenine/tryptophan ratios, and brain chemistry via BDNF immunofluorescent staining. The second experiment was conducted by colonizing gnotobiotic mice with the same GAD or HC donors and delivering weekly infliximab injections for three weeks. These mice then underwent the same psychometric and tissue analysis as described in experiment 1.

2. MATERIALS AND METHODS

2.1 Human Biomarker Analysis

All human samples used in this project were obtained from an ongoing clinical study headed by Dr. Rebecca Anglin at McMaster University. Stool samples were collected from each GAD patient (n= 71) and healthy control (n= 87) and serum samples were collected from 53 GAD patients and from 78 HCs. Samples were kept in a -80 C freezer until time of analysis. Stool samples were used to measure β-defensin2 and calprotectin concentrations whereas serum samples were used to measure C-reactive protein (CRP) and kynurenine/tryptophan ratios.

2.1.1. β-defensin

β-defensin analysis was conducted to assess whether patients and healthy controls presented with mucosal innate immune activation. Stool Human β-defensin-2 was measured in GAD patients (n=69) and in age and sex-matched healthy controls (n= 87) via an enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostik-K6500, Bensheim, Germany), according to manufacturer's instructions. Each sample was run in duplicate. Optical density was read at 450nm using ELx808 absorbance reader (BioTek, Winooski, VT, US) and the results were expressed in ng/ml.

2.1.2 Calprotectin

Calprotectin analysis was conducted to assess whether patients and healthy controls presented with overt intestinal inflammation. Stool Calprotectin was measured in GAD patients (n= 69)

and in age and sex-matched healthy controls (n= 87) with BIOHIT HealthCare Calprotectin ELISA (602260, Ellesmere Port, UK) according to manufacturer's instructions. Each sample was run in duplicate. Optical density was read at 450nm using ELx808 absorbance reader (BioTek, Winooski, VT, US) (brand, etc..) and the results were expressed in ng/ml.

2.1.3 C-Reactive protein

CRP analysis was conducted to assess whether patients and healthy controls presented with inflammation. Serum CRP was measured in GAD patients (n= 26) and in age and sex-matched healthy controls (n= 51) via an ELISA purchased from Life Technologies-KHA0031(Carlsbad, CA, US). Each sample was run in duplicate. Optical density was read at 450nm using ELx808 absorbance reader (BioTek, Winooski, VT, US) and the results were expressed in ng/ml.

2.2 Human Donor Selection

One GAD patient and one HC were selected from the GAD group (n=71) and HC group (n=87), of the aforementioned clinical study. These donors were well characterized by a psychiatrist and selected based on their anxiety level, as determined by their Depression, Anxiety, and Stress Scale (DASS) scores. The selected GAD patient, a 19-year-old female, had a DASS Anxiety score of 36 and was thus within the range of very severe anxiety (scores >20). This patient also had co-morbid depressive symptoms, with a DASS Depression score of 22 and DASS Stress score of 26. The selected healthy control was age and sex-matched to the GAD patient; the subject was a 20-year-old female and had a DASS anxiety score of 2, which as within the range

of normal anxiety, as well as DASS Depression score of 0 and DASS Stress score of 6. These two selected donors provided fecal and blood samples, in which the fecal samples were used to colonize the GF mice.

2.3 Colonization of Germ-free Mice

Germ-free NIH Swiss mice (n=27, 13 males, and 15 females) (9-32 weeks old) were obtained from the Axenic Gnotobiotic Unit of McMaster University. Mice from the first experiment (n=15) were gavaged with 200 µl of diluted human fecal samples (1:10 with pre-reduced saline) that was prepared in an anaerobic chamber. Mice from the second experiment (n=12) received a 1:1 mixture of human fecal sample and colonized-mouse fecal sample from the first experiment, which was diluted and administered as described above. The mixture of human and mouse fecal material was used due to the insufficient residual quantities of the human samples. All mice were housed for three weeks in sterilized ventilated racks, on a 12h:12h light-dark cycle with free access to food and water, and were handled in a Level 2 hood by a dedicated technician to minimize bacterial colonization. Immediately before microbiota colonization, blood was collected via facial bleeding for assessment of baseline metabolomics.

All experiments were approved by the McMaster University Animal Care Committee.

2.4 Microbiota Analysis of Colonized Mice

Microbiota analysis of both human donors and colonized mice were be performed by graduate student Ryan Potts, under the supervision of Dr. Surette. Fecal pellets were collected weekly for 5 weeks from each colonized mouse to assess microbiota changes over time. Genomic DNA was isolated from stool samples of donors and colonized mice and V3 fragments of 16S rRNA gene were amplified and sequenced via the Illumina platform (San Diego, CA, USA) to assess microbial diversity and monitor microbial variation over time. From this analysis, Principal coordinates analysis (PCoA) plots were generated to visualize the microbiota profiles of the colonized mice and their respective human donors.

2.5 Behavioral Analysis of Colonized Mice

A total of six validated psychometric tests were used to assess individual mouse behaviour. Five tests were used to measure anxiety-like behaviour in mice: the light preference, step-down, open-field, marble-burying, and digging test. In the light preference test, each mouse was placed in the center of an illuminated box connected to a smaller dark box and the mouse's preference to each compartment was measured for 10 minutes. Higher preference for the illuminated compartment was indicative of more exploratory and less anxious behaviour (Bourin & Hascoet, 2003). The step-down test measured the latency of each mouse to step down from an elevated platform, in which slower latency to step down was indicative of greater anxiety-like behaviour

(Haller & Alicki, 2012). The open field test measured movement of a mouse in an open field over a 10-minute period, in which greater time spent in the center of the field was suggestive of greater exploratory and less anxious-like behaviour (Bailey & Crawly, 2009). We also assessed the neophobic aspect of anxiety via the marble-burying test, which measured the total number of marbles buried by each mouse in a 30-minute period (Deacon, 2006). Lastly, neophobic and obsessive tendencies in anxiety was measured by the digging test, which measured the total time spent digging by each mouse during 3 minutes. Greater time spent digging was indicative of greater anxiety-like behaviour (Deacon, 2006). For measuring depressive-like behaviour in mice, the tail-suspension test was conducted. The tail suspension test assessed learned helplessness by measuring the total time spent immobile when suspended by the tip of the tail for six minutes, in which greater time spent immobile suggested higher depressive-like behaviour (Steru *et al*., 1985).

2.6 Mouse Biomarker Analysis

Following behavioral assessments, at week 5 from colonization, GAD and HC-colonized mice were sacrificed to harvest tissues for analysis. Blood was collected via orbital bleeding and serum was separated through centrifuge spinning at 4 rmp for 20 minutes. Whole brains were harvested and immediately frozen in cold 2-methylbutane. Ceca were also dissected and snap-frozen in liquid nitrogen. Blood, brain, and cecum samples were stored at -80°C until time of analysis.

2.6.1. β-defensin

Cecum samples were collected from colonized mice at the time of sacrifice and used to measure β-defensin concentrations. β-defensin-3, the homolog of human β-defensin-2 (Bals *et al.,* 1999; Burd *et al.,* 2002), was measured to assess whether the innate immune activation profiles of the GAD and HC colonized mice mirrored those of GAD and healthy subjects. β-defensin-3 was measured via ELISA (My BioSource-MBS034940, San Diego, CA, US). Each sample was run in duplicate. Optical density was read at 450nm using ELx808 absorbance reader (BioTek, Winooski, VT, US) and the results were expressed in pg/ml.

2.6.2 Kynurenine/tryptophan ratio

Kynurenine and tryptophan were analyzed in the serum of colonized mice collected at the time of sacrifice to assess tryptophan metabolism and serotonin bioavailability and whether they matched the trends observed in GAD and healthy subjects. Kynurenine and tryptophan concentrations were measured via ELISA (ImmuSmol-ISE2227, Pessac, France), according to manufacturer's instructions. Each sample was run in duplicate. Optical density was read at 450nm using ELx808 absorbance reader (BioTek, Winooski, VT, US) and the results were expressed in ng/ml. The average kynurenine concentration was divided by the average tryptophan concentration to obtain the ratio.

2.7 Immunofluorescent Staining

BDNF immunofluorescent staining was conducted following an established protocol in the laboratory. Briefly, 10 μm-thick coronal slices containing the amygdala and hippocampus were mounted onto Aptex-coated slides. The slides were fixed with cold methanol for five minutes immediately prior to staining. The primary antibody anti-BDNF (Novus Bios, Oakville, ON, Canada), was then applied overnight in a 1/500 dilution. Lastly, the fluorophore conjugated secondary antibody (Invitrogen, Burlington, ON, Canada) was applied in a 1/1000 dilution for one hour and mounted with Prolong Gold Antifade Reagent with DAPI (ThermoFisher Scientific, Mississauga, ON, Canada). To obtain a control for staining, one coronal slice per group received only the secondary antibody. The staining was visualized using Nikon fluorescent microscope and Nikon Instrument Software (NIS)-Elements software (Minato, Tokyo, Japan), in which the images were captured at a consistent gain and exposure to achieve an external control. The images were then quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) by % area of intensity. Image intensity was calculated using consistent thresholds across images to maintain an internal control.

2.8 Brain gene expression

200 μm coronal sections of the hippocampus and amygdala were prepared and stored in 300 μl of RNase-Free solution. RNA extraction was performed using the Micro RNA kit (Qiagen, Toronto, ON, Canada) according manufacturer's instructions. The extracted RNA was used for

gene expression analysis using a custom Nanostring (Seattle, WA, US) code-set developed in house, including 72 genes of interest, as listed in the table below. The results obtained were analyzed with the analysis software nSolver 2.6 (NanoString Technologies, Seattle, WA, US). The Log2 ratios built from the data, which were normalized using the geometric mean, were obtained and uploaded into Ingenuity Pathway analysis software (Qiagen, Redwood, CA, US) for further analysis. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test.

Gene Type	Gene	Target Sequence
Housekeeping	Gapdh	AGGTTGTCTCCTGCGACTTCAACAGCAACTCCCACTCTTCCACCTTCGATGC CGGGGCTGGCATTGCTCTCAATGACAACTTTGTCAAGCTCATTTCCTG
Housekeeping	Pgk1	CCGGCATTCTGCACGCTTCAAAAGCGCACGTCTGCCGCGCTGTTCTCCTCTT CCTCATCTCCGGGCCTTTCGACCTCACGGTGTTGCCAAAATGTCGCTT
Housekeeping	Gusb	AATACGTGGTCGGAGAGCTCATCTGGAATTTCGCCGACTTCATGACGAACC AGTCACCGCTGAGAGTAATCGGAAACAAGAAGGGGATCTTCACTCGCCA
Immune	C3	AAGACTTCCTAAAGAGGCAAGTGCTGACCAGTGAGAAGACAGTGTTGACA GGAGCCAGTGGACATCTGAGAAGCGTCTCCATCAAGATTCCAGCCAGTAA
Immune	T-bet	CACTAAGCAAGGACGGCGAATGTTCCCATTCCTGTCCTTCACCGTGGCTGG GCTGGAGCCCACAAGCCATTACAGGATGTTTGTGGATGTGGTCTTGGTG
Immune	Mapk1	TGCTGAAGCGCCATTCAAGTTTGACATGGAGTTGGACGACTTACCTAAGGA GAAGCTCAAAGAACTCATTTTTGAAGAGACTGCTAGATTCCAGCCAGGA
Immune	CXCR3	GTTGTATGGGGTCTCTGTCTGCTCTTTGCCCTCCCAGATTTCATCTACCTATC AGCCAACTACGATCAGCGCCTCAATGCCACCCATTGCCAGTACAACT
Immune	Cxcr4	GTTTCAATTCCAGCATATAATGGTGGGTCTCGTCCTGCCCGGCATCGTCATC CTCTCCTGTTACTGCATCATCATCTCTAAGCTGTCACACTCCAAGGGC
Immune	CD11b	ATCCCTGTTCAGATCAACAATGTGACCGTATGGGATCATCCCCAGGTCATCT TCTCCCAGAACCTCTCAAGTGCCTGTCACACTGAGCAGAAATCCCCCC
Immune	Gpr44	GAAGCCGCTCTGTCCACTCTTGGAGGAGATGGTCCAGCTTCCAAACCACAG CAACTCTAGCCTCCGCTACATCGACCACGTGTCGGTGCTGTTGCACGGG
Immune	Cd86	CAAAACATAAGCCTGAGTGAGCTGGTAGTATTTTGGCAGGACCAGCAAAA GTTGGTTCTGTACGAGCACTATTTGGGCACAGAGAAACTTGATAGTGTGA
Immune	Myd88	GCTGCAGGCTCAGCTGTTTTCTCCCCAGCAGCGAGGTTTGCATCTTCTTATT CCTTTCACGTTCTCTACCATAGAGGCAATGTCATGGTCCCTCTCAGGG
Immune	Nfkb1	GTCTTACACTTAGCCATCATCCACCTCCACGCTCAGCTTGTGAGGGATCTGC TGGAAGTCACATCTGGTTTGATCTCTGATGACATCATCAACATGAGAA
Immune	GATA-3	CATGCGTGAGGAGTCTCCAAGTGTGCGAAGAGTTCCTCCGACCCCTTCTAC TTGCGTTTTTCGCAGGAGCAGTATCATGAAGCCCGAAAGCGACAGATCT
Immune	Tlr2	GCAGGCGGTCACTGGCAGGAGATGTGTCCGCAATCATAGTTTCTGATGGT GAAGGTTGGACGGCAGTCTCTGCGACCTAGAAGTGGAAAAGATGTCGTTC
Immune	Tlr4	AACGGCAACTTGGACCTGAGGAGAACAAAACTCTGGGGCCTAAACCCAGT

		CTGTTTGCAATTAATAAATGCTACAGCTCACCTGGGGCTCTGCTATGGAC
Immune	Tlr5	CTGGGGACCCAGTATGCTAACTTGACCATTGGTCCAGGGGCTTTCAGAAAC CTGCCCAATCTTAGGATCTTGGACTTGGGCCAAAGCCAGATCGAAGTCT
Immune	IL17a	ACCTCAAAGTCTTTAACTCCCTTGGCGCAAAAGTGAGCTCCAGAAGGCCCT CAGACTACCTCAACCGTTCCACGTCACCCTGGACTCTCCACCGCAATGA
Immune	IL23r	AAGTATTTGGTATGGGTCCAAGCTGTCAATTCCCTAGGCATGGAGAACTCA CAACAACTACACGTCCATCTGGATGATATAGTGATACCTTCTGCGTCCA
Immune	IL22ra2	TAAGCATTGCCTTCTAGGTCTCCTCATCATACTCTTGAGCAGTGCAACAGAA ATACAACCAGCTCGTGTATCTCTGACGCCCCAGAAGGTCCGATTTCAG
Immune	Tollip	CTCAGCATCACTGTGGTACAGGCAAAATTGGCAAAGAATTATGGCATGACT CGTATGGACCCTTACTGCCGTCTGCGTCTGGGCTATGCTGTTTATGAAA
Immune	CCR6	CTACCGTTCTGGGCAGTTACTCATGCCACCAACACTTGGGTTTTCAGCGATG CACTGTGTAAACTGATGAAAGGCACATATGCGGTCAACTTTAACTGTG
Immune	CCR2	ATGAACTAACATAGACAGCTCAGGATTAACAGGGACTTGTGGTTTGTGGTC TGTGGGCTTATCCAAGCATGGTGATTTAGACTCTAAGGTCCGTCTGGAT
Barrier	Defcr1	TGAATGGAACCTGCAGAAAGGGTCATTTATTGTACACGCTCTGCTGTCGCT GAACATGGAGACCACAGAGAACAAGACGAGCATGAGTACTGAGGCCACT
Barrier	Lyz1	TCAGTCTGTTTAACCCTTGAGAGGATGTTCCCAGTGTCATGAGGCATTCAG GAGGACTAGTGAGCTGTGCCTGTCCTGATCTTTCTTAAGTTCCTTCATT
Barrier	Cnlp	AACCCGGCCGCTGATTCTTTTGACATCAGCTGTAACGAGCCTGGTGCACAG CCCTTTCGGTTCAAGAAAATTTCCCGGCTGGCTGGACTTCTCCGCAAAG
Barrier	Reg3g	TACATCAACTGGGAGACGAATCCTTCCTCTTCCTCAGGCAATCACTGTGGTA CCCTGTCAAGAGCCTCAGGATTTCTGAAGTGGAGAGAGAATTATTGTA
Barrier	Muc2	CTATGGGTGCCCAGATTTTGATCCTCCCAGACAGGTTAATGAGACCTGGTG GCTGTGTAACTGTACTATGGCTATTTGCAATCATGACAACGTAGTGGAG
Barrier	Cdh1	TCGAAGTGCCCGAAGACTTTGGTGTGGGTCAGGAAATCACATCTTATACCG CTCGAGAGCCGGACACGTTCATGGATCAGAAGATCACGTATCGGATTTG
Barrier	Cldn2	TCACTTTTCACACTTGAGTCATCGCCCATCAGAAGATACTCTCACAGGTATG CAAAGTCTCCAGTGTGGAACATCTGTGACCAAGGTGTTGTGTTCATGA
Barrier	Cldn1	TTGCTACATCTAGACAACGGGTTCATTAACTCTTGTCCTGCCTATGTTGCAT GAGTCAAGAGGGGGCACAGGCTAACATGGTTTTATATTGGAAAACATG
Barrier	Tjp1	GAGCAGCCGTCATACAGGTATGAGGTCTCAAGCTACACAGACCAGTTTTCT CGGAACTATGACCATCGCCTACGGTTTGAAGATCGAATCCCTACCTATG
Barrier	Ocln	CGCTTATCTTGGGAGCCTGGACATTTTGCTCATCATAAAGATTAGGTGACC AGTGACATCAGCCATGTCCGTGAGGCCTTTTGAAAGTCCACCTCCTTAC
Barrier	Casp8	TTTCATTCAGGCTTGCCAAGGAAGTAACTTCCAGAAAGGAGTGCCTGATGA GGCAGGCTTCGAGCAACAGAACCACACTTTAGAAGTGGATTCATCATCT
Barrier	F2rl1	GCTGGCCATGTACTTCATCTGCTTTGCTCCTAGCAACCTTCTGCTCGTAGTG CATTATTTCCTAATCAAAACCCAGAGGCAGAGCCACGTCTACGCCCTC
Barrier	Tff3	CAGAGCCCTCTGGCTAATGCTGTTGGTGGTCCTGGTTGCTGGGTCCTCTGG GATAGCTGCAGATTACGTTGGCCTGTCTCCAAGCCAATGTATGGTGCCG
Barrier	Rae1	TTGTTGAGCATCAGCACAGCATCCTGGCGACTTGCCGTCTCTCCATTCAGCT CCCTGCTGCGGTTTTCCTGAAACTGAGATTCTCAACTGGAGTCAGATC
Barrier	Pparg	ACCAAGTGACTCTGCTCAAGTATGGTGTCCATGAGATCATCTACACGATGC TGGCCTCCCTGATGAATAAAGATGGAGTCCTCATCTCAGAGGGCCAAGG
Barrier	Nod2	TCCTTCTTGACCATCCTGACCGTGTCCTGTTAACCTTTGATGGCTTGGACGA GTTCAAGTTCCGGTTCACCGACCGGGAGCGCCACTGCTCTCCAATTGA
Barrier	Hif1a	ACCATGATATGTTTACTAAAGGACAAGTCACCACAGGACAGTACAGGATGC TTGCCAAAAGAGGTGGATATGTCTGGGTTGAAACTCAAGCAACTGTCAT
Barrier	TGR5	TCACCTGGGTCAGCTCCCTGTTCTTTGCCAGCCTGCCTGCTCTGGGCTGGAA CCATTGGAGCCCTGATGCCAACTGCAGCTCCCAAGCTGTCTTCCCAGC

Barrier	Trpv1	GAGAAGATGATCCTCAGAGACCTGTGTCGGTTTATGTTCGTCTACCTCGTG TTCTTGTTTGGATTTTCCACAGCCGTAGTGACACTGATCGAGGATGGGA
Barrier	Mylk3	TGGAATTGTCCGTAGCAATCGACAGAATCAGCGAGGTCCTCACTAGCCTCA AGATGTCACAAGGTGGTGGTCAAGAAACCTCATCCAGCAAGCCTGACTG
ENS	GDNF	CAGAGGGGCAAAAATCGGGGGTGCGTTTTAACTGCCATACACTTAAATGT CACTGACTTGGGTTTGGGCTATGAAACCAAGGAGGAACTGATCTTTCGAT
ENS	BDNF	AGTCCCGTCTGTACTTTACCCTTTGGGGTTAGAAGTCAAGTTGGAAGCCTG AATGAATGGACCCAATGAGAACTAGTGTTAAGCCCATTTCCCTAGTCAG
ENS	NOS	GCTGGAAGAGGAATAAATTCCGCCTCACTTATGTGGCAGAAGCTCCAGAG CTGACCCAAGGTCTTTCCAATGTTCACAAAAAGCGAGTCTCAGCCGCCCG
ENS	VIP	GAAATGCCAGGCATGCTGATGGAGTTTTCACCAGCGATTACAGCAGACTTC TGGGTCAGATTTCTGCCAAAAAATACCTTGAGTCACTCATTGGCAAACG
ENS	NPY	GACACTACATCAATCTCATCACCAGACAGAGATATGGCAAGAGATCCAGCC CTGAGACACTGATTTCAGACCTCTTAATGAAGGAAAGCACAGAAAACGC
ENS	SUBP	TGGACATGGCCAGATCTCTCACAAAAGGCATAAAACAGATTCCTTTGTTGG ACTAATGGGCAAAAGAGCTTTAAATTCTGTGGCTTATGAAAGAAGCGCG
ENS	CHAT	TTGGGTCTCTGAATACTGGCTGAATGACATGTATCTAAACAACCGCCTGGC CCTGCCAGTCAACTCTAGCCCTGCTGTGATCTTTGCTCGGCAGCACTTC
ENS	NFAT	GGGCTCACATTGTCCTTGAAGTTCCTCCCTATCATAACCCAGCAGTTACATC TGCCGTGCAGGTGCACTTTTATCTTTGCAATGGCAAGAGGAAAAAAAG
ENS	P75	AACAGGGAGATCAATTGTAGGCTGACACACTCCTTTCTGAATGAGGGCGTC AAGTGCTTGTTGGCAGGGATGGAGTGACTTTCAGGGAAACATCTGGAAA
ENS	CALB	ATGGAAAAATAGGAATTGTAGAGTTGGCTCACGTCTTACCCACAGAAGAG AATTTCTTGCTGCTCTTTCGATGCCAGCAACTGAAGTCCTGCGAGGAATT
ENS	CGRP	CTCCAGGCAGTGCCTTTGAGGTCAATCTTGGAAAGCAGCCCAGGCATGGC CACTCTCAGTGAAGAAGAAGTTCGCCTGCTGGCTGCACTGGTGCAGGACT
ENS	5HT	AGTCCTAAAGACTATGGTGCCCAGACTCTTGTGGGTTCCAACCACTTCTTTC CATGAACTCTCTTGGACTTACTGCCACATTAGCTGGTGACACGGCTGA
ENS	CALRET	GCACCAAGAAGGTTCATGTCATCTTTAACTACAAGGGCAAGAATGTGCTGA TCAACAAGGATATCCGGTGTAAGGATGATGAATTCACACACCTATACAC
ENS	GFAP	CACCCTGGCTCGTGTGGATTTGGAGAGAAAGGTTGAATCGCTGGAGGAGG AGATCCAGTTCTTAAGGAAGATCTATGAGGAGGAAGTTCGAGAACTCCGG
ENS	S100BETA	ACCGAGAATCAAAATTCTGCTCGGCAGACTTCTCCTTTCAGGATGATCGCTT TGTTCCTGGAGGACAGAGGAGGGGAATGGCCAGAGTCTTTTTCTAGTT
ENS	I-FABP	GAGGCCAAGCGATTCTTTAAGAAGGAATAAGTCAACTTCTCAGAGCCTGG AGCAACGCTGAAGAGCTAAGCTGATGTCAGATTTCTTTCTCCATCATGCT
ENS	TAAR1	CGTTTTCCTCGTATGCTGGTGCCCGTTCTTTCTCTGCACGGTCCTGGACCCTT TCCTGGGCTATGTTATCCCACCCTCTCTGAATGACGCACTGTATTGG
ENS	TAAR4	TCTGGGGAGTTCTGGCTTCCTTCATAGCTTTCTTTCTGCCTGGGACAGTCAT GGTAGGGATTTACATACACATTTTCACAGTTGCCCAGAAACATGCCAG
ENS	SEMAPHORIN	GAGAACTCCCTGGTTCTGTTTGAAGGAGATGAAGTGTACTCTACCATCCGG AAGCAGGAATACAACGGGAAGATCCCTCGGTTTCGACGCATTCGGGGCG
ENS	SOX10	GGGGCCCGTGTGCTACTGTGTCATTGCCTAAGAGCTAAGCTGCCCAGGGA CCTGCCTCAAAGCCTGGAGCTGGTTCTGTCCTGTCACTAAGGACGCACTG
ENS	MMP9	CCTCTACAGAGTCTTTGAGTCCGGCAGACAATCCTTGCAATGTGGATGTTTT TGATGCTATTGCTGAGATCCAGGGCGCTCTGCATTTCTTCAAGGACGG
ENS	GABAA	GCTTCAAGGTTATGATAACAAACTTCGTCCAGATATAGGCGTGAGACCCAC AGTGATTGAAACAGATGTTTATGTAAACAGCATTGGACCTGTGGATCCC
ENS	GABAB	TGCCTGTTAAAAACCTGAAGCGTCAAGATGCTCGAATCATCGTGGGACTTT TCTATGAGACCGAAGCCCGGAAAGTTTTTTGTGAGGTCTATAAGGAACG
ENS	NR2B	GGGAAAGCTCTTCGTATAAGGCTTTGTGAAAGAGCCATTACAGTAGGGTG

		AGAGAGGGGGATGTTTTTAGTCATTAACGGTAGGGTTAGTGAGAAAGGG
ENS	NR2D	ACATCACCTGGGATAACCGAGACTACTCCTTCAATGAGGATGGCTTTCTGG TAAACCCGTCACTGGTAGTCATCTCCCTCACCAGAGACAGGACGTGGGA
ENS	HMGB1	GTGGGACTATTAGGATCAAGCAATCTGAACGTCTGTCCTTGAAGGACTGAT AGAAAAGTACCTTCTAATCCTTACACGAGGACTCTCCTTTAACCGCCAT
ENS	PGP9.5	CCTGCTCAGGCTTTCCCAGTGAGCGAGGCCGGCGCTTTATAACAGCAGCCT GGGCGGCTCCACCGGCTGTTTTTTCGGCTCCTCGGGTTTGTGTCTGCAG

2.9 Statistical Analysis

All statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, CA, USA). Figures of group comparisons were presented as box plots displaying the 10^{th}-90^{th} percentile of data variance. Two groups comparisons were done with unpaired Student t-test or Mann-Whitney's test where appropriate. Comparisons between more than two groups were done with one-way ANOVA. Microbiota β-diversity analysis was conducted using the PARMANOVA test on the Bray-Curtis dissimilarity distance matrix. A two-tailed significance criterion of $p= 0.05$ was used for all statistical tests conducted.

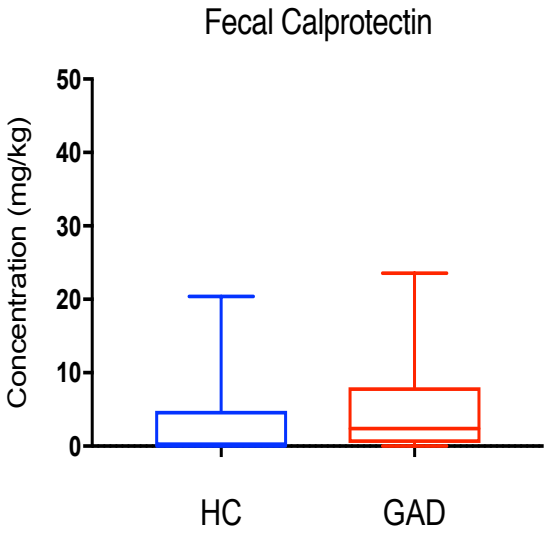
3. RESULTS

3.1 Human Biomarkers

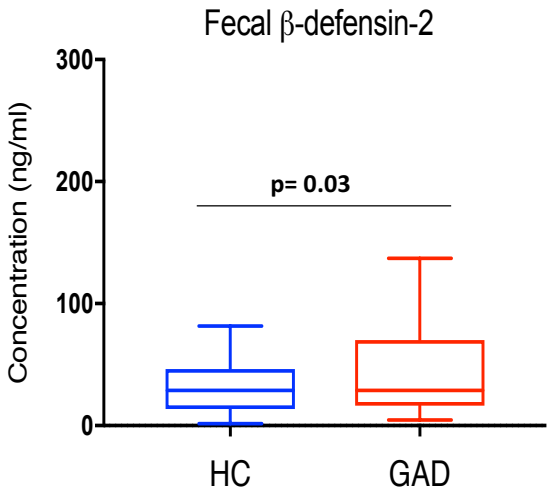
A cohort of GAD and healthy controls (HC) from a larger clinical study were assessed in this project to identify biomarker alterations in patients with anxiety. Calprotectin, β-defensin-2, and CRP were the three biomarkers that were assessed in this study. Calprotectin is a commonly used biomarker to identify overt intestinal inflammation (Von Roon *et al*., 2007; Van Rheenen *et al*., 2010; D'haens *et al*., 2012). There were no significant differences in calprotectin concentrations between GAD (n=69) and HC (n=87) subjects (Unpaired t-test, p= 0.572; Fig.1A), which eliminates overt intestinal inflammation as a confounding variable in anxiety and immune activation. β-defensin-2 was also measured to assess innate immune activation in the subjects (Langhorst *et al*., 2009; Aldhous *et al*., 2009; Zilbauer *et al*., 2010) and potential intestinal dysbiosis. GAD patients (n=69) showed increased β-defensin-2 concentrations than healthy controls (n=87) (Unpaired t-test, p= 0.026; Fig. 1B). This suggests that GAD patients, or at least a subset of them, have greater innate immune activation, which may contribute to anxiety generation. Lastly, CRP was measured to assess systemic inflammation in the subjects (Koenig *et al*., 1999). GAD patients (n=26) had increased CRP levels than healthy controls (n=51) (Unpaired t-test, p= 0.018; Fig. 1C), which further supports the notion that low grade inflammation (CRP of 1000-3000 ng/ml) can contribute to the pathophysiology of depression and anxiety (Howren *et al*., 2009; Valkanova *et al*., 2013; Liukkonen *et al*., 2011).

Figure 1.

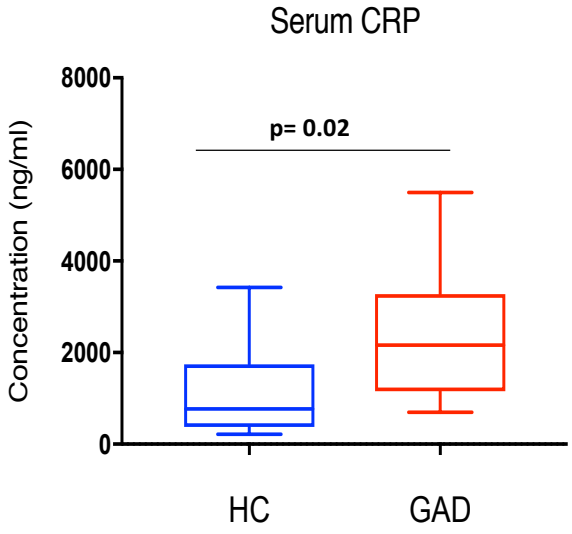
A)


Fecal Calprotectin
Concentration (mg/kg)
50
40
30
20
10
0
HC
GAD

B)


Fecal β-defensin-2
Concentration (ng/ml)
300
200
100
0
p= 0.03
HC
GAD

C)


Serum CRP
Concentration (ng/ml)
8000
6000
4000
2000
0
p= 0.02
HC
GAD

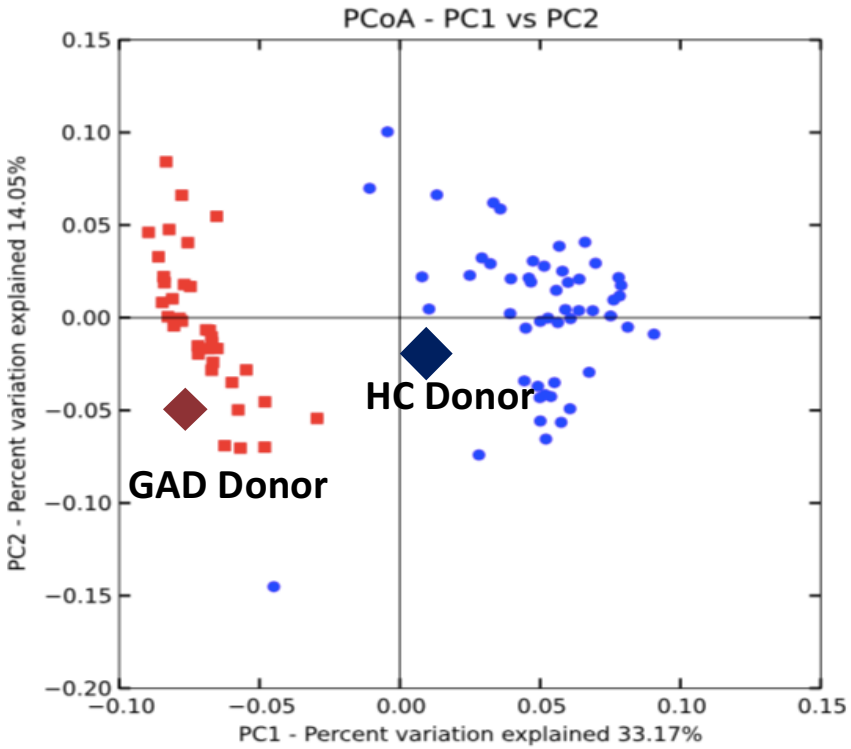
3.2 Behavioural and Biological Profile of Colonized Mice

3.2.1 Microbiota Profile

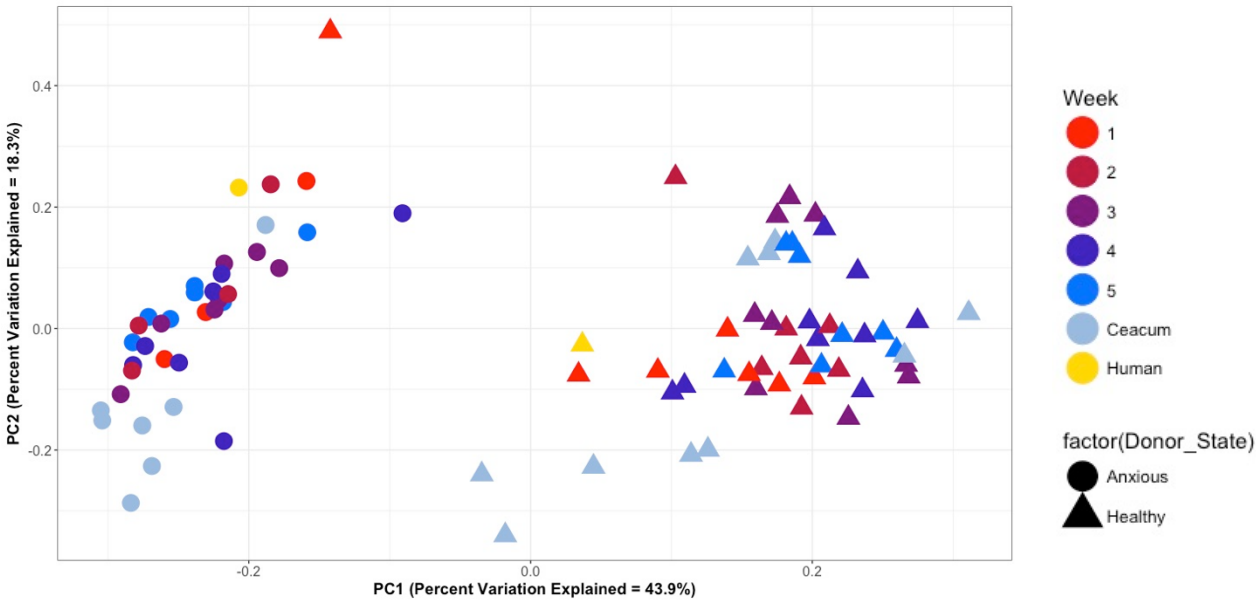
Fecal samples were collected from each GAD and HC participant in this study to obtain microbiota data, as previous studies have identified microbiota differences between depressed and healthy subjects (Naseribafrouei *et al*., 2014; Jiang *et al*., 2015) as well as between IBS patients with comorbid anxiety (IBS-A) and healthy controls (De Palma *et al*., 2017). Microbiota differences have also been found in mice colonized with human microbiota; mice colonized with IBS-A microbiota displayed distinct microbiota than mice colonized with healthy microbiota (De Palma *et al*., 2017). In this study, one GAD patient and one healthy control were selected as donors and their fecal samples were used to colonize germ-free mice (n=15). After this colonization, fecal pellets from each mouse was collected weekly for five weeks. Human and mouse microbiota was assessed for β-diversity by generating PCoA plots using the Bray Curtis dissimilarity metric. GAD-colonized mice (n=7) were found to have different microbiota than HC-colonized mice (n=8) (PERMANOVA Test, R^2=0.41, p=0.001; Fig.2A), that clustered around respective donors. This clustering indicates that recipient mice maintained a microbiota composition similar to that of their human donors. As fecal pellets of colonized mice were collected weekly, week-to-week β-diversity changes were also tracked (Fig. 2B).

Figure 2.

A)


PCoA - PC1 vs PC2
PC2 - Percent variation explained 14.05%
PC1 - Percent variation explained 33.17%
HC Donor
GAD Donor
0.15
0.10
0.05
0.00
−0.05
−0.10
−0.15
−0.20
−0.10
−0.05
0.00
0.05
0.10
0.15

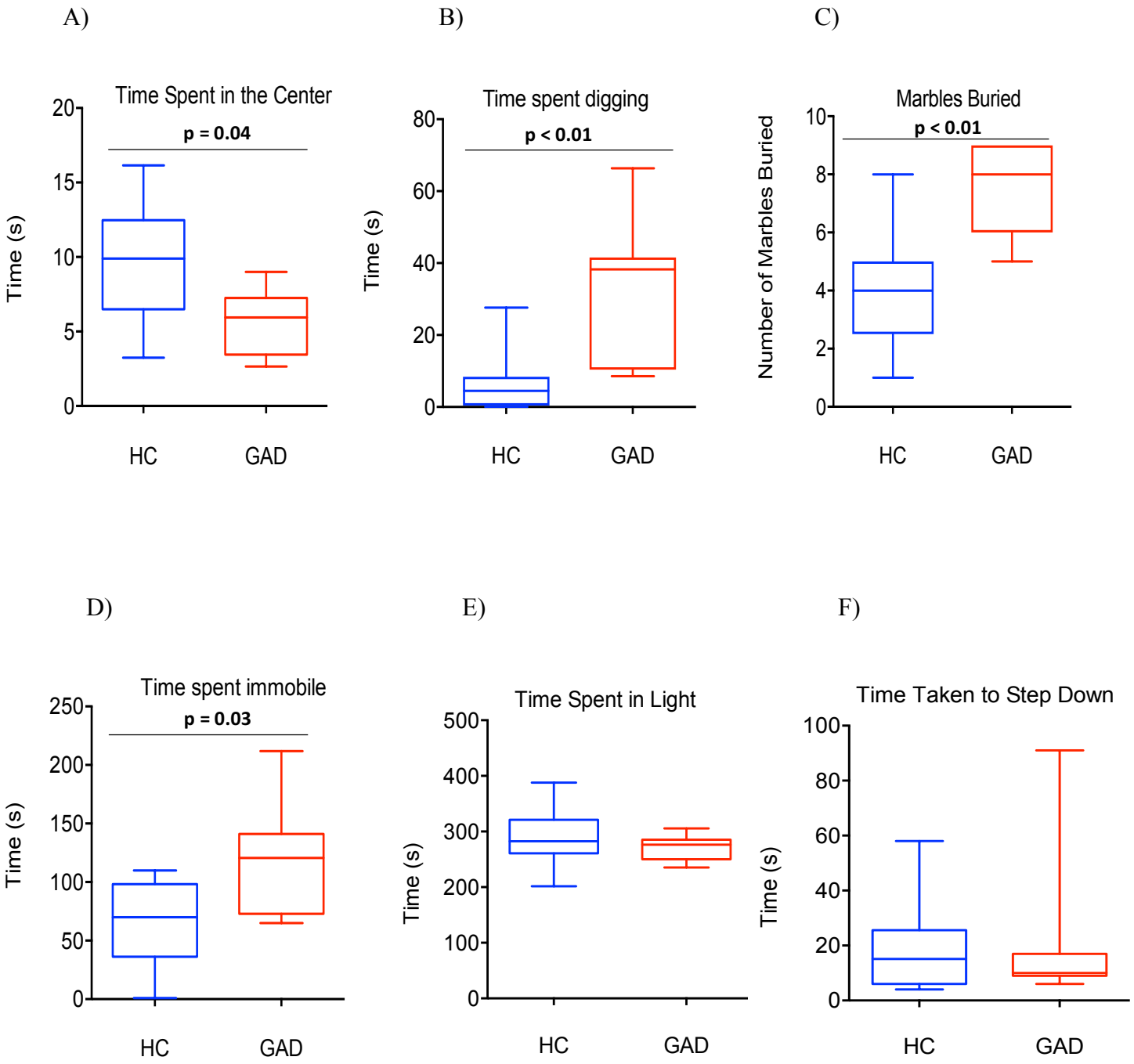
B)


PC2 (Percent Variation Explained = 18.3%)
PC1 (Percent Variation Explained = 43.9%)
0.4
0.2
0.0
-0.2
-0.2
0.0
0.2
Week
1
2
3
4
5
Ceacum
Human
factor(Donor_State)
Anxious
Healthy

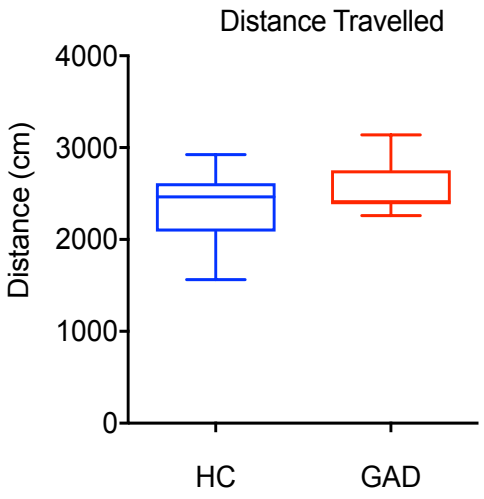
3.2.2 Behavioral Assessment

Three weeks post-colonization, GAD and HC-colonized mice were assessed for anxiety and depressive-like behaviour using six standard psychometric tests. In four of these tests, GAD-colonized mice (n=7) exhibited greater anxiety and depressive-like behavior than HC-colonized mice (n=8). In comparison to HC-colonized mice, GAD-colonized mice spent less time in the center in the Open Field Test (Mann Whitney's test, p=0.04; Fig. 3A), greater time digging in the Digging Test (Mann Whitney's test, p=0.002; Fig. 3B), and buried more marbles in Marble-Burying Test (Mann Whitney's test, p= 0.003; Fig. 3C), all of which indicate greater anxiety-like behaviour. Likewise, GAD-colonized mice expressed greater depressive-like behaviour, as indicated by a greater time spent immobile during the Tail-Suspension Test (Mann Whitney's test, p= 0.03; Fig. 3D). These findings are in agreement with research showing that mice colonized with MDD microbiota exhibit greater anxiety and depressive-like behaviour than HC-colonized mice (Kelly *et al*., 2016). GAD and HC-colonized mice did not, however, exhibit significant differences in two psychometric tests, as they spent a similar amount of time in the brightly lit compartment in the Light/Dark Box Test (Mann Whitney's test, p= 0.35; Fig. 3E) and similar time to step down in the Step-Down Test (Mann Whitney's test, p= 0.73; Fig. 3F). Distance travelled was also measured in mice to eliminate motor deficits or sickness-like behaviour as confounding variables to anxiety-like behaviour. GAD and HC-colonized exhibited no significant differences in distance travelled during the Open Field Test (Mann Whitney's test, p= 0.53; Fig. 3G), which implies that differences in anxiety-like behaviour were not due to motor deficits or sickness.

Figure.3


A)
Time Spent in the Center
p = 0.04
Time (s)
20
15
10
5
0
HC
GAD
B)
Time spent digging
p < 0.01
Time (s)
80
60
40
20
0
HC
GAD
C)
Marbles Buried
p < 0.01
Number of Marbles Buried
10
8
6
4
2
0
HC
GAD
D)
Time spent immobile
p = 0.03
Time (s)
250
200
150
100
50
0
HC
GAD
E)
Time Spent in Light
Time (s)
500
400
300
200
100
0
HC
GAD
F)
Time Taken to Step Down
Time (s)
100
80
60
40
20
0
HC
GAD

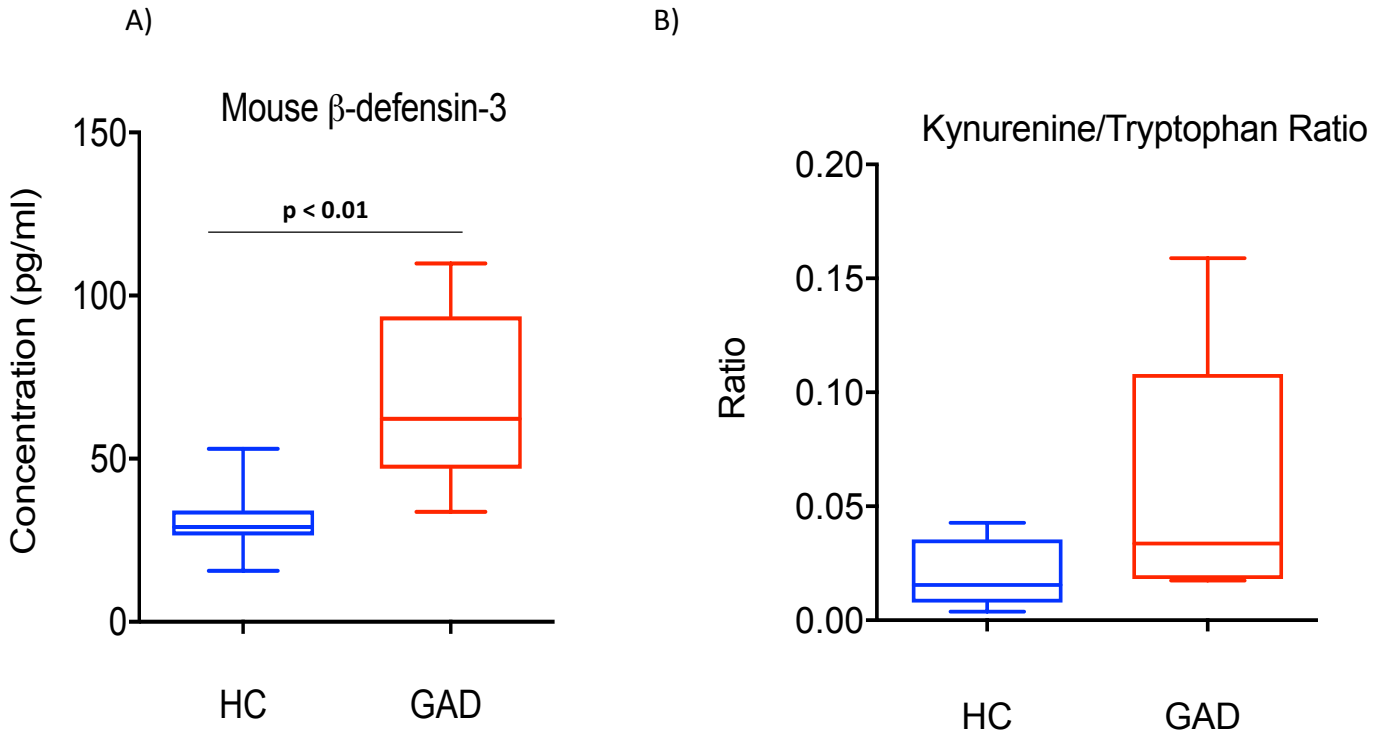
G)



3.2.3 Biomarkers

At the time of sacrifice, cecum and serum samples were collected from each mouse (n=15) to assess for biomarker differences. The two biomarkers measured in the mice were β-defensin-3 in cecum and the kynurenine/tryptophan ratio in serum. Mouse β-defensin-3 is the homolog of human β-defensin-2 (Bals *et al*.,1999; Burd *et al*., 2002) and was measured to see if GAD (n=7) and HC-colonized (n=8) mice showed similar differences as that observed in GAD patients and healthy controls. GAD-colonized mice displayed greater β-defensin-3 concentrations than HC-colonized mice (Unpaired Student's t-test, p= 0.003; Fig. 4A), which mirrored GAD and HC differences in humans. As in human subjects, the increase in β-defensin in GAD-colonized mice indicated an increase in innate immune activation (Langhorst *et al*., 2009; Aldhous *et al*., 2009; Zilbauer *et al*., 2010). The kynurenine/tryptophan ratio was also measured in mice to assess for alterations in tryptophan metabolism and serotonin availability (O' Connor *et al*., 2009). GAD-colonized mice showed a borderline significantly increased kynurenine/tryptophan ratio compared to HC-colonized mice (Unpaired Student's t-test, p= 0.06).

Figure 4.

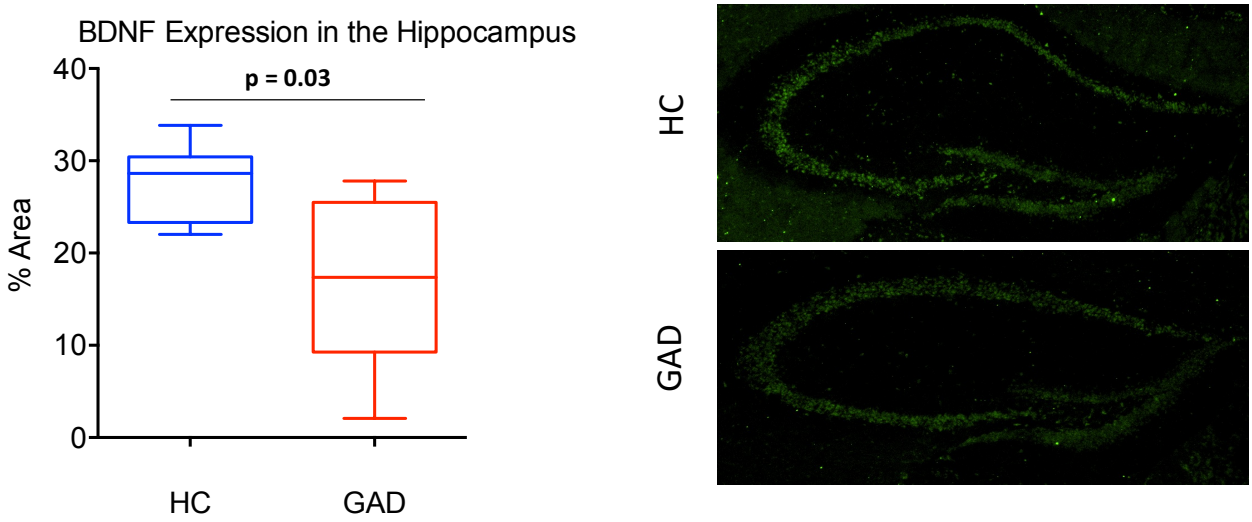

A)
Mouse β-defensin-3
Concentration (pg/ml)
150
100
50
0
p < 0.01
HC
GAD
B)
Kynurenine/Tryptophan Ratio
Ratio
0.20
0.15
0.10
0.05
0.00
HC
GAD

3.2.4 BDNF Immunofluorescent Staining

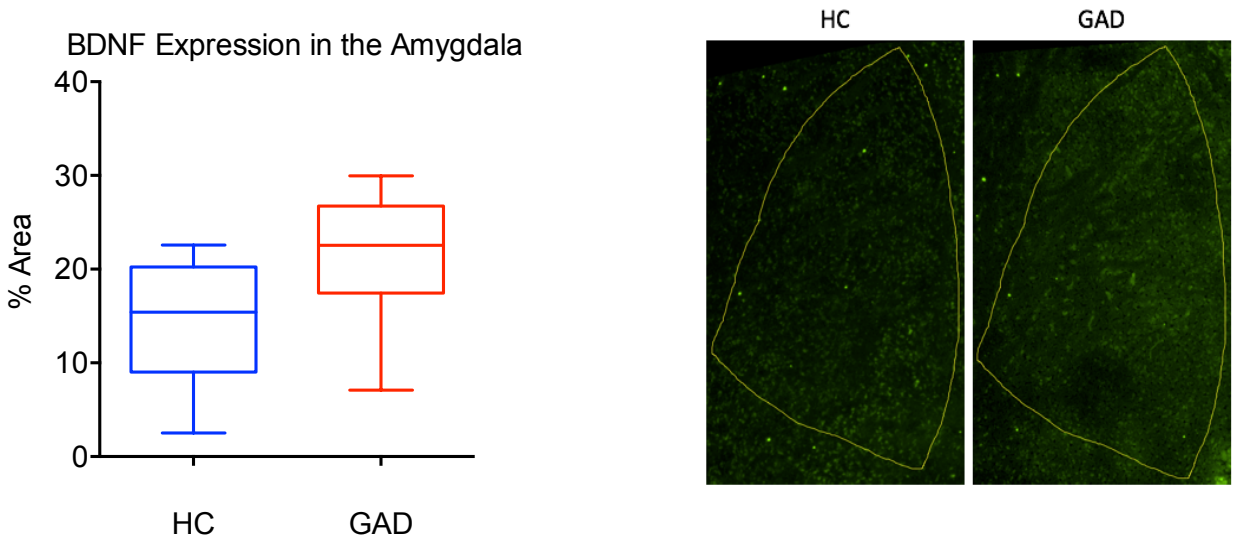
At the time of sacrifice, brains were harvested from all colonized mice (n=15) to assess for differences in central neurotrophins. BDNF was the selected neurotrophin for assessment as it is ubiquitous and has been associated with depression and stress (Smith *et al*., 1995; Duman and Monteggia, 2006; Castren *et al*., 2007). In this study, BDNF expression was measured via immunofluorescent staining. GAD-colonized mice (n=7) showed lower BDNF expression in the hippocampus than HC-colonized mice (n=8) (Unpaired Student's t-test, p= 0.032; Fig. 5A). This finding suggested that GAD-colonized mice had impaired hippocampal neurogenesis, which has been implicated in the pathogenesis of depression (Duman *et al*., 2000; Kempermann *et al*., 2003). In the amygdala, there were no significant differences in BDNF expression but GAD-colonized mice tended to have increased BDNF expression, as compared to HC-colonized mice (Unpaired Student's t-test, p= 0.091; Fig. 5B).

Figure 5.

A)


BDNF Expression in the Hippocampus
p = 0.03
% Area
40
30
20
10
0
HC
GAD
HC
GAD

B)

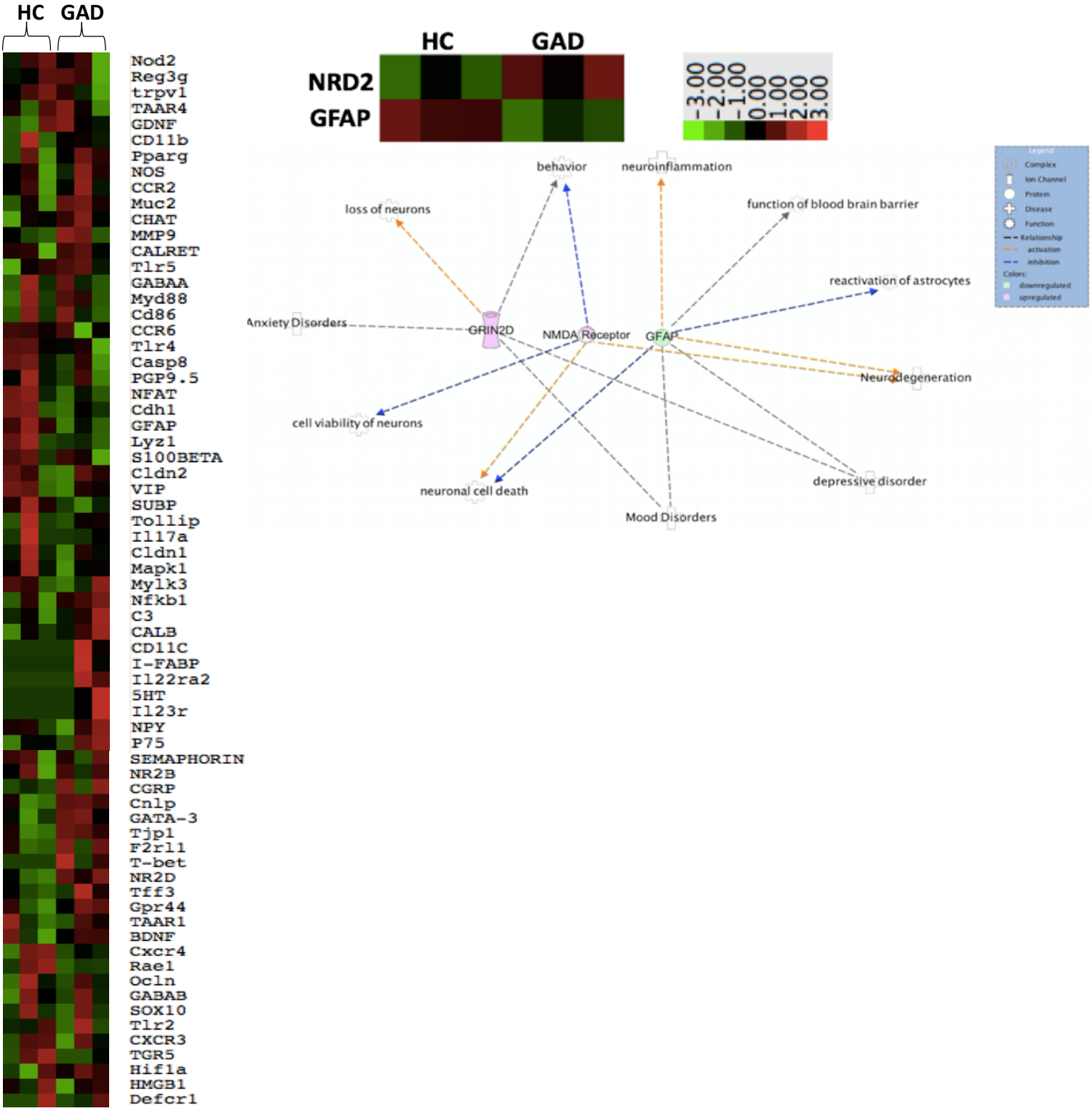

BDNF Expression in the Amygdala
% Area
40
30
20
10
0
HC
GAD
HC
GAD

3.2.5 Brain gene expression

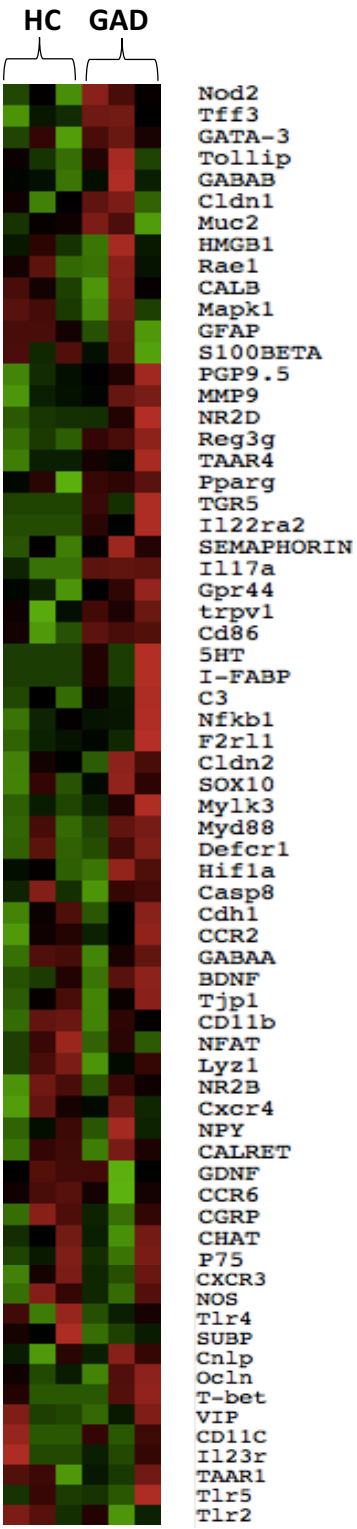
To further assess functional changes in the brain of colonized mice, a brain gene expression assay was conducted. A custom Nanostring assay, which included 72 genes of interest (Fig. 6A), revealed four genes that were statistically different ($p < 0.05$) between GAD (n=3) and HC (n=3) mice in the hippocampus and amygdala (each GAD and HC sample was a mixture of 2-3 mouse brain samples from each respective group). Afterwards, the canonical pathways of the significantly different genes were delineated using IPA software. In the hippocampus, GAD-colonized mice had upregulated Glutamate receptor, ionotropic, N-methyl-o-aspartate 2D (GRIN2D) but downregulated glial fibrillary acidic protein (GFAP), both of which were found to have canonical pathways related to aspects of behaviour and neurogenesis (Fig. 6B). The increased expression of GRIN2D, a NMDA receptor subunit, and decreased expression of GFAP, the primary intermediate filament in astrocytes, is congruent with past studies that have found the same pattern in MDD patients (Wilson *et al*. 2000; Fatemi *et al*., 2004; Si *et al*., 2011; Pergadia, 2012). In the amygdala, GAD-colonized mice, out of the 72 genes read (Fig. 6C), had significantly upregulated expression of Interleukin 17a (IL17a) and regenerating islet-derived protein 3 gamma (reg3g). IL17a and reg3g were both found to have canonical pathways related to aspects of inflammation (Fig. 6D). Our finding of IL17a, a pro-inflammatory cytokine, is in accordance to previous research showing that acute stress in mice increases IL17a gene expression in the amygdala (Vecchiarelli *et al*., 2016). However, the finding of increased reg3g was initially surprising, as reg3g is commonly known as antimicrobial peptide in intestinal cells. Recent research, however, indicates that reg3g can act as macrophage chemoattractant in nerve injury (Namikawa *et al*., 2006).

Figure 6.

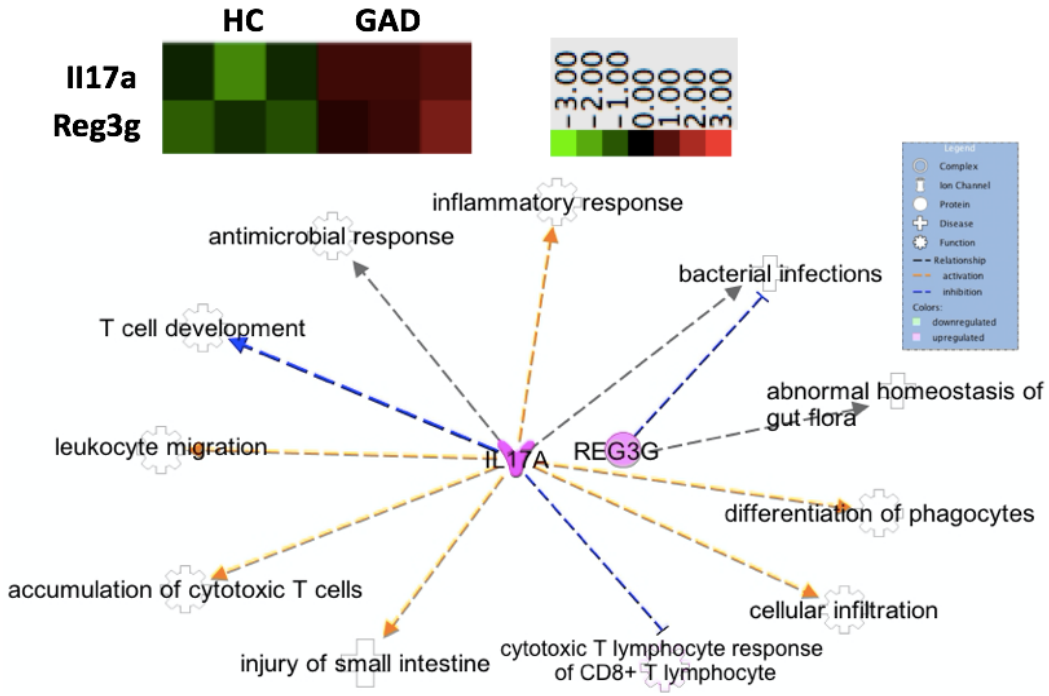
A) B)


HC
GAD
Nod2
Reg3g
trpv1
TAAR4
GDNF
CD11b
Pparg
NOS
CCR2
Muc2
CHAT
MMP9
CALRET
Tlr5
GABAA
Myd88
Cd86
CCR6
Tlr4
Casp8
PGP9.5
NFAT
Cdh1
GFAP
Lyz1
S100BETA
Cldn2
VIP
SUBP
Tollip
Il17a
Cldn1
Mapk1
Mylk3
Nfkb1
C3
CALB
CD11C
I-FABP
Il22ra2
5HT
Il23r
NPY
P75
SEMAPHORIN
NR2B
CGRP
Cnlp
GATA-3
Tjp1
F2rl1
T-bet
NR2D
Tff3
Gpr44
TAAR1
BDNF
Cxcr4
Rae1
Ocln
GABAB
SOX10
Tlr2
CXCR3
TGR5
Hif1a
HMGB1
Defcr1
HC
GAD
NRD2
GFAP
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
behavior
neuroinflammation
loss of neurons
function of blood brain barrier
reactivation of astrocytes
Anxiety Disorders
GRIN2D
NMDA Receptor
GFAP
Neurodegeneration
cell viability of neurons
neuronal cell death
depressive disorder
Mood Disorders
Legend
Complex
Ion Channel
Protein
Disease
Function
Relationship
activation
inhibition
Colors:
downregulated
upregulated

C)


HC
GAD
Nod2
Tff3
GATA-3
Tollip
GABAB
Cldn1
Muc2
HMGB1
Rae1
CALB
Mapk1
GFAP
S100BETA
PGP9.5
MMP9
NR2D
Reg3g
TAAR4
Pparg
TGR5
Il22ra2
SEMAPHORIN
Il17a
Gpr44
trpv1
Cd86
5HT
I-FABP
C3
Nfkb1
F2rl1
Cldn2
SOX10
Mylk3
Myd88
Defcr1
Hif1a
Casp8
Cdh1
CCR2
GABAA
BDNF
Tjp1
CD11b
NFAT
Lyz1
NR2B
Cxcr4
NPY
CALRET
GDNF
CCR6
CGRP
CHAT
P75
CXCR3
NOS
Tlr4
SUBP
Cnlp
Ocln
T-bet
VIP
CD11C
Il23r
TAAR1
Tlr5
Tlr2

D)


HC
GAD
Il17a
Reg3g
3.000
2.000
1.000
0.0000
1.000
2.000
3.00
Legend
Complex
Ion Channel
Protein
Disease
Function
Relationship
activation
inhibition
Colors:
downregulated
upregulated
inflammatory response
antimicrobial response
bacterial infections
T cell development
abnormal homeostasis of gut flora
leukocyte migration
IL17A
REG3G
differentiation of phagocytes
accumulation of cytotoxic T cells
cellular infiltration
injury of small intestine
cytotoxic T lymphocyte response of CD8+ T lymphocyte

3.3 Behavioral and Biological Profile of Colonized Mice Treated with Infliximab

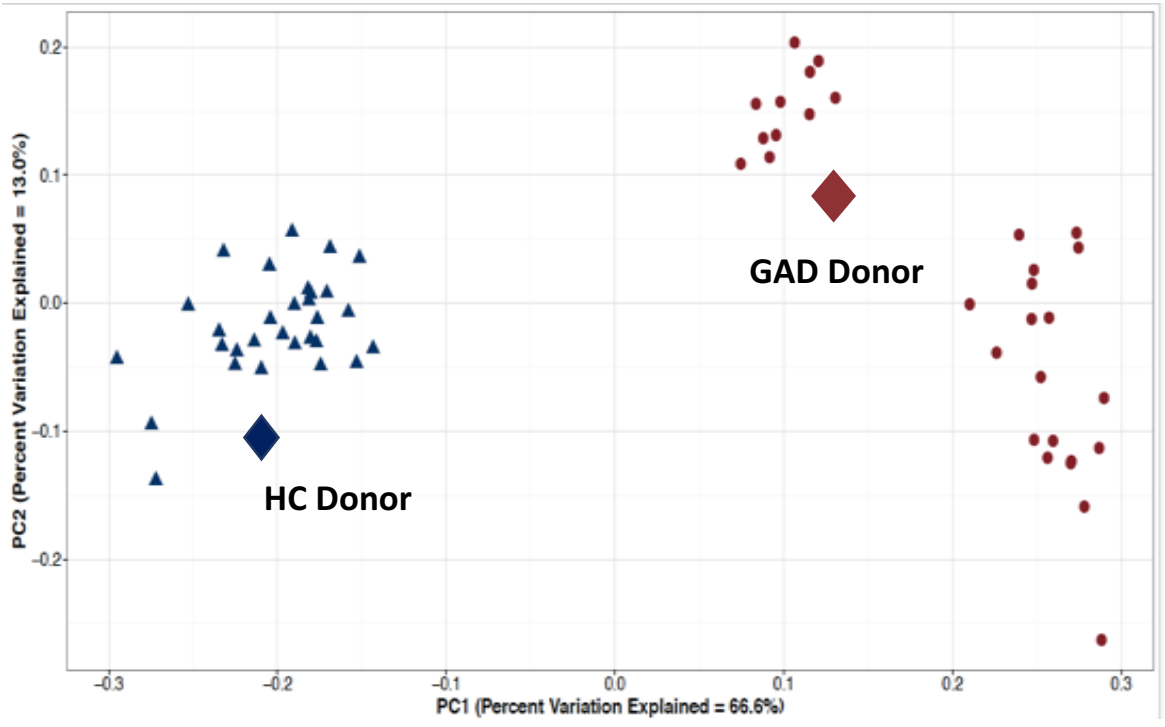
3.3.1 Microbiota Profile

In this second experiment, mice were colonized with microbiota from the same GAD or HC donor from the previous experiment but treated with weekly infliximab injections (5mg/kg) for three weeks. As before, weekly fecal pellets were collected from all colonized, infliximab-treated mice (n= 12). To gauge the microbiota profiles of colonized, infliximab-treated mice, β-diversity was analyzed by generating PCoA plots using the Bray Curtis dissimilarity metric.

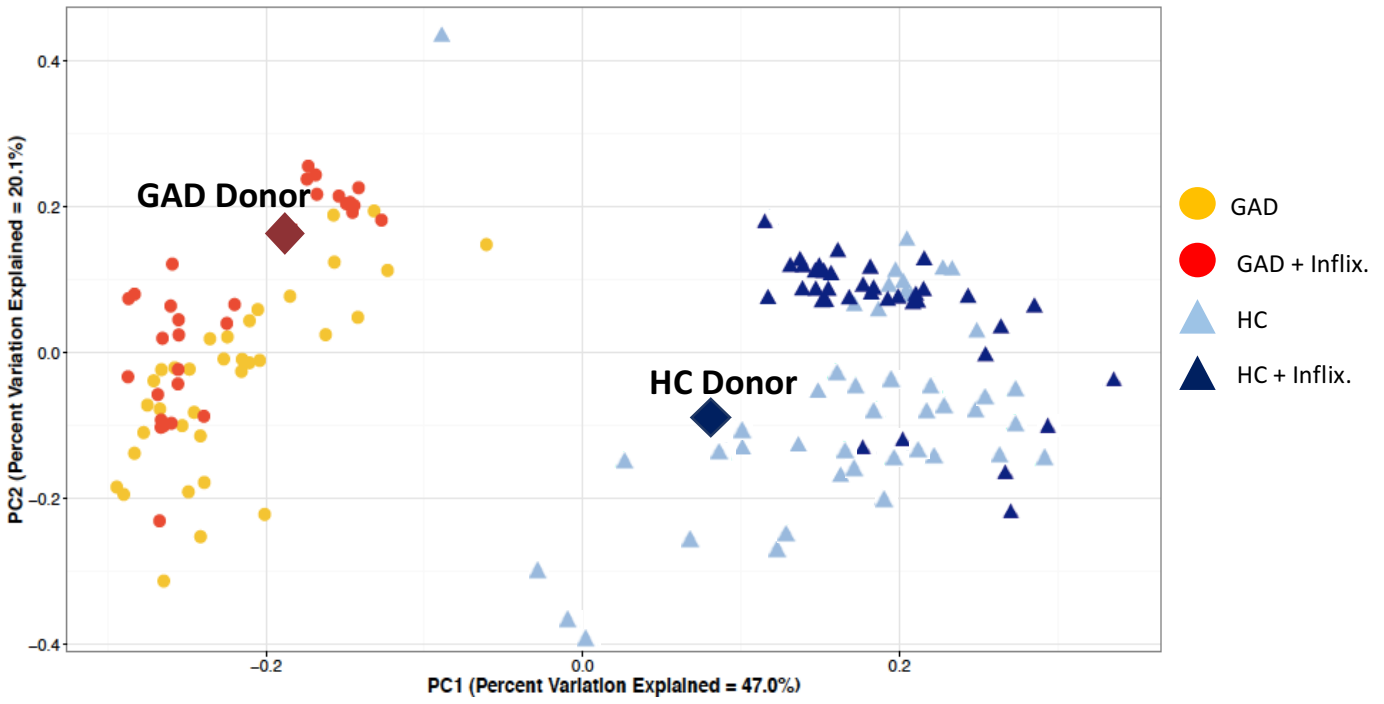
GAD-colonized mice (n=6) were found to have different microbiota than HC-colonized mice (n=6) (PARMANOVA Test, R^2=0.62, p=0.001; Fig.7A), that clustered around the respective human donors. The β-diversity analysis of the previous experiment was overlapped with this experiment's for comparison (Fig. 7B). Week-to-week β-diversity changes of colonized mice of this experiment were also tracked (Fig. 7C).

Figure 7.

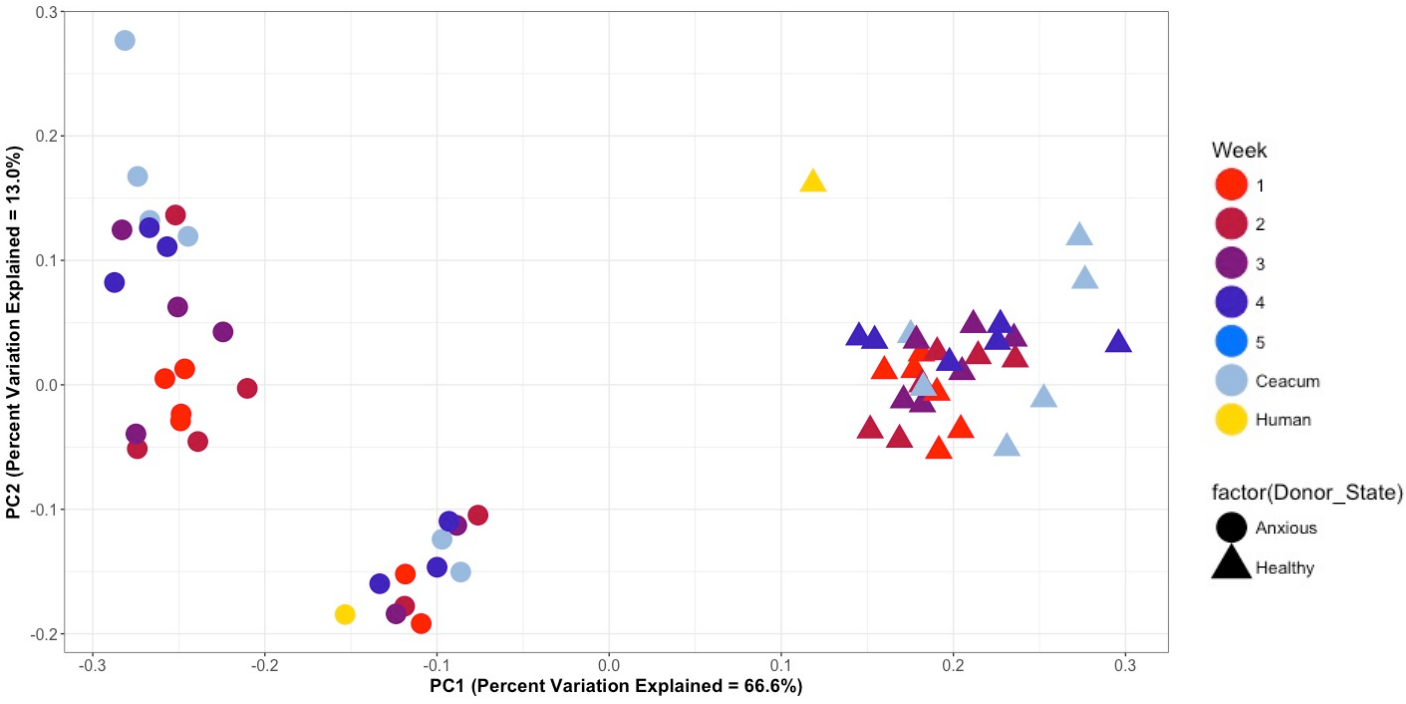
A)



B)


GAD Donor
HC Donor
PC2 (Percent Variation Explained = 20.1%)
PC1 (Percent Variation Explained = 47.0%)
0.4
0.2
0.0
−0.2
−0.4
−0.2
0.0
0.2
GAD
GAD + Inflix.
HC
HC + Inflix.

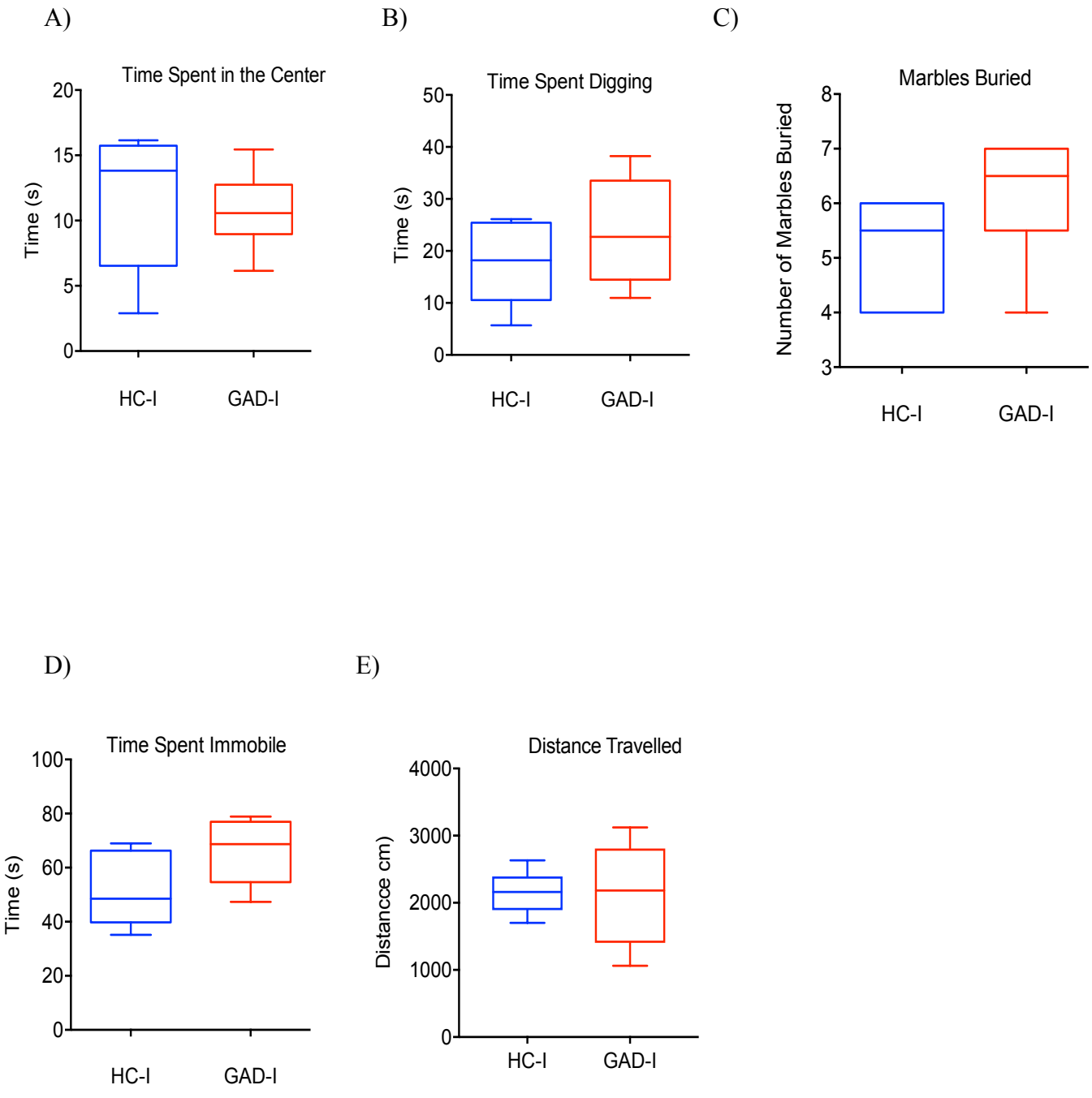
C)


PC2 (Percent Variation Explained = 13.0%)
PC1 (Percent Variation Explained = 66.6%)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
Week
1
2
3
4
5
Ceacum
Human
factor(Donor_State)
Anxious
Healthy

3.3.2 Behavioral Assessment

As in the previous experiment, behavioral assessment of colonized, infliximab-treated mice began three weeks post-colonization. Infliximab was the choice of treatment as research has shown that infliximab can alleviate symptoms of anxiety and depression in both humans (Raison *et al*., 2013; Horst *et al*., 2014) and animal models (Karson *et al*., 2013). To assess whether infliximab treatment could attenuate anxiety and depressive-like behaviour in this experiment, the four psychometric tests that previously yielded significant results were repeated in this set of mice. Unlike the previous experiment, HC (n=6) and GAD (n=6) colonized mice did not significantly differ in time spent in the center of the Open Field Test (Mann Whitney's test, p=0.485; Fig. 8A), time digging in the Digging Test (Mann Whitney's test, p=0.394; Fig. 8B), marbles buried in the Marble-Burying Test (Mann Whitney's test, p=0.194; Fig. 8C), nor in time spent immobile during the Tail Suspension Test (Mann Whitney's test, p=0.093; Fig. 8D). These results indicate that infliximab treatment can normalize anxiety and depressive-like behaviour in GAD-colonized mice. This is in agreement with a previous study which found that chronically stressed rats exhibited reduced anxiety and depressive-like behaviour when given infliximab treatment (Karson *et al*., 2013). To ensure that infliximab treatment did not induce sickness-like behaviour, which could confound anxiety-like behaviour, distance travelled was tracked in all colonized, infliximab-treated mice. However, no significant differences in distance travelled were found between GAD and HC infliximab-treated mice (Mann Whitney's test, p=0.999; Fig. 8E).

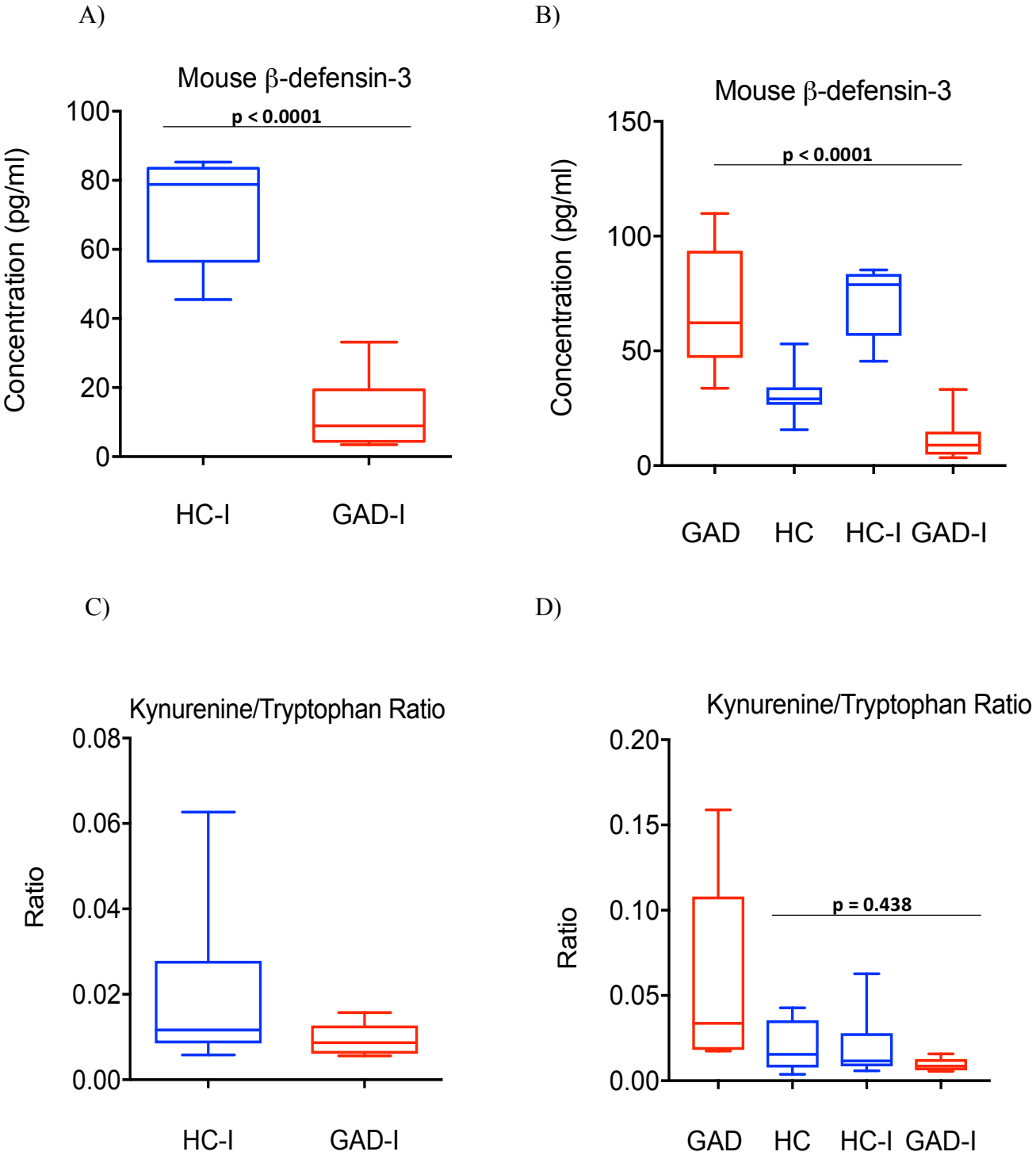
Figure 8.


A)
Time Spent in the Center
Time (s)
20
15
10
5
0
HC-I
GAD-I
B)
Time Spent Digging
Time (s)
50
40
30
20
10
0
HC-I
GAD-I
C)
Marbles Buried
Number of Marbles Buried
8
7
6
5
4
3
HC-I
GAD-I
D)
Time Spent Immobile
Time (s)
100
80
60
40
20
0
HC-I
GAD-I
E)
Distance Travelled
Distancce cm)
4000
3000
2000
1000
0
HC-I
GAD-I

3.3.3 Biomarkers

As described previously, β-defensin-3 and the kynurenine/tryptophan ratio was measured to assess innate immune activation and tryptophan metabolism, respectively. HC infliximab-treated mice (n=6) showed higher β-defensin-3 concentrations than GAD infliximab-treated mice (n=6) (Unpaired t-test, $p < 0.0001$; Fig. 9A). When compared to the colonized mice in Experiment 1, infliximab treatment decreased β-defensin levels in GAD colonized mice but increased β-defensin levels in HC-colonized mice (One-way ANOVA, $p < 0.0001$; Fig. 9B). These results suggest that infliximab treatment can attenuate elevated β-defensin-3 in GAD-colonized mice. Tryptophan metabolism became normalized with infliximab treatment as HC and GAD infliximab-treated mice had similar kynurenine/tryptophan ratios (Unpaired t-test, $p=0.319$; Fig. 9C) and all infliximab-treated mice had similar ratios as HC-colonized mice that did not receive infliximab treatment (One-way ANOVA, $p=0.438$; Fig. 9D). This finding further suggests that infliximab treatment can divert tryptophan metabolism from the kynurenine pathway into the serotonin pathway, as TNF-α induces kynurenine production through IDO (Dantzer *et al*., 2008).

Figure 9.

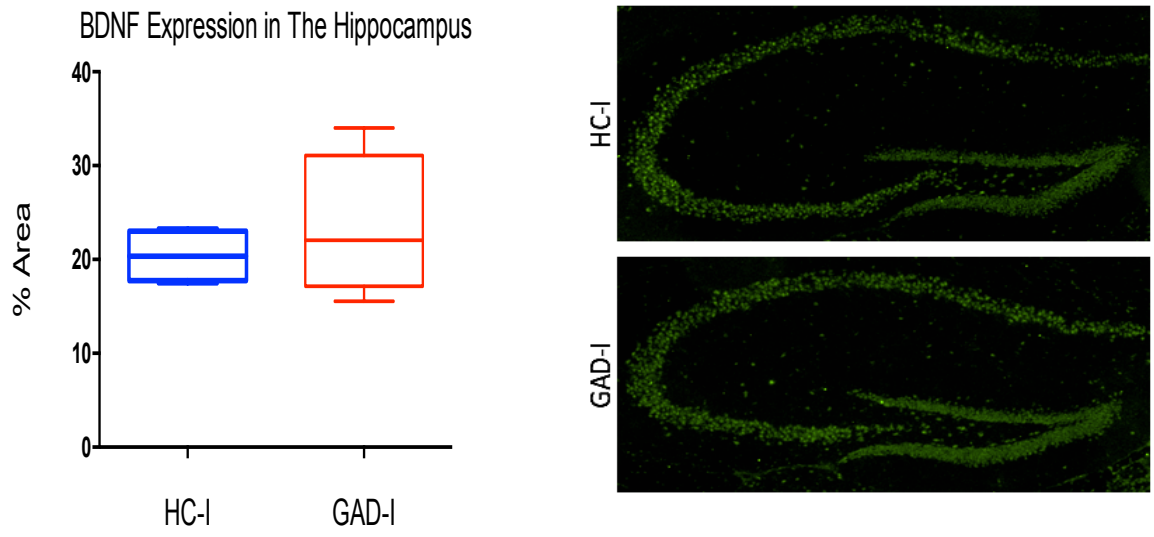

A)
Mouse β-defensin-3
p < 0.0001
Concentration (pg/ml)
100
80
60
40
20
0
HC-I
GAD-I
B)
Mouse β-defensin-3
p < 0.0001
Concentration (pg/ml)
150
100
50
0
GAD
HC
HC-I
GAD-I
C)
Kynurenine/Tryptophan Ratio
Ratio
0.08
0.06
0.04
0.02
0.00
HC-I
GAD-I
D)
Kynurenine/Tryptophan Ratio
p = 0.438
Ratio
0.20
0.15
0.10
0.05
0.00
GAD
HC
HC-I
GAD-I

3.3.4 BDNF Immunofluorescent Staining

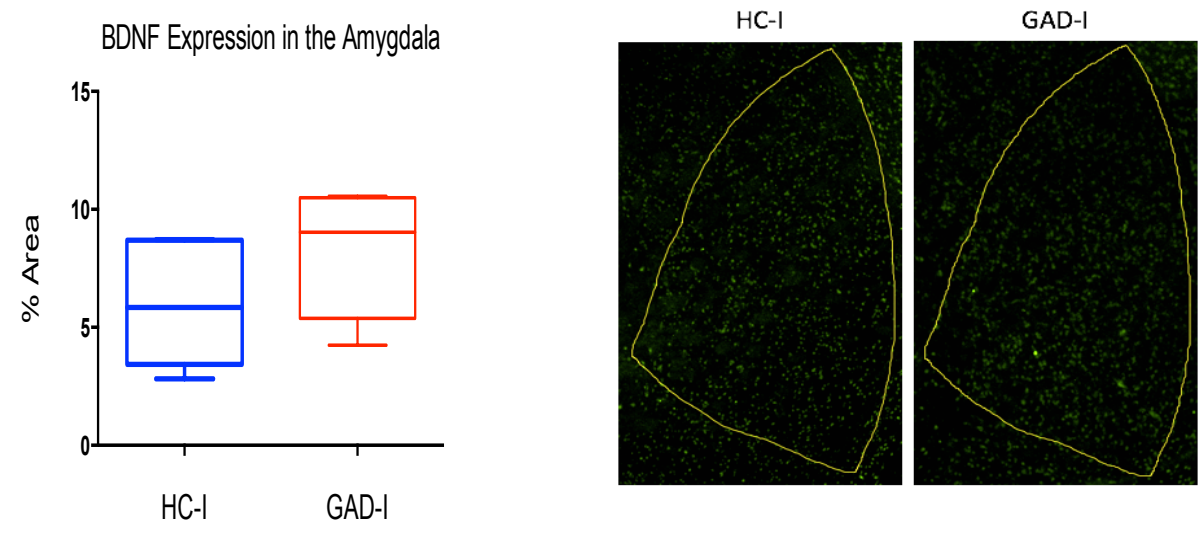
To evaluate whether infliximab treatment could alter central neurotrophin levels, brain BDNF expression was measured as described in the first experiment. Immunofluorescent staining was used to measure BDNF expression in the hippocampus and amygdala of infliximab-treated mice. GAD (n=6) and HC (n=6) infliximab-treated mice showed no significant differences in hippocampal BDNF expression (Unpaired Student's t-test, p=0.484; Fig. 10A). This finding suggests that infliximab treatment can attenuate BDNF expression in the hippocampus, which has been implicated as an important factor in alleviating depression (Saarelaimen *et al*., 2003). Likewise, in the amygdala, GAD and HC infliximab-treated mice showed no significant differences in BDNF expression (Unpaired Student's t-test, p=0.247; Fig.2B), which suggests that infliximab treatment may also help attenuate BDNF expression in the amygdala.

Figure 10.

A)


BDNF Expression in The Hippocampus
% Area
40
30
20
10
0
HC-I
GAD-I
HC-I
GAD-I

B)


BDNF Expression in the Amygdala
% Area
15
10
5
0
HC-I
GAD-I
HC-I
GAD-I

4. DISCUSSION

Depression and anxiety are highly prevalent; depression is the leading cause of disability worldwide and anxiety disorders affect up to 29% of people (WHO, 2012; Swinson *et al*., 2006). However, depression and anxiety are etiologically heterogeneous disorders and their pathophysiology remains poorly understood (Hasler, 2010). In recent years, studies have shown that the intestinal microbiota can affect brain function and behaviour (Goehler *et al.,* 2008; Bercik *et al*., 2011; Clarke *et al.,* 2013). In the present study, we investigated the functional relationship between the gut microbiota and the expression of anxiety and depression. Our results revealed that GAD patients and healthy controls had similar levels of calprotectin but differing levels of β-defensin-2 and CRP. When we colonized GF mice with microbiota from GAD patients and healthy controls, we observed that GAD-colonized mice exhibited different microbiota, increased anxiety and depressive-like behaviour, increased mouse β-defensin-3, altered kynurenine/tryptophan ratios, and altered brain BDNF and brain gene expression, as compared to mice colonized with HC microbiota. However, the majority of these changes were attenuated when mice were treated with infliximab.

4.1 Biological Measurements in Human Subjects

Three different biomarkers were measured in fecal and blood samples of GAD patients and healthy controls. Both GAD and HC subjects both had low levels of fecal Calprotectin with no significant differences between the groups. This implies that neither group had overt intestinal

inflammation (Konikoff *et al*., 2006; D'haens *et al*., 2012) that could account for their differences in anxiety and depression scores. Overt GI inflammation was an important confounding factor to eliminate, as studies have shown that patients with IBD often present with psychiatric comorbidities, namely depression and anxiety (Folks, 2004; Fuller Thomson & Sulman, 2006; Walker *et al.,* 2008). However, GAD patients did have significantly higher β-defensin-2 concentrations than healthy controls. Such a difference in β-defensins suggests that GAD patients have increased innate immune activation originating in the gut (Langhorst *et al*., 2009; Aldhous *et al*., 2009; Zilbauer *et al*., 2010), likely due to microbial dysbiosis. This result is consistent with the finding that the immune system is regulated by the intestinal microbiota (Macpherson & Uhr 2004) and as such, perturbations of the intestinal microbiota can lead to immune activation (Dinan *et* al., 2013). CRP was also measured in the serum of the study subjects. Compared to healthy controls, GAD patients had higher CRP levels, which is consistent with the finding that CRP is associated with symptoms of anxiety (Liukkonen *et al*., 2011) and depression (Liukkonen *et* al., 2006; Howren *et al*., 2009; Valkanova *et al*., 2013).

It is important to note, however, that the β-defensin-2 difference between GAD and healthy controls was primarily driven by a subset of GAD patients with very high β-defensin-2 levels. This subset of GAD patients may represent a subgroup of patients whose microbial induced immune activation plays a significant role in their anxiety disorder. It is thus important for future studies to include a GAD donor with a low β-defensin-2 concentration to test whether β-defensin-2 affects the level of anxiety expressed. This future experiment could better elucidate whether innate immune activation is necessary for the expression of anxiety.

The difference in β-defensin-2 levels between GAD and HC subjects was smaller compared to that of CRP, which likely suggests that β-defensin-2 is not as inclusive marker for anxiety as CRP is. Although CRP likely captures a larger range of patients with depression and anxiety, it provides little specificity, as it is a general marker for inflammation originating in different organs (Koenig *et al*., 1999; Emerging Risk Factors Collaboration; 2010). On the contrary, β-defensin-2 is highly specific as it represents innate immune activation in the intestinal epithelium (Langhorst *et al*., 2009; Aldhous *et al*., 2009; Zilbauer *et al*., 2010). High β-defensin-2 concentrations in GAD patients thus may serve as a useful tool for identifying patients with innate immune activation driven by gut dysbiosis.

4.2 Effects of the Gut Microbiota in the Expression of Anxiety and Depression

16S rRNA gene analysis revealed that GAD and HC-colonized mice had different microbiota profiles that clustered around their respective human donors. These results indicate that the microbiota profiles were successfully transferred from the human donors into the recipient mice. Furthermore, the microbiota analysis revealed that GAD and HC colonized mice had distinct microbiota profiles, which may contribute to the observed behavioral variances. GAD and HC-colonized mice underwent the same experimental interventions, behavioral testing regimen, and diet.

4.2.1 Microbiota Effects on Behaviour

In comparison to HC-colonized mice, GAD-colonized mice exhibited increased anxiety and depressive-like behaviour, as measured by the open field, digging, marble-burying, and tail-suspension tests. These behavioral results are consistent with published literature. For example, mice colonized with microbiota from IBS patients with comorbid anxiety exhibited an increase in anxiety-like behaviour, as compared to mice colonized with microbiota from healthy controls or from IBS patients without anxiety (De Palma *et al*., 2017). Likewise, mice colonized with pooled microbiota from MDD patients displayed increased depressive-like behaviour (Kelly *et al*., 2016). However, our study is the first to show that microbiota from patients with clinical anxiety increases anxiety-like behaviour in a murine host. Our results lend support to the hypothesis of "bottom-up" communication of the gut-brain axis, in that the gut can communicate with the brain via the microbiome and induce behavioral changes (Bercik *et al*., 2012; Foster *et al*., 2013). Currently, however, it is unknown how the microbiome can modify behaviour although studies suggest that it may be mediated through inflammation (Petra *et al*., 2015), neurochemical alterations (Neufeld *et al*., 2011), neurotransmitter metabolism (O' Mahony *et al*., 2015) or through communication via the vagus nerve (Konsman *et al*., 2000; Bravo *et al*., 2011). Our study therefore included immune, neural, and tryptophan metabolism measurements to study the functional effects of microbiota in the expression of anxiety.

4.2.2 Microbiota Effects on Biomarkers

β-defensin was measured in the cecum of GAD and HC-colonized mice to assess whether the recipient mice displayed similar β-defensin concentrations as their respective donors. Like their human donors, GAD-colonized mice had significantly increased β-defensin concentrations than

HC-colonized mice. The high β-defensin concentrations in GAD-colonized mice suggests that GAD microbiota can induce greater innate immune activation than HC microbiota (Langhorst *et al*., 2009; Aldhous *et al*., 2009; Zilbauer *et al*., 2010). The finding that more anxious mice have higher β-defensin levels is relatively novel, as only one other study has observed this same relationship (De Palma *et al*., 2017).

When we analyzed kynurenine/tryptophan ratios GAD colonized mice showed a trend for a higher ratio than HC colonized mice. Although the difference did not reach statistical significance, there was visual distinction between the mean ratios, in that GAD-colonized mice exhibited an elevated kynurenine/tryptophan ratio than HC-colonized mice. A higher kynurenine/tryptophan ratio implies that GAD-colonized mice have altered tryptophan metabolism, in which more tryptophan has been converted into kynurenine rather than into serotonin (Borre *et al*., 2014). Furthermore, a higher kynurenine/tryptophan ratio suggests possible inflammation-driven depression, as the conversion of tryptophan to kynurenine is catalyzed by cytokine-induced IDO (Slyepchenko *et al*., 2014). Our results align with previous a previous finding that mice colonized with MDD microbiota displayed greater kynurenine/tryptophan ratios than mice colonized with HC microbiota (Kelly *et al*., 2016).

4.2.3 Microbiota Effects on Central Neurotrophins

GAD colonized mice also exhibited altered BDNF expression in limbic structures of the brain. In comparison to HC-colonized mice, GAD-colonized mice had decreased BDNF expression in the hippocampus, a neural structure responsible for retention of episodic and spatial memory

(Burgess *et al*., 2002). Lower BDNF in this region suggests decreased neurogenesis and synaptogenesis in the hippocampus (Huang and Reichardt, 2001; Poo, 200; Lu, 2003). Indeed, impaired hippocampal neurogenesis is hypothesized to contribute to the pathogenesis of depression (Duman *et* al., 2000; Kempermann *et al*., 2003). Supporting evidence for this hypothesis includes the finding that people with depression often suffer from memory loss and hippocampal volume decrease (Burt *et* al., 1995; Frodl *et al*., 2002; Hickie *et* al., 2005). Moreover, it is known that depression is associated with decreased BDNF levels in the hippocampus (Chen *et al*., 2001) and that BDNF is necessary for the therapeutic effects of antidepressants (Saarelaimen *et al*., 2003). GAD-colonized mice also exhibited a pattern of increased BDNF expression in the amygdala, a limbic structure specialized for emotional processing and fear conditioning (Phelps, 2004). Overall, these results are consistent with the finding that microbial alterations in mice can lead to changes in BDNF levels (Bercik *et al*., 2011). However, our study is the first to show that microbiota from GAD patients can result in altered BDNF expression in limbic structures of the brain.

4.2.4 Microbiota Effects on Brain Gene Expression

Neurotrophin measurements were supplemented by a brain gene expression assay, to further investigate the effect of gut microbiota on the brain. A Nanostring gene expression assay revealed that GAD-colonized mice had upregulated Glutamate receptor, ionotropic, N-methyl-o-aspartate 2D (GRIN2D) but downregulated GFAP expression in the hippocampus. GRIN2D is a subunit of the NMDA receptor, which is the receptor for the primary excitatory neurotransmitter glutamate (Dingledine *et al*., 1999). Over-activation of glutamate receptors can lead to

excitotoxicity, in which central neurons die as a result of overexcitation (Choi, 1992). NMDA receptors are primary players in this excitoxicity due to their high calcium permeability (Sattler & Tymianski, 2001). The increase of NMDA gene expression, and likely excitotoxicity, in GAD-colonized mice is also congruent with the observed decreased hippocampal BDNF levels. Interestingly, the NMDA receptor has also been implicated in the neurobiology of depression and anxiety, supporting our findings of HC and GAD-colonized mice having different GRIN2D and NMDA gene expression. For example, MDD patients have been found to have an increase in glutamate receptor genes, including GRIN2D (Pergadia, 2012). Moreover, NMDA receptor antagonists can reduce depressive symptoms at a faster rate than typical antidepressants (Chandley *et al*., 2014). Likewise, multiple studies have shown that NMDA receptor antagonists lead to anxiolytic effects in rodents (Dunn *et al*., 1989; Corbett & Dunn, 1993; Plaznik *et al*., 1994). Glial fibrillary acidic protein (GFAP) gene expression was also decreased in GAD-colonized mice. GFAP is the primary intermediate filament found in astrocytes and contributes to the structural integrity of astrocytes and the blood-brain-barrier (Eng *et al*., 2000; Liedtke *et al*., 1996). Numerous studies have also identified GFAP to be associated with psychiatric diseases, as MDD patients show decreased levels of GFAP than healthy controls (Johnston-Wilson *et al*., 2000; Si *et al*., 2011; Fatemi *et al*., 2004). Our results are in line with these findings, especially as the GAD donor had comorbid depression.

The Nanostring gene expression assay also revealed gene expression differences in the amygdala of colonized mice. GAD-colonized mice had significantly increased IL17a and Reg3g gene expression, as compared to HC-colonized mice. Interleukin 17a (IL17a) is a pro-inflammatory cytokine produced by activated T_H17 cells, a particular subtype of T-cells. (Schmidt-Weber *et*

al., 2007). IL17a participates in fighting infection and in the pathogenesis of immune-related disorders (Marwaha *et al*., 2012). Our finding that GAD-colonized mice have increased IL17a expression is congruent with the inflammation hypothesis of depression (Maes *et al*., 2008; Maes *et al*., 2009). Animal models of depression have shown upregulation of IL17a (Tallerova *et al*., 2011; Kim *et al*., 2012) and depressed patients have also exhibited increased serum levels of IL17a (Chen *et al*., 2011). Moreover, antidepressant treatment has led to decreased IL17a levels in depressed patients (Munzer *et al*., 2013; Brunoni *et al*., 2014). Although most studies have measured serum levels of IL17a, one recent study has demonstrated that acute psychological stress increases gene expression of IL17a in the amygdala of mice (Vecchiarelli *et al*., 2016). GAD-colonized mice also exhibited an increase in reg3g gene expression. Regenerating islet-derived protein 3 gamma (reg3g) (also named PAPIII) is commonly known as an antimicrobial peptide released by intestinal Paneth cells (Kolls, 2008). It was thus surprising to find increased reg3g expression in a limbic structure of the brain. However, several studies have found that reg3g also assists in the survival of injured brain regions. For example, reg3g has been identified as a macrophage chemoattractant that is released by injured nerves (Namikawa *et al*., 2006). Reg3g is also upregulated following an increase in pro-inflammatory cytokines in the brain (Kunz *et al*., 2009). These observations suggest that reg3g is increased in response to inflammation-driven brain injury and helps provide a suitable environment for neuronal growth. It is possible that reg3g was upregulated in GAD-colonized in response to the increase in IL17a, a pro-inflammatory cytokine.

4.3 Attenuating Effects of Infliximab Treatment on Anxiety and Depression

As GAD-colonized mice showed increased immune activation and inflammation, as suggested by their increased β-defensin, brain gene expression, and altered kynurenine/tryptophan ratio, we conducted a follow-up experiment in which GAD and HC-colonized mice were treated with the anti-inflammatory drug infliximab. Mice were colonized with the same GAD and HC donors as in the first experiment but received three weeks of infliximab treatment. As described previously, behavioral, biomarker, and BDNF measurements were taken from the colonized mice. We found that infliximab treatment attenuated previously found changes; infliximab-treated mice no longer exhibited significant differences in behaviour, kynurenine/tryptophan ratio, or in central neurotrophin expression. These findings suggest that the behavioral, tryptophan metabolism, and neurotrophin differences observed in GAD-colonized mice are likely immune driven and specifically TNF-α dependent.

4.3.1 Attenuating Behaviour

Although the microbiota profiles of GAD and HC colonized mice with and without infliximab treatment were similar, GAD and HC colonized mice without infliximab displayed significant differences in behaviour whereas infliximab-treated mice did not. GAD-colonized mice that received infliximab treatment showed similar anxiety and depression-like behaviour as HC infliximab-treated mice, as measured in the open-field, marble-burying, digging, and tail-suspension tests. As infliximab is a TNF-α antibody, our results indicate that depression and anxiety-like behaviour may be mediated through TNF-α pathways. Our results are in line with

the inflammation hypothesis of depression (Maes *et al*., 2008; Maes *et al*., 2009) and congruent with the study that found that infliximab treatment decreased anxiety and depression-like behavior in a rat model of depression (Karson *et al*., 2013). Our findings provide further support to the notion that infliximab, among other anti-TNF drugs, may act as alternative treatment to decrease psychiatric symptoms. Indeed, several clinical studies have found that anti-TNF-α drugs reduced symptoms of anxiety and depression in patients with a chronic inflammatory disease (Tyring *et al*., 2006; Bassukas *et al*., 2008; Yip *et al*., 2008; Menter *et al*., 2010; Horst *et al*., 2014) or with MDD (Raison *et al*., 2013).

4.3.2 Attenuating Tryptophan Metabolism

Infliximab treatment normalized the kynurenine/tryptophan ratio in GAD-colonized mice as they displayed a similar ratio as HC-colonized mice with and without infliximab but the level was significantly lower than in GAD-colonized mice without infliximab treatment. These results suggest that the increased kynurenine/tryptophan ratio in GAD-colonized mice was likely driven via TNF-α activation and induction of IDO activity (Dantzer *et al*., 2008). Our results provide further support to the inflammation hypothesis of depression (Sutcigil *et al*., 2007; Yang *et al*., 2007).

4.3.3 Attenuating Central BDNF Expression

GAD-colonized mice had previously shown significantly lower BDNF expression in the hippocampus than HC-colonized mice. However, this difference was no longer observed in infliximab treated mice colonized with the same donors. GAD-colonized mice that received infliximab treatment appeared to be protected from the BDNF reduction observed in GAD-

colonized mice in the previous experiment. This observation is consistent with the known literature. For example, in a rat model of depression, chronic infliximab treatment prevented the hippocampal BDNF decrease that was exhibited by rats that only received saline (Sahin *et al*., 2015). In clinical studies, a reduction in hippocampal BDNF has been associated with increased TNF-α expression (Mondelli *et al*., 2011). The exact mechanism by which TNF-α regulates BDNF is yet to be elucidated. Nevertheless, studies have shown that TNF-α affects hippocampal development and function by changing levels of growth and neurotrophic factors in the brain (Aloe *et al*., 1999; Golan *et al*., 2004). In the amygdala, TNF-α inhibition attenuated BDNF changes but in the opposite direction. In our first experiment, GAD-colonized mice displayed a trend for increased BDNF expression in the amygdala than HC-colonized mice; however, GAD and HC infliximab-treated mice showed no differences. It is interesting and, initially, seemingly contradictory that inhibition of TNF-α resulted in increased BDNF in the hippocampus but decreased BDNF in the amygdala in GAD-colonized mice. However, the hippocampus and amygdala play different roles in anxiety and display opposite BDNF expression in mice with anxiety-like behavior, as observed in our first study. Moreover, TNF-α has been recognized as a double-edge sword as it possesses both neuroprotective and neurodegenerative abilities (Saha and Pahan, 2003). It has been proposed that TNF-α can exert its dual and opposing effects via crosstalk of signaling pathways and other cytokines (Perry *et al*., 2002). No study to date, however, has investigated the direct relationship of BDNF levels in the amygdala as a result of altered TNF-α activation. Despite opposing trends in the hippocampus and amygdala, what is consistent in our results is that inhibition of TNF-α attenuates the maladaptive BDNF alterations seen in GAD-colonized mice. This attenuation may occur in opposing directions in the

hippocampus and amygdala via differential signaling pathway crosstalk, but further investigation is warranted.

4.4. Limitations

Although this germ-free mouse study provided valuable insights, it carries limitations that must be considered. Due to the restrictive breeding and upbringing of germ-free mice, litter sizes are small and relatively few mice are available to meet experimental demand. Thus, the first and second experiment of this study employed a small sample size. It is possible that differences between GAD and HC-colonized mice may have been more pronounced had the experiments had a greater sample size and, with that power, trends may have reached statistical significance. Moreover, as this project was a pilot and proof of principle study only one set of donors were used. It is thus possible that the results we observed are limited to this particular set of donors. Repetitions of our experiments with different set of donors are needed to increase validity. It is possible that the microbiota may not contribute to the expression of anxiety in all people but, perhaps, only in those with signs of immune activation. As an animal study, our results also carry the inherent limitation that the observed physiological differences may not be the same in the human body. Future clinical studies, however, can verify whether these behavioral and physiological differences are also seen in patients. Lastly, future studies will need to verify the role of microbiota (and specific OTUs) in relation to TNF-α, innate immune activation, and central neurotrophin expression.

4.5 Conclusion

Our results suggest that GAD microbiota can contribute to the expression of anxiety and depression, accompanied by altered biomarkers, central neurotrophins, and neural gene expression. These findings are consistent with the concept of "bottom-up" communication within the gut-brain axis: that the gut microbiome can influence brain function and behavior. Our results also revealed that inhibition of TNF-α, via infliximab, can attenuate anxiety and depressive-like behaviour as well as central neurotrophin expression. These findings add to the mounting evidence that inflammation, primarily via pro-inflammatory cytokines, is associated with depression and anxiety. Altogether, our results demonstrate that the gut microbiota may serve as a novel avenue in which to advance our knowledge of mental illnesses and explore alternative treatments for depression and anxiety.

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