

THE GUT MICROBIOME IN OBSESSIVE-COMPULSIVE DISORDER

AN EXAMINATION OF THE GUT MICROBIOME IN PATIENTS WITH
OBSESSIVE-COMPULSIVE DISORDER VERSUS HEALTHY CONTROLS

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ABSTRACT

Obsessive-Compulsive Disorder (OCD) is a debilitating, chronic neuropsychiatric disorder estimated to affect approximately 1-2% of the Canadian population. Our understanding of the pathophysiological mechanisms involved in OCD is unclear, as evidenced by the moderate response associated with treatments targeting these putative pathways. As such, there is a need to explore novel mechanisms of disease. Recent research has focused on the gut-brain axis and highlighted the potential role of the gut microbiota in psychiatric conditions. Further, the role of inflammation is also gaining traction in psychiatric research. This thesis investigates the role of these commensal gut bacteria in OCD, by examining stool samples of unmedicated, non-depressed OCD outpatients and healthy community controls. Given that systemic inflammation is a suggested pathway by which gut bacteria affect behaviour, morning levels of C-reactive protein (CRP), interleukin-6 (IL-6) and tumor-necrosis factors- α (TNF- α) were also examined. To our knowledge, this thesis is the first investigation of the gut microbiome in OCD. This thesis describes: (1) a critical review of the literature developing a theoretical basis for a role of microbial dysbiosis in OCD; that (2) three specific genera and species richness/diversity are lower in OCD patients compared to controls; (3) mean CRP, but not IL-6 and TNF- α , is elevated in this sample of OCD patients; and (4) gastrointestinal symptom severity and prevalence of irritable bowel syndrome is higher in OCD. Taken together, this thesis is the first study to provide evidence for microbial dysbiosis in OCD. Although systemic inflammation may not mediate the relationship between reduced diversity and OCD symptomatology, these results provide evidence for mild systemic

inflammation. Further gastrointestinal and psychiatric symptom severity are positively correlated, but not specific to patients with IBS. These results suggest the gut microbiome may be a potential pathway of interest for future OCD research, clinical implications are also made.

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LIST of ABBREVIATIONS

5-HTT	Serotonin Transporter
5-HTTLPR	Serotonin Transporter Polymorphism
5-HT2A	Serotonin 2A Receptor
ADHD	Attention Deficit/Hyperactivity Disorder
APA	American Psychiatric Association
ASD	Autism Spectrum Disorder
BMI	Body Mass Index
BDD	Body Dysmorphic Disorder
CBT	Cognitive Behavioural Therapy
COMT	Catechol-O-Methyltransferase
CRP	C-Reactive protein
CSTC	Cortico-striatal thalamic-cortical
DASS-21	Depression Anxiety and Stress Scale
DAT1	Dopamine Active Transporter 1
DDOCS	Dutch Dimensional Obsessive-Compulsive Scale
DRD3	Dopamine receptor D3
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Edition 5
FDR	False Discovery Rate
FFQ	Food Frequency Questionnaire
GABA	Gamma-aminobutyric acid
GAD	Generalized Anxiety Disorder
GAS	Group A β -hemolytic streptococcal infection
GF	Germ-free
GSRS	Gastrointestinal Symptom Severity Scale
HPA	Hypothalamic-Pituitary-Adrenal
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
MADRS	Montgomery Asberg Depression Rating Scale
MAO-A	Monoamine Oxidase A
MDD	Major Depressive Disorder
MINI	Mini International Neuropsychiatric Interview
NMDA	N-methyl-D-aspartate
OCD	Obsessive-Compulsive Disorder
OCI-R	Obsessive-Compulsive Inventory – Revised
OTU	Operational Taxonomic Unit
PANDAS	Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infections
PANS	Pediatric Acute-Onset Neuropsychiatric Syndrome
PCoA	Principle coordinate analysis

PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PTSD	Post-Traumatic Stress Disorder
qPCR	Quantitative Polymerase Chain Reaction
rTMS	Repetitive Transcranial Magnetic Stimulation
SRI	Serotonin Reuptake Inhibitor
SSRI	Selective Serotonin Reuptake Inhibitor
TSPO	Translocator Protein
SAD	Social Anxiety Disorder
SD	Standard Deviation
SF-LDQ	Short-Form Leeds Dyspepsia Questionnaire
TNF- α	Tumour Necrosis Factor-alpha
Y-BOCS	Yale Brown Obsessive-Compulsive Scale

CHAPTER 1: OBSESSIVE-COMPULSIVE DISORDER

1.1 Definition

Obsessive-Compulsive Disorder (OCD), is a chronic and disabling neuropsychiatric condition which often arises in late adolescence or early adulthood (Abramowitz, Taylor & McKay, 2009). It is characterized by repetitive, intrusive thoughts, urges or mental images that cause anxiety (obsessions) typically paired with repetitive behaviours (compulsions) aimed at reducing this distress or according to rules that must be applied rigidly (American Psychiatric Association, 2013). Individuals may suffer from either obsessions or compulsions, but more commonly suffer from both. OCD is a clinically heterogeneous condition, in which various kinds of obsessions and compulsions exist. However, research suggests that obsessions and compulsions typically form four main symptom dimensions: (1) obsessions about being responsible for causing/failing to prevent harm; checking and reassurance-seeking compulsions; (2) symmetry-based obsessions and ordering and counting rituals; (3) contamination obsessions, washing and cleaning rituals; and (4) repugnant obsessions concerning sex, violence and religion (APA, 2013). Previously, hoarding behaviours or thoughts about acquiring and retaining objects were considered the final symptom dimension; however, the development of the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) created a new diagnostic category of hoarding disorder, which falls under the newly formed Obsessive-Compulsive and Related Disorders section.

1.1.1 DSM-5 Criteria

Previously categorized as an anxiety disorder, the development of the DSM-5 led to the creation of the Obsessive-Compulsive and Related Disorders section. The DSM-5 diagnostic criteria for OCD requires (A) the presence of obsessions, compulsions or both. Obsessions are characterized as recurrent and persistent thoughts, urges or images, that are experienced at some time during the disturbance as intrusive and unwanted, and that in most individuals, cause marked anxiety or distress. The individual may also attempt to ignore, suppress or neutralize such thoughts with some other action or behaviour.

Compulsions are defined as repetitive behaviours or mental acts the individual feels driven to perform in response to an obsession or to a set of rules. However, these behaviours must also aim to prevent or reduce anxiety/distress or prevent some dreaded situation. They should not be connected, in a realistic way, with what they are designed to neutralize or they must be excessive. The obsessions and compulsions must also be (B) time-consuming (>1 hour/day) or cause clinically significant distress or impairment in social, occupational or other important functioning. Finally, they must (C) not be attributed to some other physiological effects of a substance or medical condition or (D) not be better explained by the symptoms of another mental disorder.

1.1.2 Specifiers.

“With poor insight” was previously the sole specifier for OCD in the DSM-IV-TR. The development of the DSM-5 also incorporates this insight related specifier, however, it provides graded options including: “good or fair insight”, “poor insight” or “absent

insight/delusional OCD beliefs”. With up to 30% of individuals with OCD also presenting a lifetime tic disorder, most common in males with OCD onset in childhood, a tic-related specifier has also been included (APA, 2013). This specifier extends to individuals with a current or past history of tic disorder.

1.2 Prevalence and Course

Estimated lifetime and 12-month prevalence rates of OCD have been reported between 1.0%-2.3% and 0.7%-1.2% respectively in adults (Kessler et al., 2005a; Kessler et al., 2005b; Ruscio et al., 2010; Adam, Meinschmidt, Gloster & Lieb, 2012). It has been suggested that approximately 1% to 2% of the Canadian population will experience an OCD episode; with onset typically presenting a bi-modal distribution with a peak at 12-14 years and another at 20-22 years (Rodriguez et al., 2017). Between 30-50% of patients begin to develop OCD symptoms in childhood (Dell’Osso et al., 2016; Stewart et al., 2004), leading to the suggestion that childhood-onset OCD may be a distinct form of the disorder with a different pathophysiological mechanism fueling it (Dell’Osso et al., 2016). Mean onset age has been reported to be approximately 20 years (Rosario-Campos et al., 2001; Kessler et al., 2005a; Ruscio et al., 2010). Meta-analytic evidence suggests that early-onset OCD is associated with the male sex, greater OCD global severity and comorbidity with tics and other obsessive-compulsive spectrum disorders (Taylor, 2011); while a preponderance of late-onset cases are female (Ruscio et al., 2010; Frydman et al., 2014). The condition is also very persistent and chronic in nature with those developing it reporting an average duration of 8.9 years (standard error=1.1) of life (Ruscio et al., 2010). Left untreated, as it is in 14-56% of sufferers (Veldhuis et al., 2012; Torres et al.,

2006), individuals often experience a waxing and waning of symptoms, with few reporting deteriorating symptoms (APA, 2013). Rates of spontaneous remission are lower in adult-onset patients than those with symptom onset in childhood or adolescence, up to 40% of whom report remission by early adulthood (APA, 2013). The course of OCD is often complicated through the presence of comorbid conditions.

1.2.1 Comorbidity

More than 50% of individuals with OCD also have at least one other comorbid psychiatric disorder (Torres et al., 2006; Ruscio et al., 2010). Common comorbidities include a lifetime anxiety (76%) or mood (63%) disorder (Ruscio et al., 2010). Typically, the onset of anxiety disorders but not mood disorders, precedes OCD onset (APA, 2013). Up to 30% of OCD patients also have a lifetime tic disorder, which is most common in male patients with OCD onset in childhood. Obsessive-compulsive related disorders are also more common in patients with OCD than in the general population. These disorders include body dysmorphic disorder (BDD), trichotillomania, and excoriation disorder (APA, 2013). Rates of OCD are also elevated in eating disorders and Tourette's disorder populations. The high prevalence of comorbid conditions with OCD contributes to the increased impairment of the disorder (Ruscio et al., 2010).

1.3 Impairment

While prevalence rates are lower than other anxiety and mood disorders, the impairment associated with OCD is quite severe. This condition is associated with reduced quality of life and the associated impairment extends to many domains of life (Ruscio et al., 2010).

Greater symptom severity has also been related to increased impairment. The observed impairment in OCD can be related to numerous factors, including time occupied by the obsessions and compulsions or avoidance of situations related to symptoms which severely restricts functioning (Ruscio et al., 2010). The symptoms themselves also impair social and occupational functioning by creating unnecessary obstacles in relationships or at school/work. Past-year OCD has been associated with an average of 45.7 days (standard error = 25.9) out of role, with more severe cases reporting considerably more lost days (Ruscio et al., 2010). Negative health consequences can also present as a result of the avoidance of medical facilities, having symptoms that interfere with treatment or development of other medical problems (Ruscio et al., 2010). OCD onset during adolescence can also result in developmental difficulties, particularly when young individuals fail to socialize or function independently.

1.4 Existing Treatments

First-line treatments for OCD include pharmacological and psychological interventions, or a combination of the two.

1.4.1 Pharmacological treatments

Pharmacotherapy recommended for OCD includes serotonin reuptake inhibitors (SRIs) like clomipramine and antidepressant medications (i.e. selective SRIs [SSRI]). Evidence from meta-analyses support the use of SSRIs (Soomro et al., 2008; Ackerman & Greenland, 2002; Piccinelli, Bellantuono & Wilkinson, 1995) with rates of response generally twice that of placebo (Soomro et al., 2008). Approximately 40% to 60% of

patients respond to SRI treatment with a mean improvement in symptoms of 20% to 40% (Jenike, 2004). Selective noradrenaline reuptake inhibitors and other antidepressant medications are typically seen as second- and third-line treatment options (Katzman et al., 2014). Treatment response is commonly delayed, taking between 2 to 6 months to occur (Marazziti & Dell’Osso, 2015). While side effects have been suggested by some to serve as a positive predictor for response, hoarding symptoms, comorbid tics, and schizotypal personality disorder act as negative predictors (Ravizza et al., 1995; Mataix-Cols et al., 1999; Stein 2002). Unfortunately, 40-60% of patients show no or only partial response ($\leq 25\%$ reduction in Y-BOCS) to SRI treatment, as such adjunctive strategies are explored in these treatment-resistant groups (Katzman et al., 2014). Atypical antipsychotics, like risperidone and aripiprazole, are first-line adjunctive treatment options (Katzman et al., 2014). Long-term pharmacological therapy has been examined in relapse prevention and naturalistic follow-up studies, with a meta-analysis of 6 studies revealing a significant reduction in relapse rates with continued SSRI treatment compared to placebo over 6 to 12 months (Donovan et al., 2010).

1.4.2 Psychological treatments

Meta-analytic evidence supports the beneficial effects of psychological treatment, primarily cognitive-behavioural therapy (CBT) for OCD (Gava et al., 2007; Rosa-Alcazar et al., 2008; Jonsson & Hougaard, 2009; Olatunji et al., 2015). Behavioural therapy is useful in adult cases and suggested as the initial choice for pediatric cases of OCD prior to using medication (Katzman et al., 2014). Numerous studies of behavioural therapy, involving 10 to 20 treatment sessions, report that OCD symptoms were at least

“improved” in 85% of patients, while 55% report target symptoms to be “much improved” or “very much improved” (>50% improvement) (Jenike, 2004). Exposure and response prevention is a key component of behavioural therapy and is thought to normalize the cortico-striatal-thalamic-cortical (CSTC) circuitry (Stein 2002). Cognitive interventions have also been thought to play a role given that numerous belief domains are thought to be involved in the disorder. In practice, cognitive-behavioural approaches are most often used either individually or in groups within contexts ranging from self-help instruction (online or books) to the level of treatment in an inpatient intensive care unit (Stein, 2002). Meta-analyses have also revealed no significant differences in efficacy between group and individualized CBT (Gava et al., 2007; Rosa-Alcazar et al., 2008; Jonsson & Hougaard, 2011). Other effective psychological techniques include acceptance and commitment therapy (ACT; Twohig et al., 2010), mindfulness training (Hanstede, Girdon & Nyklicek, 2008), modular cognitive therapy addressing OCD beliefs (Wilhelm et al., 2009) and obsessional doubt (O’Connor et al., 2005), organizational training (Park et al., 2006) and internet-based CBT (Andersson et al., 2012). Follow-up studies also suggest that benefits of CBT are maintained at 1 to 5 years of follow-up (Anand et al., 2011; Whittal et al., 2008; van Oppen et al., 2005).

1.4.3 Alternative Therapies

In patients who have not responded to CBT or multiple medication trials, alternative therapies have been explored. Deep brain stimulation has been approved by the US Food and Drug Administration for treatment of refractory OCD; however, existing studies possess small sample sizes and a sham-control is difficult to achieve in a research setting

(Kohl & Baldermann, 2018). Repetitive transcranial magnetic stimulation (rTMS) may be a promising option, with meta-analytic evidence suggesting protocols using low frequency rTMS and targeting the orbitofrontal cortex or supplementary motor cortex as most promising (Berlim, Neufeld & Van den Eyde, 2013). Capsulotomy or cingulotomy may also be effective at reducing symptoms of severe, refractory OCD, however, this is considered a last resort (Katzman et al., 2014).

1.5 Aetiology

The pathophysiological mechanism behind OCD remains largely unknown; an issue often associated with the heterogeneous symptomatology of the disorder. An integrative model proposed by Pauls et al. (2014) suggested that individuals with OCD may be genetically vulnerable to the impact of environmental factors that may trigger modification of the expression of neurotransmitter systems through epigenetic mechanisms, resulting in specific imbalances between the direct and indirect loops of the CSTC.

1.5.1 Genetics

Given several lines of evidence suggesting a biological basis of OCD (discussed below), a genetic contribution to etiology is plausible. In addition to the environmental factors that may be at play, it has been suggested that the condition likely has a complex pattern of inheritance (Nestadt, Grados & Samuels, 2010). Family studies support the notion of familial transmission in OCD (Nestadt et al., 2010). Rates of OCD are known to be higher within families, with adult family studies reporting OCD prevalence in 6.2% -11.7% in first-degree relatives compared to 0% - 3.3% in relatives of normal controls (Pauls et al., 1995; Alsobrook et al., 1999; Nestadt et al., 2000; Albert et al., 2002; Fyer et al., 2005;

Grabe et al, 2006). Rates of affected relatives with OCD also tend to be higher when the proband presents with comorbid tics and earlier age of onset (Hanna et al., 2005; Miguel et al., 2005). A meta-analysis of adult and pediatric family OCD studies reported an odds ratio for OCD of 4.7 (99% CI, 2.4-9.2) and 25.6 (99% CI, 9.3-70.6) respectively (Taylor, 2013). Similarly, family members are also likely to have the types of obsessions and compulsions displayed by the proband (Alsobrook et al., 1999; Mataix-Cols et al., 2004). Unaffected parents of OCD patients have also been shown to have lower whole blood serotonin concentration and fewer platelet 5HTT (serotonin transporter) binding sites compared to unaffected parents of healthy controls (Delorme et al., 2005).

Over 100 candidate gene studies have been published in OCD (Pauls et al., 2014). These studies were designed based on the existing understanding of OCD neurocircuitry and neurotransmitter dysregulation. As such, genetic variants within pathways for serotonin, dopamine, glutamate or genes involved in immune and white matter pathways have been of primary focus. A review comprehensively outlined results of two meta-analyses of the 230 polymorphisms that have been identified in OCD (Taylor, 2013). OCD was associated with polymorphisms in two serotonin-related genes, the 5HTTLPR (serotonin transporter polymorphism) (Bengel et al., 1999; Billet et al., 1997; Carmarena et al., 2001; Di Bella et al., 2002; Frisch et al., 2000; McDougle et al., 1998; Taylor 2011) and 5HT2A (serotonin 2A receptor)(Enoch et al., 2001; Meira-Lima et al., 2004; Taylor 2013). Variants in COMT (catechol-O-methyltransferase) and MAO-A (monoamine oxidase A) genes were also shown to be associated with OCD, but only in males (Taylor, 2013). Non-significant trends were reported with polymorphisms in two dopamine related

genes, DAT1 (dopamine active transporter 1), DRD3 (dopamine receptor D3), and SLC1A1 (a glutamate system related gene) (Taylor, 2013). Genome-wide association studies revealed several potential associations, further supporting the notion that the genetic component of OCD is likely polygenic (Stewart et al., 2013; Mattheisen et al., 2014).

1.5.2 Neurocircuitry

The CSTC model (also referred to as either the frontostriatal or corticostriatal model) of OCD has been the prevailing description of the underlying neural and pathophysiological mechanisms thought to be involved in the disorder (Posner et al., 2014). The CSTC pathway projects from the cortex to the striatum, from there to the thalamus by way of the globus pallidus and back to the cortex again. There are various CSTC loops that involve different cortical regions and run in parallel through the basal ganglia and thalamus; each loop is thought to be involved in a different neurocognitive domain (Posner et al., 2014). The most agreed upon loops include the sensorimotor, associative (cognitive) and limbic CSTC loops (Posner et al., 2014). In OCD, functional and structural abnormalities have primarily been observed in the limbic CSTC, encompassing the orbitofrontal and anterior cingulate cortices and ventral striatum. These structures have been shown to be hyperactive at rest in OCD patients (adult and pediatric) compared to controls (Maia, Cooney & Peterson, 2008). This hyperactivity further increases with symptom provocation and decreases following successful treatment (Maia et al., 2008; Menzies et al., 2008; Fitzgerald et al., 2011). Gray matter density and volumetric abnormalities have also been noted in these structures (Maia et al., 2008). Finally, studies demonstrating

aberrant functional connectivity in the prefrontal and striatal regions in OCD patients, provide additional support for the CSTC model in OCD (Harrison et al., 2009; Fitzgerald et al., 2011).

1.5.3 Neurochemistry

The primary neurotransmitters implicated in OCD include serotonin, dopamine and glutamate.

1.5.3a Serotonin

The theory of neurotransmitter dysregulation in OCD, particularly of the serotonin system, has been posited on the clinical benefit observed from SRI treatment (Stein, 2002). The earliest evidence for this mechanism involved the observed efficacy of clomipramine in treating OCD (Fernandez-Cordoba & Lopez-Ibor, 1967). Similarly, provocation of OCD symptoms was also demonstrated by a serotonergic agent meta-chlorophenylpiperazine (Zohar et al., 1987). However, static measures of serotonergic function have been inconsistent, and no specific abnormality of the system has yet been identified as a cause (Stein, 2002). Fittingly, the serotonin transporter and the serotonin receptor subtypes implicated in OCD are at their highest in the brain in the ventral striatum (Pauls et al., 2014).

1.5.3b Dopamine

Dopamine has also shown some promise for a mechanistic role in OCD, as it is the primary neurotransmitter in the CSTC loop (Koo et al., 2010). Evidence for dopamine extends from preclinical studies where dopamine agonists lead to stereotypic behaviours

and exacerbation of OCD symptoms and tics in humans (Eagle et al., 2014; Szechtman, Sulis & Eilam, 1998). Similarly, augmentation of SRI treatments with dopamine blocking agents in OCD can also be of benefit in refractory patients (Stein, 2002; Katzman et al., 2014). Therefore, hyperactivity of the dopamine system has been thought to be linked to the pathophysiology of OCD. However, this abnormal activity of the dopamine system may be modulated by the serotonin system. For example, successful treatment with fluvoxamine, an SSRI, is associated with increased dopamine receptor 2 (D2) receptor binding (Moresco et al., 2007). More specifically, it is thought that serotonergic neurons may modulate the CSTC loop, with serotonin binding to 5-HT_{2A} receptors on dopaminergic neurons reducing the release of dopamine (Koo et al., 2010). Therefore, sustained serotonin signalling, as is the case in the presence of an SRI, results in a prolonged inhibitory effect on the dopaminergic system. Similarly, in the absence of serotonin, or presence of a 5-HT_{2A} receptor antagonist, the dopamine neuron may activate a dopamine signalling cascade. As such, the potential serotonin impairment in OCD may in fact also explain the dopaminergic hyperactivity characteristic of OCD (Koo et al., 2010).

1.5.3c Glutamate

The role of glutamate in OCD was first highlighted by studies reporting higher glutamate levels in the cerebrospinal fluid of OCD patients compared to controls (Chakrabarty et al., 2005; Bhattacharyya et al., 2009). Dysfunctional glutamate signalling is likely attributed to reduced glutamate brain concentrations in the anterior cingulate cortex and increased levels of glutamate in the caudate of the CSTC circuit, as demonstrated in magnetic

resonance spectroscopy studies (Rosenberg et al., 2000; Rosenberg et al., 2004). Glutamate-modulating agents have also shown benefit in OCD. Riluzole, which inhibits specific voltage-gated sodium channels, reduces glutamate release in addition to enhancing glial uptake of extrasynaptic glutamate (Coric et al., 2003). Although positive effects have been noted in an open-label study in refractory OCD patients (Coric et al., 2003), only minimal benefits have been observed in controlled trials (Grant et al., 2014; Pittenger et al., 2015). N-methyl-D-aspartate (NMDA) (excitatory glutamate) receptor antagonism has also been proposed to dampen excess glutamate signalling, and reports of memantine and ketamine (two NMDA receptor blockers) have shown positive results (reviewed in Pittenger, Bloch & Williams, 2011).

1.5.4 Neuroinflammation and autoimmunity

Several case series of OCD revealed abrupt onset, episodic course and concurrent neurological abnormalities in a subset of children following a group A β -hemolytic streptococcal (GAS) infection. Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infections (PANDAS), discussed further in CHAPTER 2, brought forth the suggestion of an autoimmune basis to OCD as there are some reports of cross-reacting antibodies against the putamen and basal ganglia in the serum of PANDAS cases (Marazziti, Mucci & Fontenelle, 2018). Although PANDAS is relevant for only a minority of cases, it posits a vulnerability to OCD and tics following infection suggesting that general states of inflammation/autoimmunity may be important in OCD (Attwells et al., 2017). Further, seropositivity of anti-basal ganglia antibodies have been associated with increased risk of primary OCD (Pearlman et al., 2014). Although the validity of this

condition and the role of GAS has been questioned (Marazziti et al., 2018), it initially brought forth the hypothesis of immune dysregulation in OCD. However, a new clinical spectrum of neuropsychiatric disorders has been posited since, termed Pediatric Acute-Onset Neuropsychiatric Syndrome (PANS). While derived from research on PANDAS, PANS is generalized to numerous triggers including environmental conditions, metabolic disorders and/or infections (Quaglieriello et al., 2018).

1.5.4b Neuroinflammation

Neuroinflammation can be measured by observing microglial activation, and translocator protein (TSPO) positron emission tomography (PET) has been utilized to measure this.

Microglia increase expression of TSPO when activated and this is an important component of neuroinflammation. Elevated TSPO binding has been reported in the basal ganglia of children with PANDAS compared to healthy adults (Kumar, Williams & Chugani, 2015). However, data suggesting neuroinflammation in pure OCD is more limited, particularly when considering astroglia density or microglial activation (Najjar et al., 2013). Animal models have suggested that dysfunction and reduction of certain microglial phenotypes may induce OCD-like behaviours (Chen et al., 2010; Antony, 2010). A recent study illustrated neuroinflammation in adult OCD by examining TSPO distribution volume (elevated in neuroinflammatory states) in the CSTC circuit of OCD patients and controls (Attwells et al., 2017). In particular, greater TSPO distribution volume (elevated by more than 30%) was noted throughout the CSTC circuit which has previously been implicated in OCD (Attwells et al., 2017). Notable brain regions included the orbitofrontal cortex, dorsal caudate, thalamus, dorsal putamen, ventral striatum and to

a lesser extent the anterior cingulate cortex (Attwells et al., 2017). Typically, autoantibody investigations have focused on the striatum, however, this study suggested that autoimmune and neuroinflammatory theories of OCD be extended to the entire CSTC circuit rather than distinct regions (Attwells et al., 2017).

1.5.4c Autoimmune Basis

There is some clinical evidence to also suggest that immune-related conditions are more common in OCD than in other psychiatric disorders. Large population studies have attempted to better elucidate the relationship between autoimmune disease and OCD. A recent study in a large Swedish cohort reported that individuals with OCD had a 43% greater risk of any autoimmune disease (odds ratio=1.43, 95% CI=1.37–1.49). Analysis of individual diseases revealed an increased risk for Sjogren’s syndrome, celiac disease, Guillain-Barre syndrome, Crohn’s disease, Hashimoto’s thyroiditis, type I diabetes, scarlet fever, ulcerative colitis, multiple sclerosis and psoriasis vulgaris (Mataix-Cols et al., 2017). First-degree relatives of OCD patients also revealed an increased risk of an autoimmune disease with the risk marginally higher among mothers and siblings compared to fathers (Mataix-Cols et al., 2017). A recent systematic review revealed rates of OCD to be higher in rheumatic fever (and those with Sydenham’s chorea); however, due to the limited studies examining other conditions, results were inconclusive (Perez-Vigil et al., 2016).

The innate immune system (including microglia) plays a pivotal role in initiating and directing the immune response and several lines of evidence suggest that dysfunction

may occur in a number of neuropsychiatric conditions. This system is also involved in neuroprotection and neurodevelopment. Data regarding anti-basal ganglia antibodies is conflicting in pure OCD (Najjar et al., 2103). The main immune cells involved in innate function are monocytes; there are at least 3 subclasses of monocytes based on their phenotypic receptors. Monocytes promote rapid response and orchestrate inflammation by modulating levels of cytokines. CD16+ monocytes (intermediate and non-classical) have been termed pro-inflammatory due to their production of inflammatory cytokines like interleukin-6 (IL-6), IL-1 β and tumour necrosis factor- α (TNF- α). Levels of pro-inflammatory monocytes have been reported to be elevated in OCD patients compared to controls as was the production of pro-inflammatory cytokines upon lipopolysaccharide stimulation (Rodriguez et al., 2017).

1.5.4a Systemic Inflammation

Cytokines describe a broad category of small proteins that are involved in cell-signalling, acting as immunomodulating agents (Schobitz, Jolsboer, & Ron de Kloet, 1994). As such, they are well-recognized markers of peripheral systemic inflammation. They can also have a variety of effects on the central nervous system by functioning through their own receptors (Schobitz, Jolsboer, & Ron de Kloet, 1994), stimulating vagal afferents (Rothwell & Strijbos, 1995) and altering neurotransmitter concentration (Chakrabarty et al., 2005). Several studies have investigated the levels of circulating cytokines in OCD patients compared to controls; however, the results have been inconsistent (Table 1.1). A previous meta-analysis of studies measuring plasma pro-inflammatory cytokine levels reported that IL-1 β is decreased in patients with OCD and demonstrated no difference

between levels of TNF- α and IL-6 (Gray & Bloch, 2012). However, a significant moderating effect of age, concurrent medication use, and comorbid mood disorders was seen. IL-6 was elevated in medication-free OCD adults compared to controls and TNF- α was higher in OCD patients with comorbid mood disorders (Gray & Bloch, 2012). A recent study illustrated significantly greater plasma levels of IL-2, IL-4, IL-6, IL-10 and TNF- α in drug-naïve, comorbidity-free OCD patients compared to sex- and age-matched healthy controls (Rao et al., 2015). Rather than suggesting an increased pro- or anti-inflammatory state these results alluded to immune dysregulation as both pro- and anti-inflammatory cytokines were elevated (Rao et al., 2015). This dysregulation may be based on alterations in innate and adaptive immune system-related parameters like circulating cytokines (Gray & Bloch, 2012; Rao et al., 2015; Simsek et al., 2016). Further, this peripheral inflammation may generate brain inflammation by activating microglia (Quan et al., 1998). However, data regarding inflammation in OCD remain inconclusive overall.

Table 1.1 Overview of immune parameters in adults with OCD from the literature

	Increase	Decrease	Normal
IL-1 β		(Brambilla et al., 1997)	(Maes et al., 1994) (Monteleone et al., 1998) (Weizman et al., 1996)*
IL-2	(Rao et al., 2015)		(Weizman et al., 1996)*
IL-2 receptor			(Maes et al., 1994) (Monteleone et al., 1998)
IL-3-LA			(Weizman et al., 1996)*
IL-6	(Rao et al., 2015) (Konuk et al., 2007)	(Denys et al., 2004) ⁺ (Fluitman et al., 2010, LPS stim)	(Maes et al., 2004) (Monteleone et al., 1998) (Carpenter et al., 2002)**
IL-6 receptor			(Maes et al., 1994) (Monteleone et al., 1998)
TNF- α	(Rao et al., 2015) (Konuk et al., 2007)	activity(Denys et al., 2004) ⁺ (Brambilla et al., 1997) (Fluitman et al., 2010, LPS stim) (Monteleone et al., 1998)	
T cell subsets			(Barber et al., 1996)
-CD8+	(Marazziti et al., 1999)		
NK-cells	(Ravindran et al., 1999) ^{males}	(Denys et al., 2004, NK activity) ⁺	(Ravindran et al., 1999) ^{females}
IL-4	(Rao et al., 2015)		
IL-10	(Rao et al., 2015)		
IFN γ			(Rao et al., 2015)

All results are expressing plasma levels, except for * in vitro cytokine production, and ** CSF levels.

⁺ denotes use of whole blood and a trend.

IL-6 and TNF- α as markers of inflammation

TNF- α and IL-6 are the most extensively studied markers of chronic low-grade inflammation in neuropsychiatric disorders due to their effects on the central nervous system. TNF- α is known to play a role in a number of infectious,

inflammatory, and autoimmune conditions even within the central nervous system where it may regulate permeability of the blood-brain barrier. TNF- α is produced by macrophages and circulating monocytes (Marazziti et al., 2018). Although the literature on systemic levels of TNF- α in OCD patients is conflicting, a functional promoter region of the TNF- α gene has been associated with OCD (Cappi et al., 2012; Jiang et al., 2018). Similarly, IL-6 acts on a variety of cells regulating immune response and has been implicated in autoimmune and inflammatory diseases. IL-6 is produced by a variety of cells, but monocytes and macrophages are primary sources (Marazziti et al., 2018). Although it is involved in some defence mechanisms, when considering chronic inflammation, IL-6 has an overall pro-inflammatory effect. The proposed pathway linking these pro-inflammatory cytokines to neuropsychiatric disorders involves their ability to activate the enzyme indoleamine-2,3-dioxygenase (IDO) (Felger & Lotrich, 2013). IDO is known to consume tryptophan diverting production to kynurenine and other neuroactive metabolites instead of serotonin (Felger & Lotrich, 2013). Neurometabolites of this pathway may go on to then alter levels of dopamine, glutamate and other neurotransmitters that have been implicated in OCD (Felger & Lotrich, 2013).

CRP as a marker of inflammation

C-Reactive protein (CRP) is an acute-phase circulating protein produced by the liver. Elevated IL-6 enhances de novo CRP synthesis by upregulating CRP mRNA transcription. TNF- α may also have similar upstream effects on CRP production

(Maccio & Madeddu, 2012). As such, CRP has been long considered a reliable marker of inflammation and has been used to assess the risk of atherosclerotic disease. CRP is known to increase with age and is related to body mass index (BMI). An emerging body of evidence suggests CRP is elevated in mood disorders and to a lesser degree anxiety disorders (Tayefi et al., 2017), the immunological alterations documented in OCD provide a basis to examine CRP.

1.6 Rationale

OCD is a debilitating, chronic psychiatric disorder and is ranked as one of the 10 most debilitating conditions by the World Health Organization. Only 40-60% of patients go on to respond to current first-line treatments for OCD, traditional agents based on our early understanding of OCD, as discussed earlier in this Chapter. Given the limited efficacy and tolerability of current treatments, it is important we move beyond current hypotheses and investigate additional plausible mechanisms, like the gut microbiome. Research has shown that the gut and brain are connected and that the gut microbiome (discussed in CHAPTER 2) is able to modulate behaviour via this bidirectional pathway. Evidence suggests that microbial dysbiosis may be involved in the putative pathophysiological mechanism of OCD and is discussed in the following Chapter.

CHAPTER 2: COULD THE MICROBIOME SERVE AS AN ALTERNATIVE PATHOPHYSIOLOGICAL MECHANISM? A REVIEW EXAMINING THE RELATIONSHIP BETWEEN THE MICROBIOME AND OCD

Forward to CHAPTER 2

This chapter is a re-print of a narrative review developing the relationship between the gut microbiome and OCD. Permission was granted by the publisher (John Wiley and Sons) to republish this material in this thesis. This manuscript was incorporated into this thesis to provide additional background and rationale to support exploration of the role of the gut microbiome in OCD. Despite the plethora of review articles discussing a role for the gut microbiome in mental health, this is a novel contribution as previous publications have not focused on this particular disorder. This manuscript comprehensively ties together previously suggested lines of evidence in anxiety and mood disorders and applies them to OCD. Existing evidence focuses on production of neurotransmitters, HPA axis dysregulation, ascending neural pathway involvement, and a strong focus on inflammation and immune dysregulation. Given that the role of the gut microbiome in psychiatric illnesses is a novel topic, several new studies have been published since the publication of this review. These are summarized in section 2.2.

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2.1 What's bugging the gut in OCD?" A review of the gut microbiome in Obsessive-Compulsive Disorder

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Abstract: The gut microbiome has become a topic of major interest as of late, with a new focus specifically on psychiatric disorders. Recent studies have revealed that variations in the composition of the gut microbiota may influence anxiety and mood and vice versa. Keeping the concept of this bidirectional “microbiota-gut-brain” axis in mind, this review aims to shed light on how these findings may also be implicated in Obsessive-Compulsive Disorder (OCD); potentially outlining a novel etiological pathway of interest for future research in the field.

Introduction

Obsessive-compulsive disorder (OCD) is a psychiatric condition marked by recurrent intrusive thoughts (obsessions) and ritualistic behaviors aimed at reducing distress (compulsions). The estimated lifetime prevalence of OCD is 2-3% in the population (Kugler et al., 2013). While imbalances in serotonin, dopamine and glutamate activity are believed to play a causative role, there is no general consensus on any of these mechanistic theories at present time. Given that current pharmacological treatments targeting these systems are paired with moderate to low rates of treatment response, it is possible that there are additional etiological pathways that have yet to be explored. One pathway that has recently gained interest involves the bidirectional signalling between the gut and brain and its relation to mental illness. The role of the gut microbiome, the collection of microbes in the human gastrointestinal tract, plays in modulating the gut-brain axis appears particularly relevant to mental health (Foster & Neufeld, 2013).

This review will discuss the literature related to the gut microbiome and psychiatric disorders, and will show how this preliminary data can be used to illustrate a possible link to OCD.

The gut-brain axis

The extensive connections between the brain and gut have gained interest in the scientific community. This communication system integrates neural, hormonal and immunological signalling between the gut and brain with neural connections involving the central, autonomic and enteric nervous system (Collins, Surette, & Bercik, 2012). Top-down modulation of gastrointestinal function by stress and emotions and bottom-up signalling

from visceral afferents to the brain in abdominal pain syndromes and emotional regulation have been reported in multiple studies. For example, emotional factors including stress or depression can influence the chronic progression of gastrointestinal illnesses via this axis (Cryan & Dinan, 2012). It has been suggested that illnesses such as inflammatory bowel disease (ulcerative colitis and Crohn's disease), and irritable bowel syndrome (IBS) are associated with compositional changes in the gut microbiota (Morgan et al., 2012). Moreover, these chronic bowel disorders are often paired with psychiatric comorbidities, including anxiety and depression (Collins et al., 2012). As such, the microbiota are now seen as a potential mediator of this axis, leading to the development of a novel conceptual model incorporating gut bacteria termed the “microbiota-gut-brain axis”.

The gut microbiome in anxiety and mood disorders

The human gut is home to numerous bacteria where the gut bacteria outnumber human cells 10-fold (Gill et al., 2006). Also known as the “gut microbiome,” these bacteria facilitate nutrient absorption by metabolizing indigestible dietary compounds, developing gut-associated lymphoid tissue, producing vitamins and preventing colonization by pathogens (Rees, 2014). The overall profile of the gut microbiome, such as the one in the human gastrointestinal (GI) tract, is largely stable in adult life (Faith et al., 2013) but can be modulated by factors including infection, antibiotics and probiotics. It has been hypothesized that dysfunction of gut flora can lead to states of chronic and acute disease. The primary inhabitants of the gut microbiome include bacteria classified under the categories (domain) of *Bacteroidetes* and *Firmicutes* (Eckburg et al., 2005).

Proteobacteria, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* phyla are also present; however they are less abundant (Eckburg et al., 2005). Changes in the gut microbiome have been observed in a number of illnesses like inflammatory bowel disease (Morgan et al., 2012), autism (Parracho, Bingham, Gibson, & McCartney, 2005), celiac disease (Nadal, Donant, Ribes-Koninckx, Calabuig, & Sanz, 2007), diabetes (Brown et al., 2011) and obesity (Turnbaugh et al., 2006). More recently, researchers have looked at the relationship between the gut and brain and its possible contribution to psychiatric illnesses, including anxiety and mood disorders.

Results of animal studies

Studies examining the gut microbiome in anxiety and mood disorders are limited to rodent models. Several studies have shown that rodent behavioral responses are affected when gut microbiota are altered. For example, germ-free (GF) mice, that is mice with no commensal intestinal microbiota, have demonstrated reduced anxiety-like behaviors (more exploratory behaviours) (Heijtz et al., 2011; Clarke et al., 2012; Neufeld, Kang, Bienenstock, & Foster, 2011a; Neufeld, Kang, Bienenstock, & Foster, 2011b). Such changes have been demonstrated in the elevated-plus maze and components of the light/dark test, both which are widely used to measure anxiety-like behaviors in mice (Heijtz et al., 2011; Clarke et al., 2012). These results present evidence for the ability of the gut microbiota to alter behaviour and the gut-brain axis. Beyond this, a seminal study transferred fecal microbiome samples between two strains of mice, the exploratory NIH Swiss mouse and less exploratory BALB/c mouse (Bercik et al., 2011). What was noted that the GF-recipient mice receiving stool samples from the donor mouse (NIH Swiss to

BALB/c and BALB/c to NIH Swiss) began to display exploratory behaviours of the donor mouse (Bercik et al., 2011).

More interestingly, re-establishment of the microbiota in GF-mice during early life has been shown to “normalize” locomotor activity and anxiety behaviors on previously mentioned rodent behavioral tests, returning them to the normal range. However, this normalization does not apply if the microbiota is reconstituted in adulthood (Heijtz et al., 2011; Clarke et al., 2012; Neufeld et al., 2011a; Neufeld et al., 2011b), suggesting there is a critical period early in life where the anxiety response is programmed. Overall these findings support the notion of the gut microbiome’s effect on behaviour. Significant bacterial depletion from early adolescence and restructuring of the depleted microbiota populations in the gut has also been shown to alter brain development and behavior in mice treated with antibiotics (Desbonnet et al., 2015). This antibiotic-mediated depletion of the gut microbiota induces significant changes in concentrations of neuromodulatory substances including tryptophan and kynurenine peripherally in serum. It can also induce changes in the main metabolite of serotonin, 5-hydroxyindoleacetic acid, in brain areas relevant to the regulation of core behaviors associated with brain–gut dysregulation. Such biological changes have also been paralleled by behavioral changes including reduced anxiety (Desbonnet et al., 2015). Further, studies administering probiotics have shown reduced anxiety (Bercik et al., 2011; Bravo et al., 2011) and depression-like symptoms in healthy mice (Desbonnet et al., 2010).

Although the aforementioned studies illustrate the bottom-up role of the microbiota-gut-brain axis, studies have also illustrated the top-down function by subjecting animals to external stressors. O'Mahony et al. (2009) reported that the fecal microbiome composition of adult rats subjected to maternal separation was significantly altered when compared to that of non-separated controls. Bangsgaard Bendtsen et al. (2012) demonstrated that the microbiota composition of mice exposed to a prolonged restraint stressor differed significantly from that of non-stressed controls. Finally, Bailey et al. (2011) observed that social stressors significantly altered the relative abundance of bacteria, particularly when the microbiota were assessed immediately following exposure to the stressor.

Results of human studies

In the past year, two studies looking at the human microbiome in major depressive disorder (MDD) have been published (Naseribafrouei et al., 2014; Jiang et al., 2015). These studies explore possible differences in the gut microbiome of depressed and healthy individuals by evaluating differences in terms of species diversity measures and abundance of operational taxonomic units (OTUs). OTU is a term used to describe a taxonomic group (species/genus) that share a given set of observed characteristics and can be used when only DNA sequence data is available (Beck, Settles, & Foster, 2011). A study by Naseribafrouei and colleagues (2014), did not find a significant relation in terms of species richness or α -diversity and depression; however, at the domain level a correlation suggesting a general underrepresentation of *Bacteroidetes* in depression was detected (Naseribafrouei et al., 2014).²⁶

In contrast to the findings of Naseribafrouei and colleagues (2014), Jiang et al (2015) showed somewhat higher fecal microbial diversity in their active-MDD group. In this sample, *Bacteroidetes* were increased in individuals with active-MDD as were *Proteobacteria*, while the proportion of *Firmicutes* was significantly reduced (Jiang et al., 2015). Parabacteroides and Alistipes were identified as the primary contributors to the increase in *Bacteroidetes*. The authors commented on their conflicting findings pointing out that the control group in Naseribafrouei's sample was recruited from an outpatient neurological unit. Although no psychiatric disorders were present in the sample, Jiang's control group was comprised of healthy subjects and believed to have served as a better control subsequently producing a more profound difference between samples. Jiang et al (2014) also looked at "responded-MDD," individuals with scores ≥ 20 on the Hamilton-Rating Scale for Depression at baseline but whose samples were collected following a 50% reduction of scores after 4-weeks of treatment. At the phylum-level, *Firmicutes*, *Fusobacteria*, and *Actinobacteria* were significantly more abundant in the fecal microbiota of healthy controls than the responded-MDD group, whereas *Bacteroidetes* and *Proteobacteria* were significantly more abundant in the fecal microbiota of the responded-MDD group (Jiang et al., 2015). No comment was made regarding the differences between individuals with active-MDD versus those responding to 4-weeks of treatment.

The gut microbiota and Central Nervous System

The influence of the gut microbiota on behavior may be explained by both direct and indirect pathways. Four candidate pathways include: 1) Production of neurotransmitters

2) Triggering gut hormone release from enteroendocrine cells (Collins et al., 2012; Cryan & Dinan, 2012). 3) Activation of the ENS and signalling of the brain via ascending neural pathways (Crumeyrolle-Arias et al., 2014). 4) Activation of the immune system via cytokine release by the mucosal immune cells (Collins et al., 2012; Cryan & Dinan, 2012). Evidence for each of these candidate pathways is described in the following sections.

Production of neurotransmitters

Bacteria of the gut microbiome are known to produce a number of neurotransmitters implicated in psychiatric disorders including: serotonin (*Candida*, *Streptococcus*, *Escherichia*, *Enterococcus*), dopamine (*Bacillus*, *Serratia*), gamma-aminobutyric acid (GABA) (*Lactobacillus*, *Bifidobacterium*), norepinephrine (*Escherichia*, *Bacillus*, *Saccharomyces*) and acetylcholine (*Lactobacillus*) (Collins et al., 2012). These secreted neurotransmitters of microorganisms in the intestinal lumen may induce epithelial cells to release molecules ultimately modulating neural signalling within the enteric nervous system or acting directly on afferent axons.

Gut hormone release and HPA axis

Activity of the hypothalamic-pituitary-adrenal (HPA) axis, the central stress response system, has long been known to affect the gut microbiota (Lyte, 2011). Animal studies have shown that emotional stressors, including but not limited to maternal separation, crowding, heat and acoustic stress can all contribute to a negative impact on microbiota composition. Alternatively, Sudo et al (2004) demonstrated the ability of the microbiota to affect the HPA axis by using the GF-mouse model (Sudo et al., 2004). In this study, the

mice elicited an enhanced HPA axis response to psychological stressors providing evidence of the gut microbiome's role in programming stress response (Sudo et al., 2004).

One proposed mechanism for these stress-induced alterations of the gut microbiota, by way of immune system activation and the HPA axis, involves the “leaky gut” (Maes, Twisk et al., 2013; Maes, Ringel et al., 2013). This phenomenon is best described as the increased translocation of bacterial products due to a compromised gut barrier. Compromised gut epithelium is thought to be the result of either a stressful event or microbiota dysbiosis where translocated metabolites directly interact with immune cells. More specifically, this intestinal permeability results in increased circulation of bacterial derived lipopolysaccharides which trigger an immunological and inflammatory response characterized by increased systemic levels of pro-inflammatory cytokines (Qin et al., 2007). Increased rates of bacterial translocation have been illustrated in depression (Maes, Twisk et al., 2013; Maes, Ringel et al., 2013) and schizophrenia (Severance et al., 2013) and low-grade immune activation or inflammation have been discussed as common underlying factors (Maes, Twisk et al., 2013; Maes, Ringel et al., 2013; Qin et al., 2007); the effects of leaky gut (increased bacterial translocation) can be reversed in mice by oral administration of probiotics (Ait-Belgnaoui et al., 2012; Ait-Belgnaoui et al., 2014; Savignac, Kiely, Dinan, & Cryan, 2014).

Activation via ascending neural pathways

The vagus nerve has both efferent and afferent functions. With almost 80% of nerve fibres being sensory, it plays a strong role in conveying information about the body's organs to the central nervous system (Thyer & Sternberg, 2009). Moreover, many of the

effects of the gut microbiota and probiotics on brain function have been shown to be dependent on the activity of the vagus nerve. For example, Bercik et al. (2011) first showed that *Bifidobacterium longum* normalizes anxiety-like behaviors in mice with infectious colitis, an illness where microbiota dysregulation has been established (Bercik et al., 2011). However following a vagotomy, mice treated with probiotics showed a greater latency period indicating that the effect of the probiotic on anxiety-like behavior was lost, and that the vagus nerve was necessary for this anxiolytic effect (Bercik et al., 2011). Similarly Bravo et al. (2011) illustrated that treatment with *Lactobacillus rhamnosus* reduced anxiety- and depression-related behaviors in mice and this effect was absent in vagotomised mice (Bravo et al., 2011).

Activation of the immune system

Research has begun to focus on another role of the gut microbiome, that is, its ability to instruct and mould the immune system of the associated host. For example, GF-mice prenatally develop in sterile uterine conditions and are subsequently delivered surgically; as such, postnatal colonization of the microbiome is inhibited by raising them in a sterile environment. These mice, interestingly, illustrate defects in the immune system by presenting virtually no immune activity. These deficits are evident at both structural and cellular levels (Round & Mazmanian, 2009). However, immune function can be activated following colonization of the gut. Colonization with certain microorganisms can even restore full function of B- and T-lymphocytes, which are major active components of the adaptive immune system (Talham, Jiang, Bos, & Cebra, 1999; Umesaki, Okada,

Matsumoto, Imaoka, & Setoyama, 1995; Umesaki, Setoyama, Matsumoto, Imaoka, & Itoh, 1999).

The gut microbiota play a major role in the modulation of both the intestinal and general immune system and are essential for preserving functions including the maturation of gut-associated lymphoid tissue, secretion of immunoglobulin A (an antibody that can initiate an inflammatory reaction in the blood) and production of important antimicrobial peptides (Kamada, Seo, Chen, & Nunez, 2013). The gut microbiota can communicate with the host through toll-like receptors (TLRs), which are present on cells of the innate immune system. Such interactions activate important intracellular signalling pathways that can modulate processes like cell survival, replication, apoptosis and an inflammatory response (Kamada et al., 2013; Kamada & Nunez, 2014). More specifically, TLR interactions elicit the first step in cytokine production; that is, molecules that are involved in the inflammatory response. Since manipulation of the microbiome profile can influence systemic cytokine levels, it is possible that the microbiota implements its effect on behavior by influencing these systemic cytokines. In reverse, the host immune system can also control microbial composition through release of molecules which defend against various pathogenic microorganisms.

Probiotics are preparations containing live microorganisms thought to confer health benefits when consumed and have been used as treatment for conditions where microbiota dysregulation has been demonstrated (Ritchie & Romanuk, 2012). The proposed beneficial effects of probiotics on mood and anxiety may be explained by their effect on the immune response consequent to changes in cytokine production. For

example, various strains of probiotics have been shown to attenuate peripheral levels of the pro-inflammatory cytokines IL-1 β , TNF- α , IL-6 and interferon- γ (Dinan & Cryan, 2013). In addition to the production of such cytokines, it has also been proposed that the immunomodulating effects of probiotic organisms may occur through the generation of T-cell populations (Dinan & Cryan, 2013).

The Role of Inflammation in Psychiatric Illness

Inflammation was once considered to be a local process involving immune cell reactions to fight bacteria or in response to injury. However, now inflammation is seen as a multi-systemic condition, involving multiple tissues and organs, including the brain, with implications on diseases ranging from diabetes, heart disease, cancer, obesity and neuropsychiatric disorders.

Neuroinflammatory and immunological abnormalities have been documented in patients with psychiatric disorders. In the past decade, accumulating data suggests a connection between inflammation and psychiatric symptoms. Reports of peripheral immune modulators having the ability to induce psychiatric symptoms have been demonstrated in animal models and humans (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Laske et al., 2008; Eisenberger et al., 2010; Haroon, Raison, & Miller, 2012; Benros et al., 2011; McNally, Bhagwagar, & Hannestad, 2008; Harrison et al., 2009; Raison & Miller, 2011). For example, healthy animals injected with the pro-inflammatory cytokines IL-1 β and TNF- α developed "sickness behavior" associated with social withdrawal, decreased motor activity and reduced food and water intake (Dantzer et al., 2008). Therapeutic administration of the inflammatory cytokine interferon- α (IFN-

α) has been found to induce depressive symptoms in as many as 50% of treated patients (Musselman et al., 2001). Similarly, pre-administration of antidepressant medications to IFN- α treatment have been shown to significantly reduce the development of depressive symptoms (Musselman et al., 2001; Raison et al., 2007). Numerous studies have suggested that major depression is accompanied by immune dysregulation; more specifically, activation of the inflammatory response system. This belief has been further confirmed by a meta-analysis conducted by Dowlati et al. (2010) demonstrating the association between elevation of two pro-inflammatory cytokines, IL-6 and TNF- α , and major depression (Dowlati et al., 2010). Diseases with immunological abnormalities like obesity, diabetes, malignancies and multiple sclerosis, are also considered risk factors for depression and bipolar disorder. This positive correlation provides additional support in favour of a widespread underlying inflammatory process which affects the brain among other organs (S. Najjar, Pearlman, Alper, A. Najjar, & Devinsky, 2013). In addition to peripheral inflammatory markers, microglial activation has also been illustrated in patients experiencing a major depressive episode as compared to healthy, age-matched controls (Setiawan et al., 2015). TSPO expression in particular increases when microglia are activated during neuroinflammation. As such, Setiawan et al (2015) demonstrated this process by illustrating high levels of translocator proteins in brain areas involved in mood regulation, such as the prefrontal cortex, anterior cingulate cortex and insula suggesting neuroinflammation (Setiawan et al., 2015).

Several mechanisms have been suggested by which pro-inflammatory cytokines may affect mood and anxiety. One possible mechanism of action involves activation of

the HPA axis. Activation of this stress response axis involves the release of TNF- α and IL-6 which directly activate the HPA axis by increasing the release of different neuroendocrine hormones (corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and cortisol) (Dantzer, Wollman, Vitkovic, & Yirmiya, 1999; Vallieres & Rivest, 1999; Chrousos, 1995; McCann et al., 1995). Consequently, it is possible that increased inflammation may explain the hyperactivity of the HPA axis typically observed in mood and anxiety disorders.

Pro-inflammatory cytokines also have the capacity to affect the tryptophan metabolic pathway as a result of their ability to activate IDO, a rate-limiting catalyst in the synthesis of kynurenine (Schrocksadel, Wirleitner, Winkler, & Fuchs, 2006; Heyes et al., 1992; Mellor & Munn, 1999). A pro-inflammatory state will therefore activate this enzyme and the metabolic fate of tryptophan begins to favour the production of kynurenine as opposed to serotonin, which may contribute to depressive symptoms (Heyes et al., 1992; Mellor & Munn, 1999) or symptoms of other conditions where a putative role for serotonin has been posited. Another product of this IDO-activated pathway is quinolinic acid, a potent NMDA receptor agonist, which can have a detrimental effect on hippocampal neurons and result in apoptosis (Schwarz, Whetsell, & Mangano, 1983; Stone & Behan, 2007). In addition to mood disorders, a growing body of evidence suggests that other psychiatric disorders like schizophrenia and OCD are also associated with inflammatory markers not only in the central nervous system but also in the periphery (Najjar et al., 2013).

Overlap in OCD

As of May 2013, with the release of the DSM-5, OCD is no longer classified as an anxiety disorder. Instead, OCD and other previously independent disorders have been categorized under the heading of “Obsessive-Compulsive Spectrum Disorders.” Although most of the current microbiome-psychiatric literature is related to anxiety and mood, many of these findings can be related to OCD as anxiety is a predominant feature of this disorder.

Although studies related to OCD specifically are lacking, there is one study in OCD using the probiotic *Lactobacillus rhamnosus* in mice (Kantak, Bobrow, & Nyby, 2014). In this study, Kantak et al. (2014) used RU24969 (a known 5-HT_{1A/1B} receptor agonist) to induce OCD-like behaviors. The RU24969 model is supported by studies showing that SRI pre-treatment attenuates the induction of OCD-like mouse behaviors (Shanahan, Velez, Masten & Dulawa, 2011). Moreover, some 5-HT_{1B} receptor agonists also exacerbate OCD symptoms in humans (Shanahan et al., 2011). In the first experiment, mice were pre-treated with probiotic (or saline) for 2- or 4-weeks (Kantak et al., 2014). When OCD-like behaviors were induced using an injection of RU24969, marble burying and locomotor behavior were significantly reduced in the probiotic treated mice compared to those treated with saline. In experiment two, an additional group of mice was included in this analysis, those pretreated with fluoxetine, a first-line treatment for OCD for 4-weeks (Kantak et al., 2014). Both groups pre-treated with fluoxetine and probiotic showed attenuation of OCD-like behaviors. Further, in healthy humans, probiotic formulations including *Lactobacillus helveticus* and *Bifidobacterium longum* administered daily for 30 days have been shown to reduce “obsessive-

compulsive” subscores on the Hopkins symptoms checklist (Messaoudi et al., 2011).

With such findings in mind, there is reason to hypothesize that dysregulation of the microbiome may be involved in OCD. Although we can only postulate for the time being, it may be that the onset of OCD can be explained by communication between the microbiome, inflammatory response and HPA axis.

The role of the immune system in OCD has long been under investigation. Numerous studies suggest that an inflammatory process due to an acute or chronic infection or a post-infectious immune response may be involved in the pathogenesis of OCD. Almost two decades ago OCD was found to be associated with streptococcal infections, and the term PANDAS was developed to describe this phenomenon. PANDAS is characterized by acute exacerbations of OCD-like symptoms and/or motor/phonic tics following a prodromal group A β -hemolytic streptococcal infection. The pathophysiology is thought to involve cross-reactivity between anti-streptococcal antibodies and basal ganglia proteins, however the specific mechanism is still under investigation (Swedo et al., 2015). Moreover, a recent imaging study revealed that in comparison to controls, children with PANDAS were found to have enhanced binding of translocator protein receptors in the bilateral caudate and lentiform nucleus suggesting microglia-activated neuroinflammation (Kumar, Williams, & Chugani, 2015). Despite the controversy associated with the etiology of PANDAS (Swedo et al., 2015; Swedo, 2010), the clinical overlap between the PANDAS OCD symptoms and pure OCD suggest a common etiological mechanism and warrants further investigation into the immune basis of OCD (Morer et al., 2008). In addition to GAS infection, other infectious diseases may

be involved in the development of OCD. Some studies have associated OCD with infections with borna virus disease (Dietrich et al., 2005) and toxoplasma gondii (Miman et al., 2010). These observations hint to the possible connection between the immune system and the development of OCD, leading to the progression of studies to assess immune parameters in OCD (Teixeira, Rodrigues, Marques, Miguel, & Fontenelle, 2014).

A series of studies have investigated the levels of circulating cytokines in OCD patients in comparison with control subjects. Although the results are inconsistent, many believe that this may be due to the confounding effects of medications and comorbid conditions present in study populations. A recent study, however, illustrated significantly greater plasma levels of IL-2, IL-4, IL-6, IL-10 and TNF- α in drug-naïve, comorbidity-free OCD patients as compared to sex- and age-matched healthy controls (Rao et al., 2015). These results can be taken to suggest a dysregulated inflammatory response as the presence of the anti-inflammatory cytokines (IL-10) may be seen in response to an overall pro-inflammatory cytokine profile. A meta-analysis of studies measuring plasma cytokine levels reported that IL-1 β is decreased in patients with OCD (Gray & Bloch, 2012). Other studies have also looked at different inflammatory mediators in OCD patients, such as chemokines, and showed differences between OCD patients and healthy individuals (Fontenelle et al., 2012; Denys, de Geus, van Megen, & Westenberg, 2004). While the inflammatory profile of OCD remains unclear, these findings suggest that the immune response may in fact be dysregulated in OCD and as an extension, the inflammatory response. Given the potential role of these cytokines in OCD, further experimental studies are needed in order to determine the effect of probiotics on systemic

cytokines and behavior. These findings may serve as an observation into the possible connection between OCD and inflammatory processes. As the gut microbiome has been shown to affect the process of inflammation, through immune dysregulation, and vice versa, it is reasonable to postulate that OCD and the microbiome are connected themselves.

Much of the gut-brain literature in anxiety focuses on the enhanced activity of the HPA axis. Interestingly, increased elevated basal activity of the HPA axis has also been reported in OCD making it reasonable to suggest that the findings in the anxiety literature may also be applicable to OCD. Increased basal activity of the HPA axis has been illustrated by studies measuring cortisol at multiple points throughout the day or by 24-hour urinary free cortisol collection (P.E. Gustafsson, P.A. Gustafsson, Ivarsson, & Nelson, 2008; Kluge et al., 2007). Further, elevated levels of cerebrospinal fluid levels of CRH and ACTH have been reported in OCD (Kluge et al., 2007; Catapano, Monteleone, Fuschino, Maj, & Kemali, 1992; Monteleone, Catapano, Tortorella, & Maj, 1997). Marked stress can increase HPA activity (Frodl & O'Keane, 2013), a trend paralleled in OCD, where increased life stressors can cause the onset or worsening of OCD (Rosso, Albert, Asinari, Bogetto, & Maina, 2012). While studies have examined the relationship between traumatic life events and onset of OCD, Real et al (2011) reported that 37.4% of their sample indicated that onset of OCD was preceded by non-traumatic stressful life events (Real et al., 2011). Such events included health, educational stressors and bereavement (Real et al., 2011).

Although there is little evidence to suggest the involvement of neurotransmitters in regulating microbiota-gut-brain axis activity, gut bacteria are capable of producing their own neurotransmitters (refer to “Production of neurotransmitters”). Moreover, probiotics also have the ability to function as a delivery vehicle for neuroactive compounds. For example, strains of *Lactobacilli* and *Bifidobacterium* produce gamma-aminobutyric acid (GABA) (Collins et al., 2012) a main inhibitory neurotransmitter of the nervous system. Oral administration of *Bifidobacterium infantis* has also been shown to increase levels of tryptophan (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008), a precursor to serotonin. This is particularly interesting as SRIs, a first-line treatment option for OCD, act to increase the availability of serotonin in the synapse. With implications of abnormal neurotransmitter levels in OCD, and as the role of microflora to modify key players in the central nervous system becomes clearer, it is not unreasonable to think that probiotics can play a role in transforming OCD to a more tolerable state potentially through the effects of neuroactive compounds.

Conclusion

With treatment methods for OCD being less than ideal, coupled with the unclear pathophysiology, there is a great need to explore additional etiological pathways possibly involved in this chronic, disabling disorder. There is a growing body of evidence suggesting that the gut microbiome and gut-brain interactions may play an important role in mental health and the development of psychiatric illness. As outlined above, there are numerous ways in which potential alterations of this axis or changes made to this axis influence behavior, particularly mood and anxiety. As such, potential treatment

modalities affecting this system may be a more effective means of treatment for many psychiatric illnesses, like OCD. It is important however to bear in mind that the field is currently in its early stages and more research is needed to first elucidate the possible role of the microbiome in mental health in general. Further, despite the possible link between the disorder and the microbiome as illustrated above, the current microbiome-OCD literature is minimal at best. Consequently, there is an urgent need for studies looking at the gut microbiome and its potential relationship with OCD. The literature as it currently stands is primarily composed of rodent microbiome data. Although useful, the human gut microbiome is increasingly more complex making it difficult to directly translate findings, thus demonstrating the need to conduct clinical studies. If a possible link is established, further investigations would logically include the potential influence of first-line OCD treatments including SSRIs and CBT on the gut flora and whether there is a difference between individuals who respond to such treatments and those that do not. Moreover, if a disruption in the microbiome is established in OCD, then the use of treatments such as antibiotics, probiotics and fecal biotherapy as a remediating therapy should also be investigated. The results of such studies could serve as a catalyst for a new direction of OCD research, a disorder which is in desperate need of improved treatments.

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2.2 Update on the gut microbiome in psychiatry

To date, the largest body of gut microbiome research has been conducted in clinical populations of autism spectrum disorder (ASD) (Vuong & Hsiao, 2017). The most consistent finding in ASD patients has been a higher abundance of the genus *Clostridium*; however, no defined microbial signature has been identified (Vuong & Hsiao, 2017).

The microbiome literature in other psychiatric conditions is also developing. A study in medicated individuals with first episode psychosis reported a positive correlation between *Lactobacillus* bacterial group numbers and symptom severity (Schwarz et al., 2017). Patients clustered with controls at baseline were also more likely to show symptom remission following 12 months of antipsychotic treatment compared to those with the “abnormal” microbial profile (Schwarz et al., 2017). A biological signature of post-traumatic stress disorder (PTSD) vulnerability has been identified and associated with Actinobacteria, Lentisphaerae and Verrucomicrobia, with decreased total abundance of these taxa associated with greater symptom severity (Hemmings et al., 2017). An abundance of Actinobacteria and reduced Firmicutes were also reported in a sample of adults with Attention deficit/hyperactivity disorder (ADHD); however, the finding was not significant once multiple comparisons had been corrected for (Aarts et al., 2017). In a sample of bipolar patients, significantly decreased levels of *Faecalibacterium* (phylum Firmicutes, family *Ruminococcaceae*) have also been observed (Evans et al., 2017).

In addition to the earlier studies (Naserbafrouei et al., 2015; Jiang et al., 2015) outlined in CHAPTER 2 of this thesis, recent studies in major depressive disorder (MDD) populations continue to suggest microbial dysbiosis in the condition. For instance, Zheng et al. (2016) found that the microbiota of MDD patients significantly differed from controls. These differences were characterized by an increased relative abundance Actinobacteria and decreased Bacteroidetes (Zheng et al., 2016). They also reported that fecal microbiome transplant of germ-free (GF) mice with microbiota from MDD patients, but not controls, led to development of depression-like behaviours which appeared to be

driven by disturbances of microbial genes and host metabolites involved in carbohydrate and amino acid metabolism (Zheng et al., 2016). Finally, another study which restricted their examination to levels of *Bifidobacterium* and *Lactobacillus* levels revealed that MDD patients had significantly lower *Bifidobacterium* counts (Aizawa et al., 2016).

Although microbial dysbiosis has still not been examined in OCD, a recent study examined the gut microbiome in 30 PANS patients (and 70 age-matched controls) using 16s rRNA analysis (Quagliariello et al., 2018). Lower α -diversity in the PANS/PANDAS group compared to controls was noted, specifically according to the Observed and Chao1 indices. Further, Bray-Curtis and Unweighted Unifrac β -diversity metrics also revealed separation between 4-6 and 7-8 year old controls from all others. At the phylum level, reduced Firmicutes were noted in the young-PANS group in addition to enrichment of Bacteroidetes (including *Odoribacter* and *Oscillospira*) (Quagliariello et al., 2018).

2.3 Comorbidity between psychiatric disorders and conditions with known microbial dysbiosis

Populations suffering from physical conditions linked to microbial dysbiosis, like inflammatory bowel disease (IBD), often present with high rates of comorbid anxiety and mood symptoms (Morgan et al., 2012). Given that the microbiota are key players in the gut-brain axis, it is reasonable to postulate that high comorbidity may suggest a common pathophysiological mechanism. A recent study examining psychiatric disorders in patients with functional bowel disorders reported that 85% of the sample was diagnosed with a psychiatric disorder, with the most prevalent being dysthymia (25%) and OCD (20%) (Fakhraei et al., 2015). Similarly, in patients with OCD, gastrointestinal conditions

were the second most prevalent medical condition (20.5%) following after endocrine/metabolic diseases (25.9%) (Aguglia et al., 2018).

Unlike most gastrointestinal conditions, irritable bowel syndrome (IBS) does not have any leading known physical causes. However, altered gut immune activation, intestinal permeability, and intestinal and colonic microbiome have been identified in some IBS patients (Kennedy, Cryan, Dinan & Clarek, 2014). Many have suggested that this condition is the product of both biological and psychological factors, and as expected there is also high comorbidity with anxiety and mood disorders. Only two studies have examined IBS in OCD specifically. One study utilized Rome I criteria and reported high prevalence of IBS in (35.1% vs. 2.5%) in their sample of OCD patients compared to age- and sex-matched control group of patients from a family practice (Masand et al., 2006). A second report revealed rates of comorbid IBS (based on Rome II criteria) in their OCD group were comparable to community samples; but higher in generalized anxiety disorder (GAD), panic disorder and MDD (Gros et al., 2009).

Given the overlapping pre-clinical evidence suggesting involvement of the gut microbiome in OCD, a cross-sectional study of medication-free, non-depressed OCD outpatients and non-psychiatric healthy controls was proposed. The study reported in this thesis is the first to examine the presence of microbial dysbiosis in OCD.

CHAPTER 3: METHODS

Forward to Chapter 3: Based on the preliminary evidence outlined in the narrative review (CHAPTER 2), a cross-sectional study was designed to examine the gut microbiome in a clinical sample of non-depressed unmedicated OCD patients and controls. Secondary objectives to clarify the potential role of the gut microbiome were also incorporated. The results of this study are discussed in CHAPTERS 4-6.

3.1 Design

This was a cross-sectional examination of the gut microbiome profile of non-depressed, non-medicated patients with OCD and a sex- and age-matched healthy control group comparator. This novel pilot study was designed to detect feasibility and a potential “signal” to premise the undertaking of further trials examining the research question. The study (protocol, consent form, advertisements) received ethics approval by the Hamilton Integrated Research Ethics Board at McMaster University (Hamilton, ON, Canada).

3.2 Sample Size

Given the pilot nature of the study and the lack of existing data on which to base a formal sample size calculation in OCD, the sample size was selected based on feasibility for recruitment. Further, differences in the gut microbiome have been detected in psychiatric clinical samples (primarily ASD and MDD) with small sample sizes ranging between 10 (DeAngelis et al., 2013) to 29 participants (Jiang et al., 2015) per group. Thus, the proposed study was considered likely to be adequately powered for a pilot study.

3.3 Participants

Males and females aged 18 to 65 years were recruited for this study. Forty-three participants were recruited from the community to participate in this study; 21 non-depressed, unmedicated individuals with a primary DSM-5 (APA, 2013) diagnosis of OCD and 22 age- and sex-matched controls. Age-matching in particular was critical to the study as the gut microbiome is known to change throughout the lifespan by default (Kim et al., 2017). OCD outpatients and controls were recruited at the MacAnxiety Research Centre (McMaster University, Hamilton, ON, Canada) using a variety of advertising strategies not limited to word of mouth, online classified/clinic websites, study posters in the community and public transit ads. Recruitment was completed between January 2015 and April 2017.

3.4 Objectives

3.4.1 Primary Objective

The primary objective of this study was to compare the gut microbiome profiles of individuals with OCD (non-depressed, unmedicated) to non-psychiatric controls as per various indices of α - and β -diversity.

3.4.2 Secondary Objectives

The secondary objectives of the study were as follows:

1. To compare differences in the gut microbiome profile and symptom severity.

2. To observe differences in the gut microbiota of OCD patients with additional comorbid anxiety disorders (as per MINI International Neuropsychiatric Interview (MINI)).
3. To determine the proportion of OCD patients with IBS, as dictated by Rome III criteria for IBS.
4. Characterize gastrointestinal function in OCD patients, compared to controls.
5. To examine whether there are differences in levels of the circulating cytokines IL-6, TNF- α and CRP between individuals with OCD and controls.

3.5 Eligibility Criteria

3.5.1 Inclusion Criteria

The study sample included males and females ages 18 to 65 years with either no current clinically significant psychiatric symptoms (controls) or a principal diagnosis of OCD (DSM-5) as per the MINI. OCD patients also required a score ≥ 20 on the clinician-rated Yale Brown Obsessive Compulsive Scale (Y-BOCS; Goodman et al., 1989), indicative of at least moderate OCD symptom severity (Maust et al., 2012). Patients with current MDD or a Montgomery Asberg Depression Rating Scale (MADRS; Montgomery & Asberg, 1979) score of ≥ 17 were excluded, as low-grade inflammation has been demonstrated in MDD (Dowlati et al., 2010).

3.5.2 Exclusion Criteria

Patients with significant suicidal ideation (MADRS item 10 ≥ 3) or those who enacted suicidal behaviors within 6 months prior to assessment were also excluded and referred for appropriate clinical intervention. Those fulfilling criteria for a lifetime history of

bipolar disorder, history of substance abuse, a history of schizophrenia or other psychotic disorders, delirium, dementia and amnesic and other cognitive disorders, or were in an agitated state at assessment were also excluded. Current substance use disorder was also excluded.

Lifetime or current autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, etc.), inflammatory bowel disease, and diabetes were excluded as these conditions typically present with an elevated inflammatory profile. Current use of any psychotropic agents; SSRIs, benzodiazepines, monoamine oxidase inhibitors, tricyclic antidepressants or herbal psychoactive treatments (e.g. St. John's Wort, Kava Kava, Chamomile Extract, Valeria), was excluded. Past pharmacotherapy or use of psychoactive compounds was permitted if discontinued 3 months prior to sampling as this has been documented as sufficient time for the gut microbiota to fully recover following a recorded disruption (e.g. antibiotics, between 4 to 12 weeks; Jernberg et al., 2007). Those receiving psychotherapy, including CBT for an anxiety or mood disorder, 3 months prior to sampling were also excluded. Similarly, antibiotic or probiotic use within 12 weeks of sampling was also considered exclusionary. However, if willing, participants were permitted into the study 12 weeks following final dose of antibiotic/probiotic, assuming all remaining study criteria continued to be met.

3.6 Study Assessments

Interested participants underwent a preliminary phone screen to eliminate those meeting any obvious exclusion criteria. Seemingly eligible participants were asked to attend 1-2 in-person assessment visits where all study assessments and biological samples were

collected. OCD and comorbid diagnoses were confirmed with a psychiatric consultation. Information regarding family and personal medical and psychiatric history were also collected.

3.6.1 Psychiatric Assessments and Measures

3.6.1a MINI International Neuropsychiatric Interview

The MINI is a semi-structured interview for psychiatric disorders. It was used to confirm diagnosis of OCD and additional comorbid psychiatric conditions in the OCD group while confirming absence of psychiatric disorders in controls. The MINI was also used to screen for lifetime bipolar disorder, schizophrenia and substance use disorders, as these individuals were excluded. The MINI was administered by either a trained doctoral student or research assistant; however, OCD study patients also underwent a consultation with a psychiatrist to confirm diagnosis.

3.6.1b Yale Brown Obsessive-Compulsive Scale

The Y-BOCS is a 10-item, clinician-rated scale designed to measure OCD symptom severity (Goodman et al., 1989). Scores for each item range from 0 to 4 and it is considered a standard severity rating scale for OCD. The original instrument showed excellent interrater reliability for the total score ($r=0.98$) and good reliability ($\alpha=0.88-0.91$) (Goodman et al., 1989). Individuals in the OCD group required a Y-BOCS score ≥ 20 (indicative of at least moderate OCD severity) to participate (Goodman et al., 1989). A score of 0 -7 is considered nonclinical, with scores ranging between 8 - 15 considered mild. Scores between 16 - 23 are moderate while scores between 24 - 31 are severe and

32 - 40 are considered extreme (Maust et al., 2012). These severity gradings were used to categorize individuals based on symptom severity.

3.6.1c Montgomery Aberg Depression Rating Scale

The MADRS (Montgomery & Asberg, 1979) is a 10-item clinician-rated questionnaire designed to measure the severity of depressive symptoms in patients. Each question is scored between 0-6, with a maximum score of 60. This scale was utilized to assess depressive symptom severity with no more than mild depressive symptoms allowed in eligible patients (MADRS score ≤ 17) (Montgomery & Asberg, 1979). If individuals presented any suicidal behaviour within 6 months prior to screening (MADRS item 10 ≥ 3) they were excluded and referred for appropriate intervention. The scale has demonstrated good internal consistency ($\alpha=0.85$ to 0.94) and high concurrent validity (Bondolfi et al., 2010; Montgomery & Asberg, 1979).

3.6.1d Dutch Dimensional Obsessive-Compulsive Scale

The Dutch Dimensional Obsessive-Compulsive Scale (DDOCS) is a 9-item semi-structured, clinician-rated interview. The first item (time) has a scale from 0 to 16 with the remaining 8 items scored on a scale from 0-4 resulting in a total score ranging from 0-50. Internal consistency of the DDOCS was high ($\alpha=0.8$) as was inter-rater reliability ($r=0.967$) with intra-class correlations of separate items ranging from 0.819 to 0.981. Convergent validity was also high with the Y-BOCS ($r=0.827$, $p<0.01$) while divergent validity suggested moderate correlation with the MADRS ($r=0.38$, $p<0.01$) (Denys et al., unpublished). This scale was used as an additional indicator of OCD symptom severity.

3.6.1e Obsessive Compulsive Inventory – Revised

The Obsessive-Compulsive Inventory (OCI-R) is an 18-item self-report measure of OCD symptoms (Foa et al., 2002). It contains information regarding hoarding, checking/doubting, ordering, mental neutralizing, obsessing and washing subscales. The subscales demonstrate good internal consistency ($\alpha=0.81-0.90$), test-retest reliability over an interval of 2 weeks ($r=0.74 - 0.91$) and moderate to high convergent/divergent validity (Foa et al., 2002). The OCI-R was utilized to provide information regarding self-reported OCD symptomatology.

3.6.1f Depression, Anxiety and Stress Scale

The Depression Anxiety Stress Scale (DASS-21) is a 21-item self-report scale that measures levels of depression, stress and anxiety. With 4 response options for each question, a maximum score of 21 on each subscale is possible (scores are multiplied by 2 to make results comparable to the full DASS-42). This tool demonstrated excellent internal consistency (0.93) (Henry & Crawford, 2005) and interpretations revealed good construct validity (Henry & Crawford, 2005). This measure was incorporated to provide information regarding mood and anxiety levels in the sample.

3.6.2 Gastrointestinal and Diet Assessments

3.6.2a Gastrointestinal Symptom Rating Scale

Participant gastrointestinal symptoms and bowel functioning were assessed using the clinician-rated Gastrointestinal Symptom Rating Scale (GSRS) (Svedlund et al., 1988).

This instrument contains 15 questions providing insight on abdominal pain, dyspepsia, reflux and bowel dysfunction (Svedlund et al., 1988). The interrater reliability (weighted kappa) revealed high agreement between users for both the separate items ($\kappa_w=0.86-1.00$) and sub-categories ($\kappa_w=0.92-0.94$) (Svedlund et al., 1988).

3.6.2b The Short-Form Leeds Dyspepsia Questionnaire

The Short-form Leeds Dyspepsia Questionnaire (SF-LDQ) assessed frequency and severity of indigestion, heartburn, regurgitation and nausea (Fraser et al., 2007). High level of internal consistency was demonstrated in primary and secondary care patients ($\alpha=0.90$), with item-total correlation for each question ranging from 0.57 to 0.75 (Fraser et al., 2007). Test-retest reliability was also high with a Pearson's correlation coefficient of 0.93 between the first and second summed scores (Fraser et al., 2007).

3.6.2c Rome III Criteria for Irritable Bowel Syndrome

The Rome III criteria for IBS was used to evaluate prevalence of IBS in the population (Drossman & Dumitrascu, 2006). Since the development of this study, the new Rome IV criteria for IBS have been published with minor modifications to relevant criteria (Drossman & Hasler, 2016). The new criteria include: “recurrent abdominal pain, on average at least 1 day/week in the last 3 months associated with 2 or more of the following: 1) related to defecation; 2) associated with a change in frequency of stool; or 3) associated with a change in form (appearance) of stool”. This criteria was fulfilled for “the last 3 months with symptom onset at least 6 months before diagnosis” (Drossman & Hasler, 2016). Rome III criteria incorporated “recurrent abdominal pain or discomfort at

least 3 days/month in the last 3 months” and required improvement with defecation and that onset be associated with a change in frequency or appearance of stool (Drossman & Dumitrascu, 2006).

3.6.2d EPIC-Norfolk Food Frequency Questionnaire

Information regarding participant diet was recorded using the EPIC-Norfolk Food Frequency Questionnaire (FFQ)(Bingham et al., 1997) as diet can effect microbiota composition (Canlon & Bird, 2015). Given that it was unfeasible to recruit participants based on diet similarity, the FFQ was used to examine whether significant differences were present in the diets of OCD patients and sex- and age-matched controls. The EPIC-Norfolk FFQ is a validated 130-item semi-quantitative FFQ where participants are required to provide information regarding their diet in the past 12 months. Respondents report the frequency of consumption of a “medium serving” from “never or once per month” to “more than 6 times per day”. Amounts of energy and individual nutrient intake were calculated from the frequency and amount of each food item represented in the FFQ and converted into grams per day using the FETA software (Mulligan et al., 2014).

3.6.3 Stool Samples

To examine the presenting gut microbiome profiles, patients and controls were instructed to collect morning stool samples at home (or in the clinic) using a protocol well-established by the Surette lab. During the recruitment visit, participants were provided a kit containing a collection hat, single screw capped sterile sample jar and a cooling pad. They were instructed to transfer several grams of feces (first bowel movement of the

day) while wearing gloves, into the vial placed in a sample bag labelled with time and date of sample collection and stored in a household freezer until delivery. The samples were then transported to the MacAnxiety Research Centre and stored in a -20°C freezer until delivery to the Surette lab where part of the sample was transferred into four 2-mL cryovials and snap frozen using liquid nitrogen for molecular analysis of the microbiota.

3.6.3a Extraction of DNA from stool samples

DNA was extracted from each fecal sample using a standard extraction and purification method for mixed clinical samples as previously described (Sibley et al., 2011). This approach involved the basic steps of mechanical lysis, chemical lysis, and DNA purification in a series of 10 steps. Approximately 300 µL of feces was placed in a 2mL plastic screw top tube containing 0.2 g of 2.0 mm diameter ceramic beads and suspended in 800 µL of 200 mM NaPO₄ (pH 8) and 100 µL of GES. The tube was homogenized at 3000 r.p.m for 3 minutes in a bead-beater instrument twice. Approximately 0.2 grams of 0.1 mm diameter ceramic beads were added and then homogenized at 1500 r.p.m for an additional 3 minutes. Samples were then subjected to a two-step enzymatic lysis. The first comprised of an incubation at 37°C water bath for 1-1.5 hours in a 110 µL solution of 50 µL of lysozyme (100 mg/mL in H₂O), 50 µL of mutanolysin (10 U/µl) and 10 µL of RNase A (10 mg/mL in H₂O). In the second stage, samples were incubated for 0.5-1.5 hours in 125 µL solution of 25 µL 25% sodium dodecyl sulfate (SDS), 25 µL Proteinase K, and 75 µL 5 M NaCl. Screwcap tubes were then centrifuged at max speed for 5 minutes and then 900 µL of supernatant was removed and transferred to a 2-mL tube containing 900 µL (equal volume) of 25:24:1 phenol-chloroform-isoamyl alcohol. The

solution was vortexed and then centrifuged at max speed for 10 minutes, and the top layer transferred to a sterile 1.5 mL tube. Purification and final elution of DNA was done using a Zymo DNA clean and concentrator 250 kit (Thermo Scientific, Waltham, MA). DNA was eluted in 50 μ L of sterile DNase/RNase free water pre-heated at 65°C. DNA concentration and quality in the extracts was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Extracted DNA was stored at -80°C until needed for PCR amplification.

3.6.3b Bacterial profiling of 16S rRNA genes using Illumina Miseq

Quantitative, real-time, PCR (qPCR) was used to measure total eubacterial load using the universal bacterial primers 8fM and Bact515R. Variable region 3 (V3) of bacterial 16S ribosomal RNA (16s rRNA) genes present in each fecal community was amplified using qPCR, and the resulting amplicons were sequenced on an Illumina MiSeq 2000 instrument (Illumina Inc, San Diego, CA) as described previously by Bartram et al (2011) with the modification that bar-codes are included in the forward primer. Samples were amplified in triplicate using a Veriti® 96-Well Fast Thermal Cycler, model 9902 (Applied Biosciences® Burlington ON). The PCR reaction mixture in a volume of 60 μ L contained 6 μ L (10 pmol/ μ L) each of V3 forward and barcoded reverse primers, 1.5 μ L magnesium chloride (MgCl₂) (50 mM) solution, 6 μ L 10 x PCR buffer, 1 μ L deoxynucleotide solution (dNTPs) (10 mM each), 34.25 μ L dH₂O, 0.25 μ L Taq Polymerase, and 5 μ L Template DNA (30 ng total) with the following cycling conditions: 30 cycles (94°C, 30 s, 50°C, 30 s; 72°C, 30 s) after an initial denaturation of 2 min at

94°C. Amplicons from the triplicate reactions were pooled together, and separated electrophoretically on a 2% agarose gel. Sequencing was carried out on a MiSeq Illumina sequencer in the MOBIX-McMaster Genome Center (McMaster University). This methodology has been used to profile the human microbiome through the gastrointestinal tract and is used extensively in the Surette lab.

3.6.3c Microbial Sequencing and Analysis

Analysis was performed using an in-house bioinformatics pipeline that generates clusters of operational taxonomic units (OTUs), taxonomic assignment and various measures of α - and β -diversity. PCR products were sequenced using the Illumina Miseq with paired-end reads. Custom Perl scripts were developed in-house to process the sequences as outlined in Whelan and colleagues (2014). First, Cutadapt (Martin 2011) was used to trim these sequences to the V3 region, ridding of any sequences surpassing this region. Next, sequences were aligned with their pair using PANDAseq (Masella et al., 2012); during this alignment, any mismatches or ambiguous bases were culled. Operational taxonomic units (OTUs) were picked using AbundantOTU and as described previously (Ye, 2011) with a clustering cut-off of 97%. Taxonomy of the resultant OTUs was assigned via comparison of a representative sequence of the unit to the Greengenes reference database (DeSantis et al., 2006) using the Ribosomal Database Project classifier.

3.6.4 Blood sample collection

Morning blood samples were collected from OCD patients and controls using standard venipuncture techniques. For the CRP sample, blood was collected in a 6 mL sodium

heparin tube, inverted to mix and immediately centrifuged for 15 minutes at 4000 rpm. A minimum of 1.0mL of heparinized plasma was extracted and transferred to a 12 x75 mm plastic tube with a cap.

Blood samples for IL-6 and TNF- α were collected in a plain, 10 mL VACUTAINER red-top tube. Tubes were maintained in an upright position for 30 minutes prior to centrifugation to allow for clotting. Samples were centrifuged for 15 minutes at 4000 rpm and whenever possible three aliquots of 1.5 mL of serum were collected and transferred into 1.8 mL cryogenic tubes. All samples were stored in a -70°C freezer until processing.

3.6.4a CRP plasma sample analysis

Plasma samples were analyzed at the McMaster University Medical Centre's Core Laboratory (Hamilton, ON, Canada). Plasma CRP levels were determined by immunoturbidimetry (MULTIGENT CROP Vario assay; Abbott Laboratories Inc., Abbott Park, Illinois, USA). The detectable range for this assay was 0.2 to 320mg/L; an intraassay coefficient of variability (CV) of 0.7%-2% and interassay CV of 0.4%-0.8% were also reported.

3.6.4b Serum sample analysis

Serum samples were analyzed for levels of IL-6 and TNF- α in the Bercik laboratory McMaster University (Hamilton, ON, Canada). IL-6 and TNF- α levels were determined using Quantikine heparin sulfate enzyme-linked immunosorbent assay (HS ELISA) (Human IL-6 and TNF- α Immunoassay; R&D Systems, Inc., Minneapolis, MN, USA).

The minimum detectable dose of human IL-6 ranged from 0.0160-0.110 pg/mL (mean: 0.039pg/mL) and intra- an inter-assay CV for the IL-6 assay were 6.9%-7.4% and 6.5%-9.6% respectively. The minimum detectable dose for the TNF- α assay was 0.011-0.049pg/mL; while intra- and inter-assay CV were 1.9%-2.2% and 6.2-6.7% respectively.

3.7 Study Procedure

At the end of the initial phone screen, conducted to exclude those meeting obvious exclusion criteria, participants were notified and scheduled to attend in-person study visits at the MacAnxiety Research Centre. Each study participant had a subject folder containing all data collected, linking them only via an assigned participant number. At the first visit, the information sheet was reviewed, and informed consent was obtained; participant demographic information, personal and family medical/psychiatric history were recorded. If no immediate exclusion criteria were met, the MINI was conducted by a doctoral student or research assistant trained in administering clinical assessments. This was followed by the Y-BOCS, MADRS, DDOCS, GSRS and Rome III criteria for IBS. Participants then completed all self-rated measures in a quiet room independently. Stool sample collection kits and detailed instructions regarding sample collection were then provided and participants were scheduled in for a second morning visit. In most instances the visit was conducted the subsequent morning, however if this was not feasible participants were asked to store the morning sample in their household freezer until the next visit which was conducted within 1 week. Participants then returned the stool sample and morning blood samples for circulating cytokines were collected. Participants were provided \$25 for their time.

3.8 Statistical Analysis

Descriptive statistics, such as number of subjects, means and standard deviations were provided to summarize demographic information, psychiatric and gastrointestinal data and cytokine data of the OCD patient group and the healthy controls.

Data were tested for normality using a Shapiro-Wilk test and Levene's test was used to examine homogeneity of variance. All data were collected using REDCap, a secure electronic case report form, which was programmed to confirm completion of questionnaires eliminating likelihood of missing data.

3.8.1 Microbiome data

Taxonomy was organized at the level of phyla, class, order, family, genus and species into bar charts. This data was used to run Wilcoxon matched pairs rank sum test ($\alpha= 0.05$) comparing OCD and control groups for each OTU. Data was rarefied to the sample with the lowest number of sequences in all samples (13,000 reads).

The primary outcome analysis of differences in the microbiome included β -diversity metrics (dissimilarity measure) and α -diversity (species richness and/or evenness). Beta-diversity was examined utilizing the Bray Curtis dissimilarity, weighted and unweighted Unifrac and Jaccard distribution methods. Using these metrics, principal coordinate analysis (PCoA) plots were created illustrating the ecological distance between each sample to identify clustering. A permutational multivariate analysis of variance (PERMANOVA) was applied to determine whether the observed distances were statistically significant. Overall, β -diversity provides a measure of the degree to which

samples differ from one another with metrics being either quantitative (use sequence abundance, e.g. Bray-Curtis or weighted Unifrac) or qualitative (considering only presence-absence of sequences, e.g. Jaccard or unweighted Unifrac) (Goodrich et al., 2014). Alpha-diversity metrics (Simpson, Inverse Simpson (InvSimpson), Shannon, Observed and Chao1 indices) examined species richness (number of different species) and evenness (relative abundance of different species) within samples. Groups were compared using either the Mann-Whitney U test or the Kruskal Wallance test given the non-parametric nature of the data, when using categorical outcomes. Paired non-normal data was analyzed using the Wilcoxon matched pairs signed rank test. Quasipoisson (log) distribution was used to examine the association between continuous variables and α -diversity (Dill-McFarland, 2017). All phylogenetic data was analysed using the vegan (Oksanen et al., 2017) and Phyloseq packages (McMurdie & Holmes, 2013) in R Statistical Software (version 3.3.3). The ANCOM function (Mandal et al., 2015) was used for the taxon analysis. IBM SPSS Version 25 was used for the remaining analyses. Multiple comparisons were corrected for using the Benjamini-Hochberg Procedure, adjusting p-values using a false-discovery rate (FDR) of 0.05 (Pike, 2011).

3.8.2 Gastrointestinal symptomatology and circulating cytokine data

Mann Whitney U test was used to evaluate statistical significance with non-normal data; otherwise a t-test was used ($p < 0.05$). Similarly, Pearson's r was used to examine correlations of normal data, while Spearman's rank correlation was utilized as the non-parametric equivalent. Paired data was analyzed using the Wilcoxon matched pairs signed

rank test or paired t-tests were used when data were normal. Frequencies were compared using a chi-squared test of independence ($p < 0.05$).

CHAPTER 4: DIFFERENCES IN THE GUT MICROBIOME PROFILE OF NON-DEPRESSED OCD PATIENTS VERSUS HEALTHY CONTROLS

Forward to Chapter 4: This Chapter is one of three describing the results of the overall study. In CHAPTER 4, a comparison of the gut microbiome in a clinical sample of non-depressed, unmedicated OCD patients and non-psychiatric controls is presented; in addition, findings associated with Secondary Objectives 1 and 2 are also reported (examining gut microbiota differences based on symptom severity and psychiatric comorbidity). CHAPTER 5 presents the results of Secondary Objective 5 related to levels of circulating cytokines. Finally, CHAPTER 6, discusses the prevalence of gastrointestinal disorders and symptomatology in OCD patients versus controls (Secondary Objectives 3 and 4). Stool sample analysis was completed by the Surette lab and circulating cytokines were analyzed by the Bercik lab.

4.1 Results

4.1.1 Participants

Forty-three participants were recruited from the community to participate in this study; 21 non-depressed, unmedicated individuals with a primary DSM-5 diagnosis of OCD [OCD Group; mean age = 31.00 (SD=8.98)] and 22 age- and sex-matched controls [control group; mean age = 29.27 (SD=11.16); $t(41) = -0.558, p=0.580$]. Age-matching in particular was critical to the study as the microbiome is known to change throughout the lifespan (Kim et al., 2017). Demographic and clinical characteristics for the OCD and control groups are presented in Tables 4.1 and 4.2 respectively.

No significant differences were observed on any demographic variable between control and OCD subjects (Table 4.1). All variables describing clinical characteristics of the sample differed significantly between both groups, with greater severity noted in the OCD group (Table 4.2). No β - or α -diversity metric differed when examined based on age, sex, BMI, height, or weight (all $p > 0.05$).

Table 4.1: Demographic characteristics of OCD and healthy control groups

Demographic Characteristic	OCD (n=21)	Controls (n=22)
Age (years, mean \pm SD)	31.00 \pm 8.98	29.27 \pm 11.16
Sex		
Male (n)	10	10
Female (n)	11	12
Height (cm, mean \pm SD)	171.18 \pm 9.16	171.55 \pm 10.36
Weight (kg, mean \pm SD)	72.49 \pm 18.84	68.64 \pm 12.64
BMI (mean \pm SD)	24.58 \pm 5.47	23.22 \pm 3.22
Marital status (n, %)		
Never Married	12 (57.1%)	14 (63.6%)
Married/Common-law	8 (38.1%)	8 (36.4%)
Divorced/Separated	1 (4.8%)	0 (0.0%)
Education (n, %)		
High school or less	2 (9.5%)	0 (0.0%)
Some college/university	4 (19.0%)	7 (31.8%)
Technical/non-university	5 (23.8%)	1 (4.5%)
University degree	5 (23.8%)	9 (40.9%)
Graduate degree	5 (23.8%)	5 (22.7%)
Employment (n, %)		
Full-time	8 (38.1%)	5 (22.7%)
Part-time	2 (9.5%)	2 (9.1%)
Unemployed	3 (14.3%)	2 (9.1%)
Retired	1 (4.8%)	2 (9.1%)
Student	7 (33.3%)	11 (50.0%)
Race (n, %)		
White	12 (57.1%)	16 (72.7%)
South Asian	4 (19.0%)	3 (13.6%)
East Asian	1 (4.8%)	2 (9.1%)
West Asian	2 (9.5%)	0 (0.0%)
Mixed race	2 (9.5%)	1 (4.5%)

Table 4.2 Clinical characteristics of the OCD and CONTROL groups

Clinical Characteristics (mean score \pm SD)	OCD (n=21)	Controls (n=22)
MADRS	9.67 \pm 4.88	1.36 \pm 1.26
OCI-R	28.43 \pm 9.70	2.32 \pm 2.66
DASS-21		
Depression	6.30 \pm 5.67	0.91 \pm 1.06
Anxiety	5.45 \pm 4.56	0.59 \pm 0.85
Stress	10.25 \pm 4.96	1.32 \pm 1.55
DDOCS	22.09 \pm 6.34	0.38 \pm 0.72
Y-BOCS	27.05 \pm 4.06	2.09 \pm 2.58
Y-BOCS Compulsion	14.48 \pm 2.27	1.18 \pm 1.53
Y-BOCS Obsessions	12.57 \pm 3.40	1.00 \pm 1.60
Comorbidity (n, %)		
Generalized Anxiety Disorder	16 (76.2%)	
MDD (Past)	13 (61.9%)	
Presence of Tics	11 (52.3%)	
Social Anxiety Disorder	10 (47.6%)	
ADHD	9 (42.8%)	
Specific Phobia	6 (28.6%)	
BDD	5 (23.8%)	
Hoarding Disorder	1 (4.6%)	
Age of Onset (n, %)		
Pediatric (\leq 12 years)	11 (52.4%)	
Adult ($>$ 12 years)	10 (47.6%)	

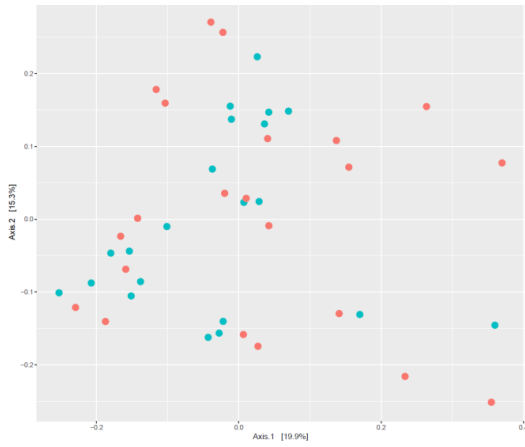
4.1.2 Gut microbiome profile OCD patients versus controls

4.1.2a Beta-diversity

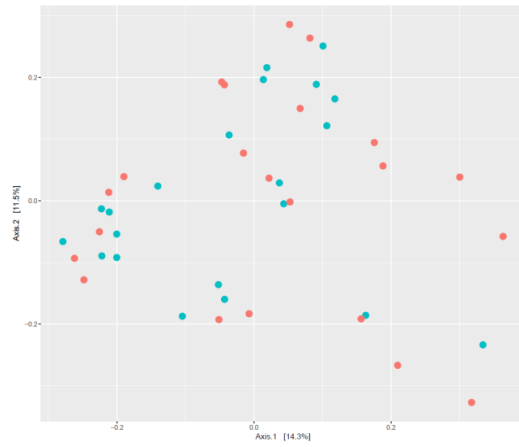
No significant differences were observed using the Jaccard ($F(1,41)=1.106$, $p=0.186$), Weighted Unifrac ($F(1,41)=0.509$, $p=0.697$), Unweighted Unifrac ($F(1,41)=1.164$, $p=0.134$) or Bray-Curtis ($F(1,41)=0.951$, $p=0.479$) metric ($p>0.05$) when comparing samples based on condition (OCD versus controls, Figure 4.1a-d).

Figure 4.1: Principle Coordinate Analysis plots illustrating β -diversity of OCD samples and healthy controls (orange CONTROL; blue OCD)

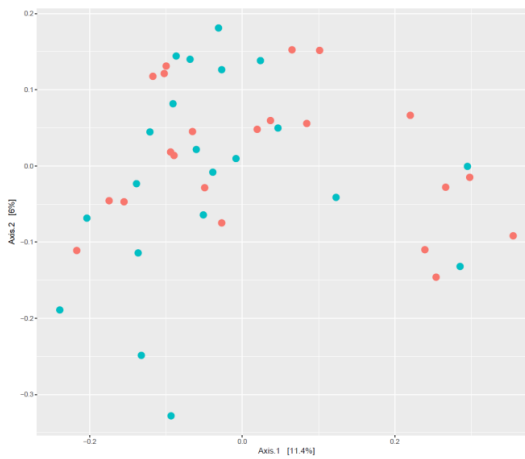
a. Bray Curtis



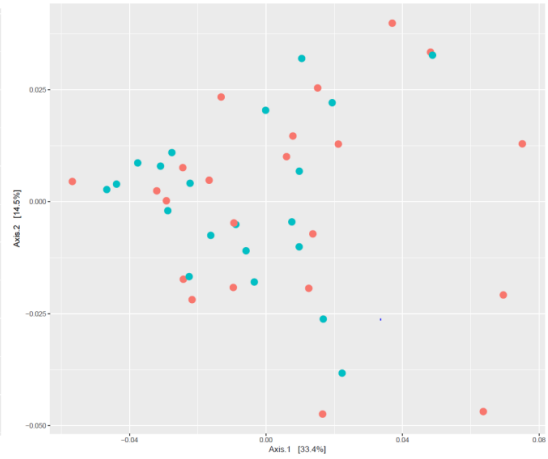
b. Jaccard



c. Unweighted unfrac



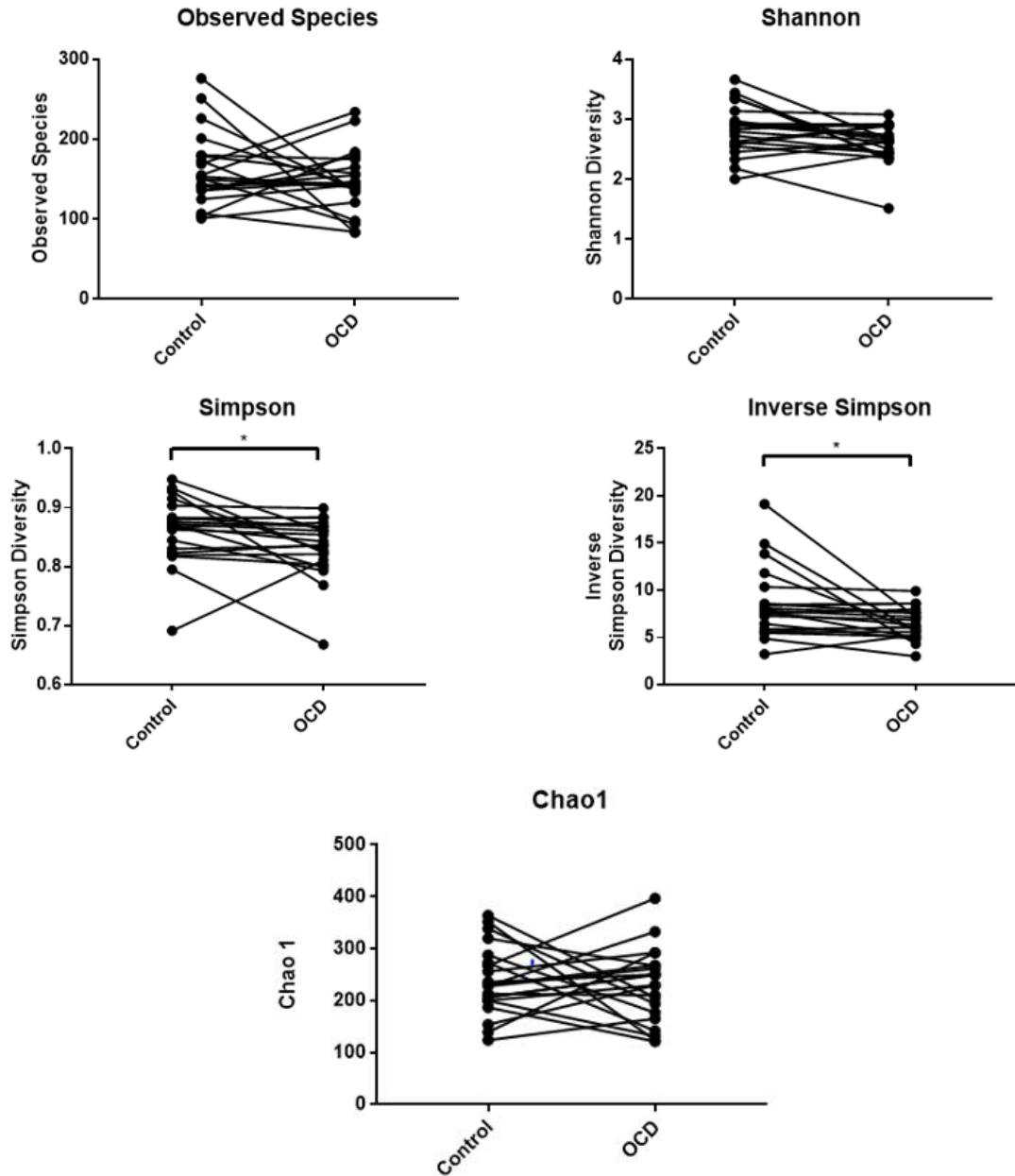
d. Weighted unfrac



4.1.2b Alpha-diversity

Significant differences were noted using the Simpson ($Z = -142$, $p = 0.0142$) and InvSimpson ($Z = -145$, $p = 0.0101$) α -diversity indices, with lower diversity noted in the OCD group (Figure 4.2) compared to age- and sex-matched controls. These differences remained significant even after correcting for multiple comparisons with FDR ($p < 0.02$). No significant differences were noted using the Shannon Index ($Z = -103$, $p = 0.0760$), Chao1 ($Z = -19$, $p = 0.7593$) or Observed Richness ($Z = -33$, $p = 0.5795$) (Figure 4.2).

Figure 4.2 Differences in the gut microbiome of OCD patients versus CONTROL as per various indices of α -diversity



4.1.2c Differences in taxa

Although no differences in β -diversity were noted in this sample, the relative abundance of specific taxa were lower in the OCD group compared to matched controls. Relative abundance of *Oscillospira* (Figure 4.3)(OTU13; Z=-145, p = 0.010), *Odoribacter* (Figure 4.4)(OTU92; Z=-111, p = 0.013) and *Anaerostipes* (Figure 4.5)(OTU137; Z=-111, p=0.014) was significantly lower in the OCD group compared to matched controls, even after FDR was applied (p<0.02). Given that these OTUs have been associated with BMI (lower in higher BMI), the data were categorized based on ‘High’ (≥ 25 kg/m²)/‘Normal’ (<25 kg/m²) BMI. A Mann-Whitney U test revealed that these OTUs were not associated with BMI in this sample (*Oscillospira*: U=207, p=0.819; *Anaerostipes*: U= 188, p=0.471; *Odoribacter*: U=183.5, p=0.400).

Figure 4.3 Lower relative abundance of OTU13 in OCD patients compared to CONTROL, identified as *Oscillospira*

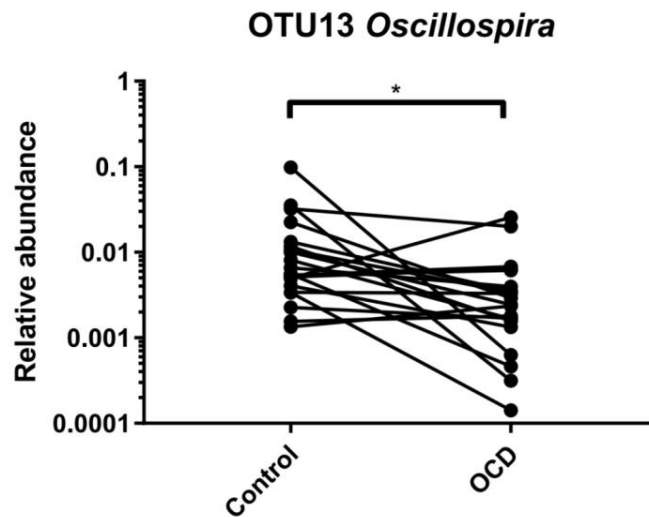


Figure 4.4 Lower relative abundance of OTU92 in OCD patients compared to CONTROL, identified as *Odoribacter*

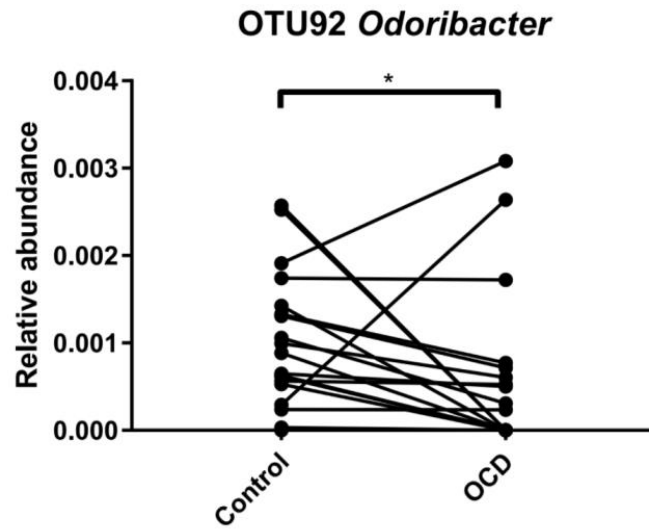
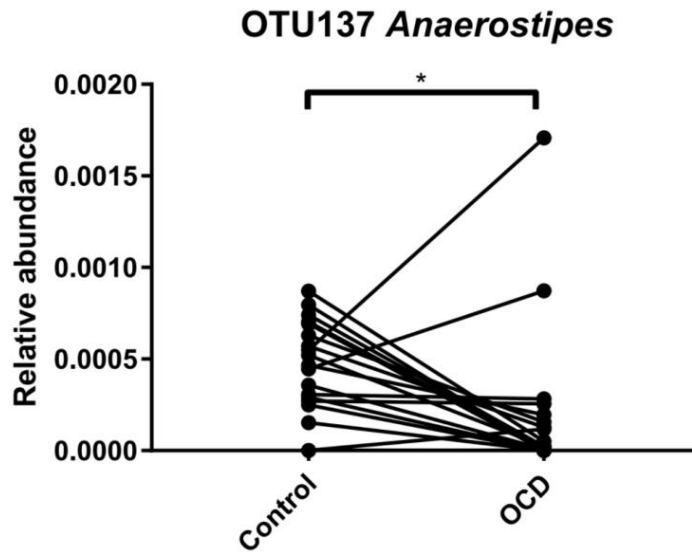


Figure 4.5 Lower relative abundance of OTU137 in OCD patients compared to CONTROL, identified at *Anaerostipes*



Given the lower relative abundance of these OTUs in OCD patients, a “dose relationship” comparing levels of each different OTU as per symptom severity was pursued. To accomplish this, OCD patients were characterized as ‘Moderate’ (n=8; Y-BOCS score ≤ 24) or ‘Severe’ (n=13; Y-BOCS score 24-31) (Maust et al., 2012). Although a Y-BOCS score of 32-40 is characterized as an “Extreme” case, given that there were only 2 individuals meeting this criterion, these data were compounded into the ‘Severe’ category. All 3 taxa followed a similar pattern with individuals in the ‘Severe’ group possessing higher levels of each OTU than the ‘Moderate group’. A Kruskal-Wallis test revealed the distribution of each OTU differed between the 3 groups (*Oscillospira*: $H(2)=7.875$, $p=0.019$; *Odoribacter*: $H(2)=6.598$, $p=0.037$; *Anaerostipes*: $H(2)=9.902$, $p=0.007$)(Figure 4.6 - 4.8). However, pairwise comparisons (Dunn’s test) revealed that only the comparison between controls and the ‘Moderate’ (*Oscillospira*: $Z=14.265$, $p=0.014$; *Odoribacter*: $Z=13.114$, $p=0.021$; *Anaerostipes*: $Z=16.583$, $p=0.004$) and ‘Severe’ (OTU13: $Z=8.432$, $p=0.045$; OTU92: $Z=7.264$, $p=0.049$; OTU137: $Z=8.417$, $p=0.043$) groups respectively, were significantly different. Once multiple comparisons were corrected for ($p<0.025$), only the difference between controls and the Moderate group persisted (FDR $p<0.025$).

Figure 4.6. Relative abundance of *Oscillospira* (OTU13) based on OCD severity (Y-BOCS score)

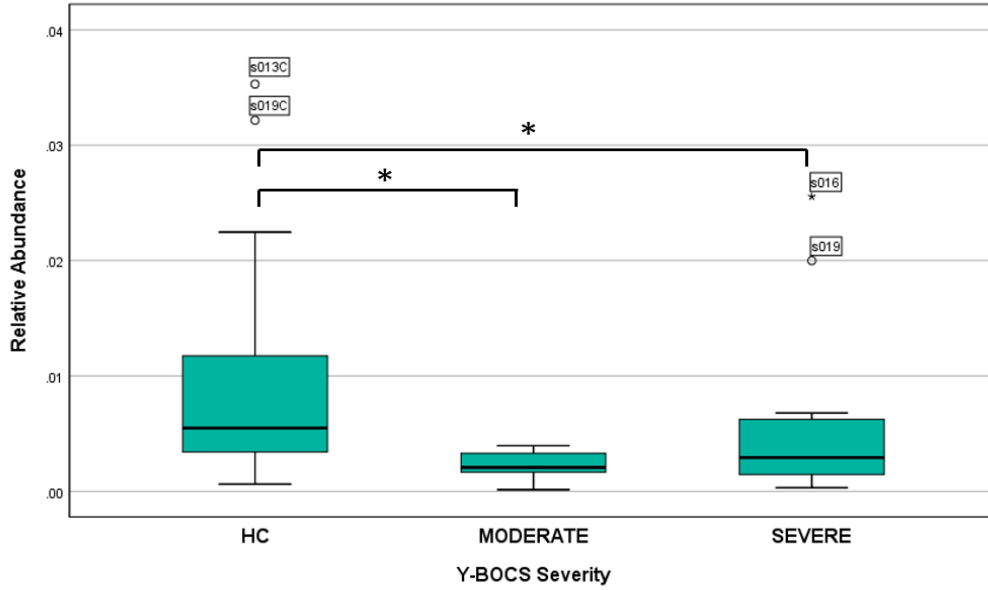


Figure 4.7 Relative abundance of *Odoribacter* (OTU92) based on OCD severity (Y-BOCS score)

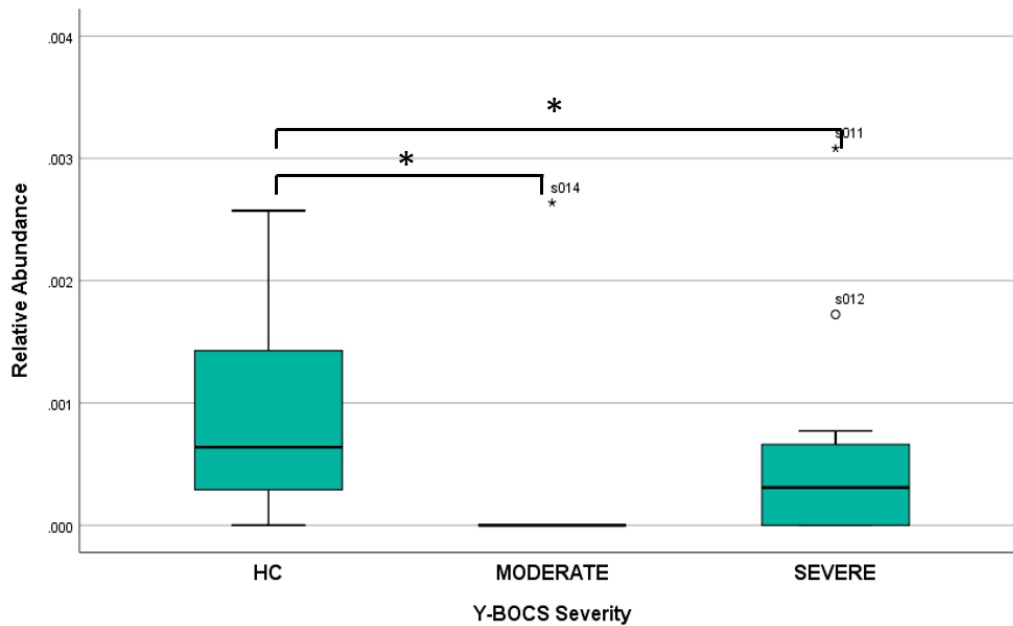
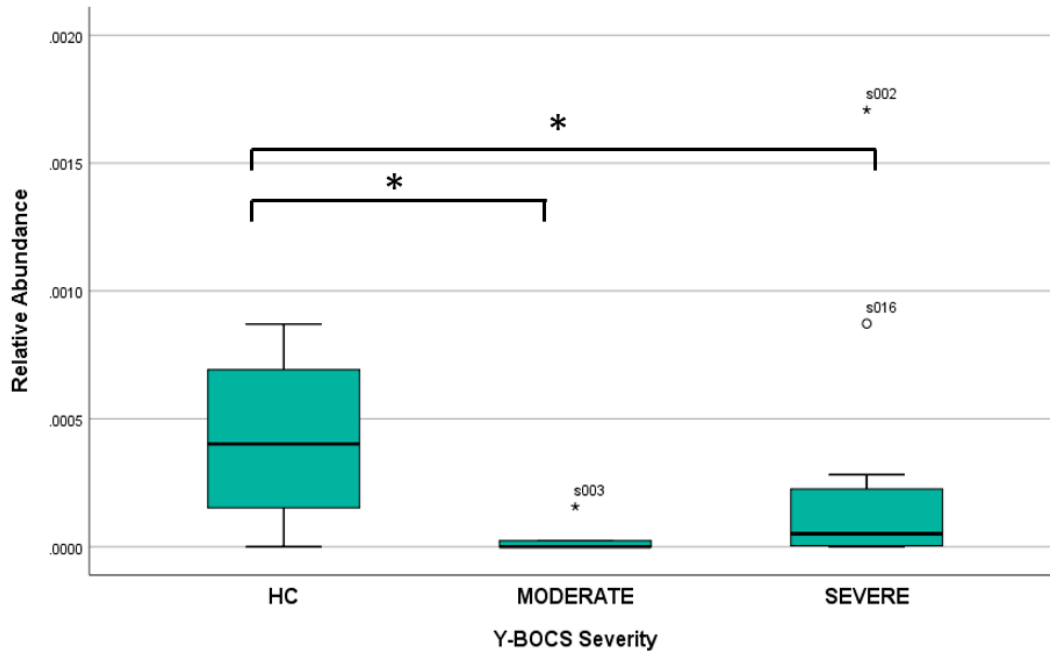


Figure 4.8. Relative abundance of *Anaerostipes* (OTU137) based on OCD severity (Y-BOCS score)



Similarly, OCI-R scores were used to categorize OCD patients as well. Although there are no existing symptom severity gradings for this scale, the sample was organized by controls, those with a score ≥ 21 (n=16) or <21 (n=5). A score of 21 is a suggested cut-off for whether patients should be considered for an OCD diagnosis (Foa et al., 2002).

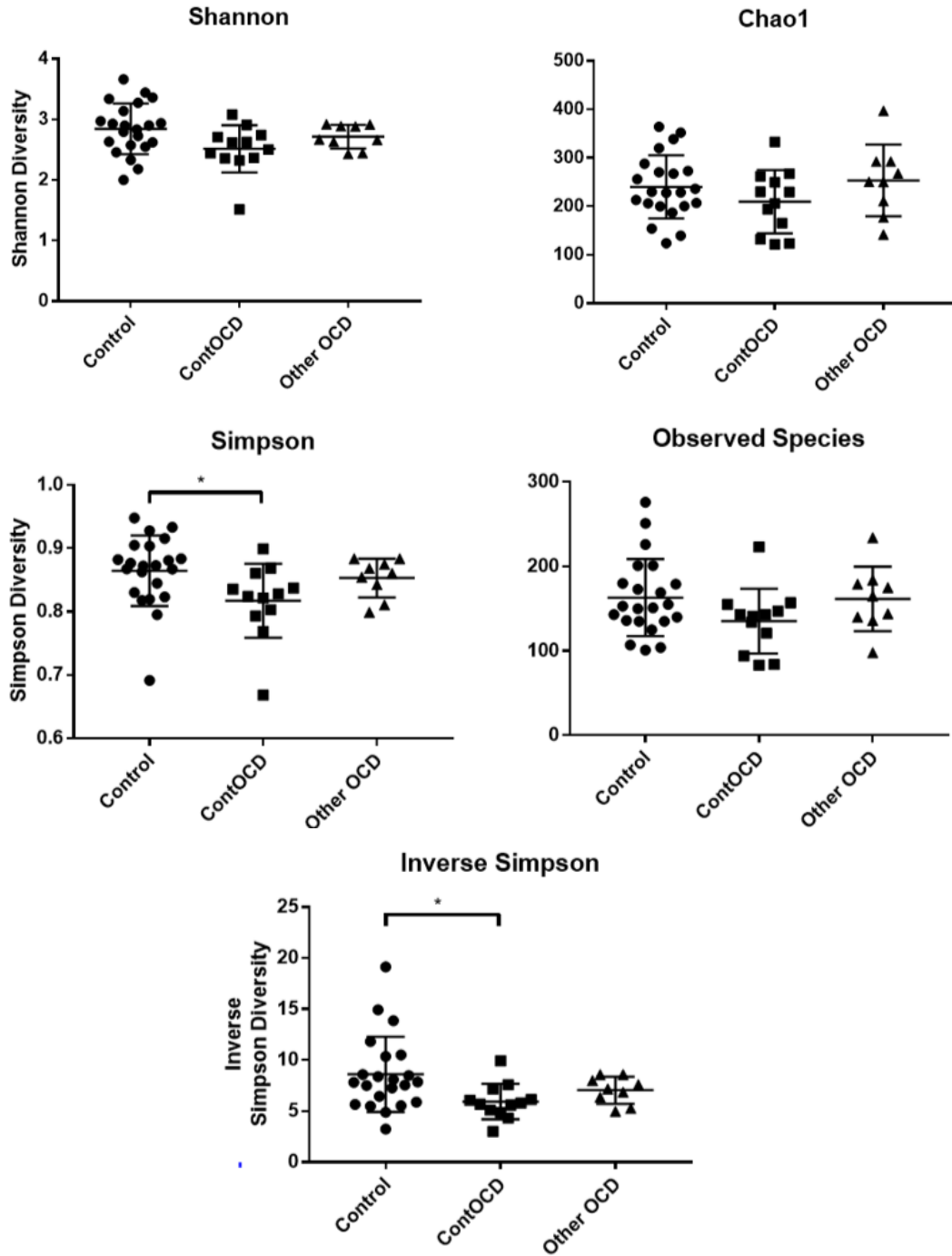
Although five OCD patients did not meet this threshold, a psychiatric consultation still resulted in a diagnosis likely due to the few but impairing and severe symptoms (mean Y-BOCS: 27 ± 3.41). A Kruskal-Wallis test revealed differences in *Oscillospira* ($H(2)=9.072$, $p=0.011$) and *Anaerostipes* ($H(2)=8.117$, $p=0.017$). However, these differences only applied to the comparison between OCD patients with a score ≥ 21 and controls

(*Oscillospira*: $Z=9.812$, $p=0.016$; *Anaerostipes*: $Z=10.274$, $p=0.011$) ($FDR<0.022$) and should be interpreted cautiously given the largely unequal groups.

4.1.2d Differences in the gut microbiome based on OCD symptom subtype and controls

Given the heterogenous nature of OCD, microbial profile differences based on primary symptom subtype were also of interest. Guided by the hygiene hypothesis, the OCD was group was categorized into patients with primary contamination-based symptoms (ContOCD, $n=12$) and those falling under all other OCD symptom subtype categories (OtherOCD, $n=9$). Considering the symptom categories of the dimensional Y-BOCS, the OtherOCD group included patients with primary aggressive obsessions and related compulsions, sexual and religious obsessions and related compulsions and also symmetry, ordering, counting and arranging obsessions. Further classification of the OtherOCD group was not possible due to an unequal and small sample size as 7 patients presented with primary symmetry/ordering symptoms, and only 2 with primary harm avoidance/checking. Lower α -diversity as per the Simpson ($H(2)=11.93$, $p=0.0243$) and InvSimpson ($H(2)=11.93$, $p=0.03$) was noted in the ContOCD group compared to controls (Figure 4.9). However, this difference was no longer significant following correction for multiple comparisons ($p<0.02$). No differences were noted in the remaining α -diversity indices (Figure 4.9).

Figure 4.9 Differences in α -diversity indices based on primary OCD symptom subtype (ContOCD and OtherOCD) and controls.



4.1.3 Gut microbiome profile and symptom severity

4.1.3a OCD Symptom Severity

Beta-diversity

Y-BOCS and OCI-R scores were used to illustrate OCD symptom severity when examining β -diversity. Patients were grouped based on the categorization method utilized in section 4.1.2c, comparing β -diversity to controls. No significant differences in β -diversity were observed using either measure ($p > 0.05$).

Alpha-diversity

Greater OCD severity was associated with lower α -diversity as per the InvSimpson metric. A quasipoisson (log) distribution model was used to examine the fit of OCD symptom severity and InvSimpson due to the non-normal nature of microbiome data. There was a significant relationship between Y-BOCS ($p = 0.0139$) and InvSimpson diversity. Similarly, the primary self-report OCD symptom severity measure, the OCI-R ($p = 0.033$), but also the DDOCS ($p = 0.0254$), revealed lower diversity as symptom severity increased. Simpson, Shannon, Observed or Chao1 metrics were not associated with any statistically significant differences.

4.1.3b Mood and Anxiety Symptoms

Increasing MADRS scores were associated with decreasing α -diversity (InvSimpson, $r_s = -0.338$, $p = 0.027$). Analyses for β -diversity could not be completed as OCD patients did not present any more than mild depressive symptoms resulting in no informed categorical

division of scores. MADRS scores were negatively correlated with *Oscillospira* ($r_s=-0.464, p=0.002$), *Odoribacter* ($r_s=-0.409, p=0.007$) and *Anaerostipes* ($r_s=-0.397, p=0.009$); while the DASS-D was only associated with *Oscillospira* levels ($r_s=-0.349, p=0.025$).

No differences were seen on any of the DASS-21 sub-scales (anxiety, stress or depression) using any α -diversity metric ($p>0.05$). However unweighted Unifrac, revealed a potential trend with DASS-A ($F(5,42)=1.172, p=0.051$) and DASS-S ($F(5,42)=1.136, p=0.074$). DASS-S only revealed a negative correlation with *Oscillospira* ($r_s=-0.367, p=0.018$) and *Anaerostipes* ($r_s=-0.380, p=0.014$).

4.1.4 Gut microbiome profile based on comorbid conditions

No significant differences were noted in any β -diversity metric when looking at comorbid GAD, ADHD, BDD, social anxiety disorder (SAD) or past MDD ($p>0.05$). The number of comorbid disorders was also not related to β - or α -diversity metrics used in this study.

OCD patients with and without comorbid GAD (Figure 4.10)(InvSimpson, $H(2)=7.148, p=0.028$) and Tics (Figure 4.11)($H(2)=6.1001, p=0.0457$) revealed lower α -diversity in the sample. However, only the comparison between controls and those with comorbid GAD ($n=16$)($Z=2.619, p=0.0264$) and no tics ($n=10$)($Z=2.418, p=0.0467$) was statistically significant (Figure 4.10 and 4.11 respectively).

Figure 4.10 Alpha-diversity (InvSimpson) in OCD patients with (red) and without (green) comorbid GAD and controls (blue)

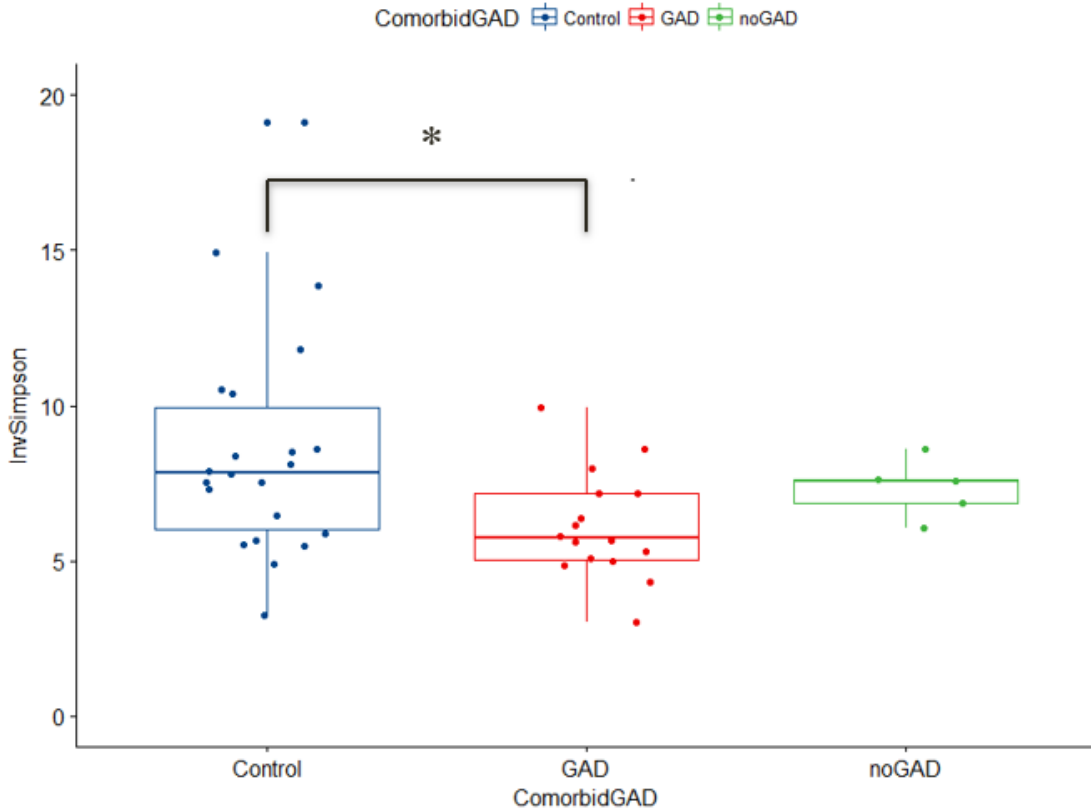
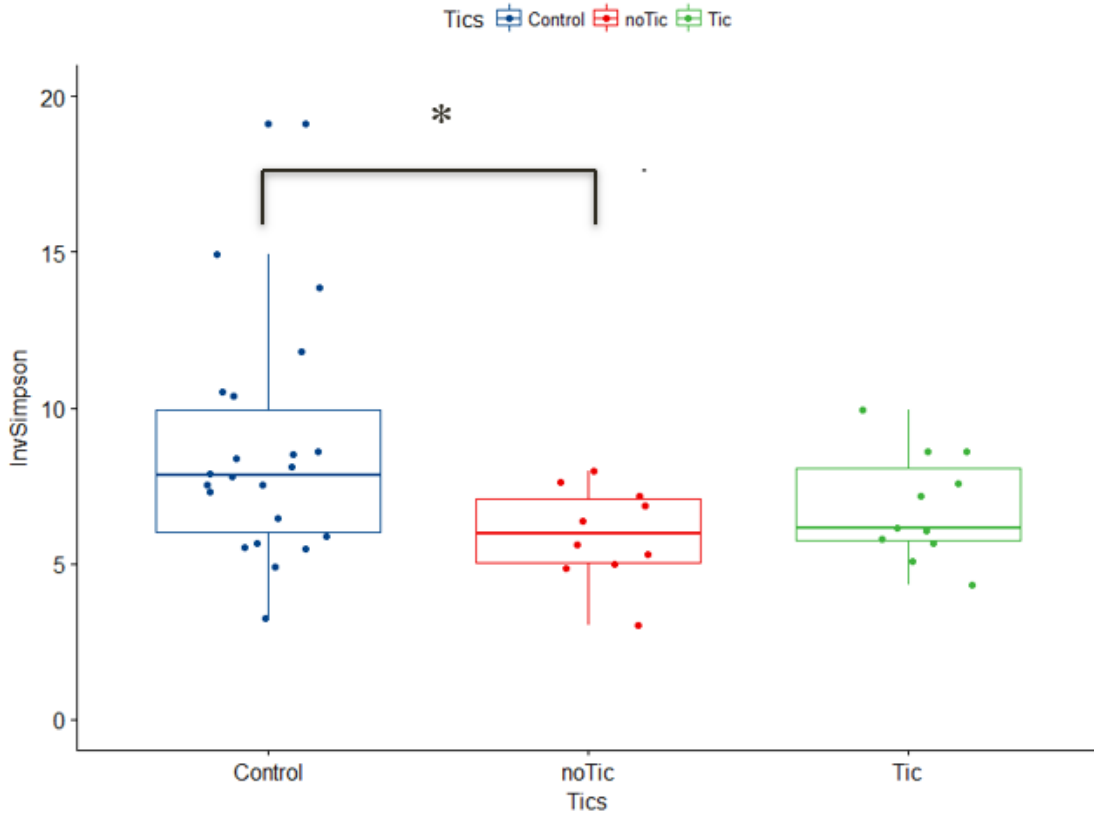


Figure 4.11 Alpha-diversity (InvSimpson) in OCD patients with (green) and without (red) tics and controls (blue)



OTU differences were not attributed to comorbid GAD, SAD or presence of tics. However, Past MDD (n=13) and Comorbid ADHD (n=9) revealed a difference in relative abundance of OTU13 (*Oscillospira*, PastMDD: $H(2)=10.298$, $p=0.006$; ComorbidADHD: $H(2)=10.982$, $p=0.004$). Pairwise comparison's (Dunn test) revealed that both past MDD ($Z=12.165$, $p=0.004$), and comorbid ADHD ($Z=-14.732$, $p=0.003$) presented lower relative abundance of *Oscillospira* than controls. Comorbid ADHD was also associated with differences in the relative abundance of OTU137 (*Anaerostipes*, $H(2)=8.862$, $p=0.012$), which was restricted to a lower relative abundance in patients with comorbid ADHD compared to controls ($Z=-13.365$, $p=0.006$). Finally, the relative abundance of OTU92 (*Odoribacter*) differed based on Comorbid BDD ($H(2)=8.577$, $p=0.014$), with lower levels in patients with comorbid BDD (n=5) compared to controls ($Z=-16.776$, $p=0.005$). However, many of these comparisons should be interpreted cautiously given the unequal groups.

4.1.5 The gut microbiome based on age of onset

With childhood onset of OCD thought to have a potentially distinct mechanism from adult onset (Dell'Osso et al., 2016), as age of onset has been associated with varying outcomes, it was of interest to examine whether such differences extended to the gut microbiome. No differences were noted in the β -diversity of OCD patients with pediatric (onset ≤ 12 years) versus those with adult onset and controls ($p > 0.05$). Similarly, no differences were noted in the α -diversity metrics, or taxa ($p > 0.05$). In line with existing research, greater OCD symptom severity was noted in those with self-reported pediatric onset of their OCD [Pediatric: mean Y-BOCS= 29.18 (SD=3.763); Adult: mean Y-

BOCS=25.40 (SD=3.373); $t(19)=-2.415$, $p=0.026$]. However, differences based on mean OCI-R [Pediatric: mean OCI-R = 31.00 (SD=10.402); Adult: mean OCI-R =25.60 (SD=8.488), $t(19)= -1.295$; $p=0.211$] or DDOCS scores [Pediatric: mean DDOCS=23.27 (SD=7.254); Adult: mean DDOCS=20.50 (SD=5.395), $t(19)=-0.987$; $p=0.152$] were not statistically significant. Depressive symptoms [MADRS: $t(19)=-0.207$, $p=0.839$; DASS-D: $t(19)=0.600$, $p=0.556$] and anxiety [DASS-A: $t(19)=0.293$, $p=0.773$; DASS-S: $t(19)=1.315$, $p=0.205$] symptoms did not differ between pediatric or adult OCD onset cases. Nor did sex distribution amongst the two groups ($\chi^2=1.173$, $p=0.395$) or number of mean comorbid disorders between the two groups (Pediatric: 3.091 (SD=1.513); Adult: 2.700 (SD=1.59); $t(19) =0.659$, $p=0.528$). A Chi-square test revealed that primary symptom subtype was not related to age of OCD onset ($\chi^2(1)=0.064$, $p=0.801$). Similarly, none of the OCI-R subscales were different between these two groups, including severity of washing symptoms ($t(19)=0.076$, $p=0.940$).

4.1.6 Diet

The EPIC-Norfolk FFQ was utilized to determine possible differences between diets of study participants. The EPIC-Norfolk FFQ assessed diet and provided consumption of several micro- and macro-nutrients and food groups (refer to Appendix I). Once multiple comparisons were corrected for ($p<0.00083$), no significant differences were noted in any nutrient or food group when comparing the OCD group to controls. While it is typically expected that ContOCD patients may consume reduced amounts of animal products due to their contamination concerns; this was not seen in the current sample. Similar amounts of egg and egg products ($H(2)=2.978$, $p=0.226$), fish and fish products ($H(2)=1.410$,

$p=0.494$) and meats and meat products ($H(2)=2.491$, $p=0.288$) were consumed by the ContOCD, OtherOCD and control groups.

4.2 Summary of findings in this Chapter

This is the first study to examine the gut microbiome profiles in patients with OCD. Although no differences in the β -diversity metrics were noted (Figure 4.1a-d), α -diversity (as per the InvSimpson and Simpson indices) was reduced in OCD patients compared to controls (Figure 4.2). These differences continued to persist when evaluated based on symptom dimension (ContOCD and OtherOCD). While α -diversity was reduced in both OCD groups, only the ContOCD group statistically differed from controls (Figure 4.9) until multiple comparisons were corrected for. Taxon analysis revealed lower relative abundance of 3 different OTUs when examined by condition. *Oscillospira* (Figure 4.3), *Odoribacter* (Figure 4.4) and *Anaerostipes* (Figure 4.5) were all lower in the OCD group compared to controls. These differences were not related to diet of study participants, as no differences were noted in the general food group and nutrient consumption in the sample.

Although increasing OCD symptom severity predicted lowering InvSimpson diversity index, the differentiating OTUs between the OCD and controls conditions did not decrease with OCD severity. Rather (Figures 4.6-4.8), the relative of abundance of each OTU seemed to increase with severity. However, the unequal sample sizes of the groups limit this interpretation. Finally, each OTU was paired with lower relative abundance in certain comorbid conditions, including BDD, ADHD and Past MDD.

Although the OTU-specific differences did not relate to Comorbid GAD or tics, α -diversity was notably lower in those with GAD (Figure 4.10) and those with no tics (Figure 4.11) compared to controls.

CHAPTER 5: CIRCULATING CYTOKINES IN NON-DEPRESSED OCD PATIENTS VERSUS CONTROLS

Forward to CHAPTER 5: Given the possible role of immune dysregulation in OCD, paired with systemic inflammation serving as a leading theoretical component of the gut-brain axis (CHAPTER 2), morning levels of circulating cytokines were examined. The effects of systemic inflammation on the presenting gut microbiome profile is also examined.

5.1 Results

5.1.1 Comparison of circulating cytokines between OCD patients and controls

Mean plasma CRP was significantly higher in OCD patients than controls (Table 5.1).

While no significant differences were noted between mean serum IL-6 and TNF- α levels between the two groups (Table 5.1). Levels of circulating cytokines were not related to sex or age, in the entire sample or the OCD group alone. Neither CRP ($r_s=0.256$, $p=0.097$) nor TNF- α ($r=0.262$, $p=0.089$) were related to BMI; however, IL-6 levels revealed moderate increases with BMI ($r_s=0.566$, $p<0.001$). Cytokines did not differ based on age at self-reported OCD onset ($p>0.05$).

Table 5.1. Mean serum and plasma levels of circulating cytokines

Circulating cytokine	OCD (n=21) (mean \pm SD)	Controls (n= 22) (mean \pm SD)	p-value
Plasma CRP (mg/L)	3.26 \pm 2.90	1.08 \pm 1.35	Z= -2.590, p= 0.01
Serum IL-6 (pg/mL)	1.210 \pm 0.936	1.436 \pm 1.632	Z= -0.191, p=0.898
Serum TNF- α (pg/mL)	0.888 \pm 0.267	0.764 \pm 0.252	t(41)=1.267, p=0.220

5.1.2 Clinical correlates of CRP

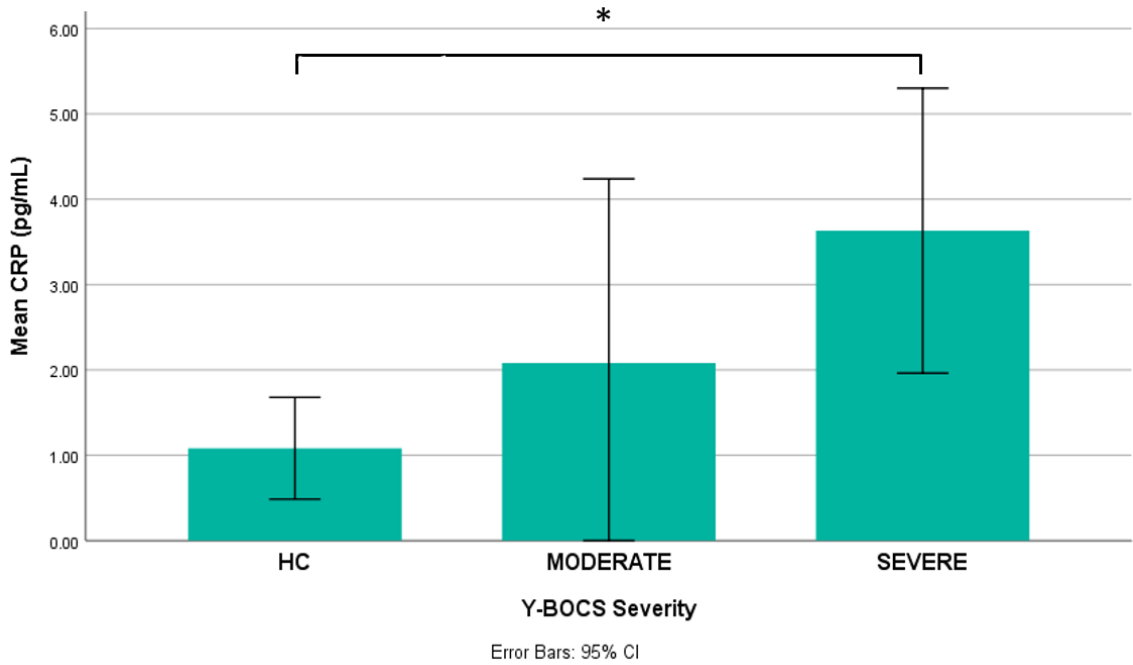
There was a significant effect of condition on mean CRP even after adjusting for Past MDD, BMI, sex and age ($F(4, 40)=43.917, p=0.004$).

5.1.2a OCD symptoms

CRP levels were positively correlated with OCD symptom severity (total Y-BOCS: $r_s=0.446, p=0.003$; total DDOCS: $r_s=0.496, p=0.001$; total OCI-R ($r_s=0.508, p=0.001$).

When categorized based on Y-BOCS severity gradings, mean plasma CRP differed among the groups [$H(2)=11.189, p=0.004$](Figure 5.1). However, only the difference between controls [mean CRP = 1.08 mg/L (SD=1.35)] and the ‘Severe’ OCD group (n=13)[mean CRP = 3.63 mg/L (SD=3.13)] was statistically significant ($Z=-13.631, p=0.003$).

Figure 5.1. Higher mean plasma CRP levels associated with greater OCD symptom severity



5.1.2b Anxiety and mood symptoms

Current MDD and significant depressive symptoms were excluded in the study, however total MADRS score was still positively correlated with CRP levels ($r_s=0.523$, $p=0.0003$). The DASS-D did not reveal a similar relationship ($r_s=0.219$, $p=0.164$). Higher anxiety was also noted with increasing CRP levels as revealed by a moderate correlation (DASS-A: $r_s=0.465$, $p=0.002$ and DASS-S: $r_s=0.433$, $p=0.004$).

5.1.2c Psychiatric and gastrointestinal comorbidity

Given that higher CRP levels were seen in OCD patients, where reduced species and evenness was observed in Chapter 4. We examined whether other conditions paired with altered α -diversity would reveal similar results. Mean CRP differed when considering

Comorbid GAD (n=16) [$H(2)=11.679$, $p=0.003$] and presence of tics (n=11) [$H(2)=12.861$, $p=0.002$]. Dunn's test for pairwise comparison revealed that CRP levels were higher in patients with Comorbid GAD (mean CRP:3.756mg/L (SD=3.121) compared to controls (mean CRP = 1.082mg/L (SD=1.346) ($Z=-14.037$, $p=0.001$). Although individuals with no GAD [n=5, mean CRP=1.680mg/L (SD=1.225)] still had higher CRP than controls, this difference did not reach statistical significance likely due to unequal sample size. Interestingly, patients with no tics illustrated significantly higher CRP [NoTics; mean CRP =4.170mg/L (SD=2.649)] than did controls as per Dunn's test ($Z=-16.768$, $p<0.001$). Although the difference did not achieve statistical significance, mean CRP was lower in patients with Tics [With Tics; mean CRP=2.436 mg/L(SD=2.993)].

Gastrointestinal symptom severity (total GSRS score) increased with CRP levels ($r_s=0.409$, $p=0.008$), as did frequency and severity of dyspepsia (total-SF-LDQ; $r_s=0.339$, $p=0.028$). Despite noting a positive correlation between CRP and gastrointestinal symptoms, this marker of systemic inflammation did not differ in participants who met Rome III criteria for IBS (n=11) compared to those who did not (n=33)($n=(U=61.5$, $p=0.654$)

5.3.3 Correlates of TNF- α and IL-6 in OCD patients and controls

5.3.3a TNF- α

Given the normal distribution of TNF- α , Pearson's correlation revealed that this pro-inflammatory cytokine increased with greater abdominal pain severity (GSRS abdominal

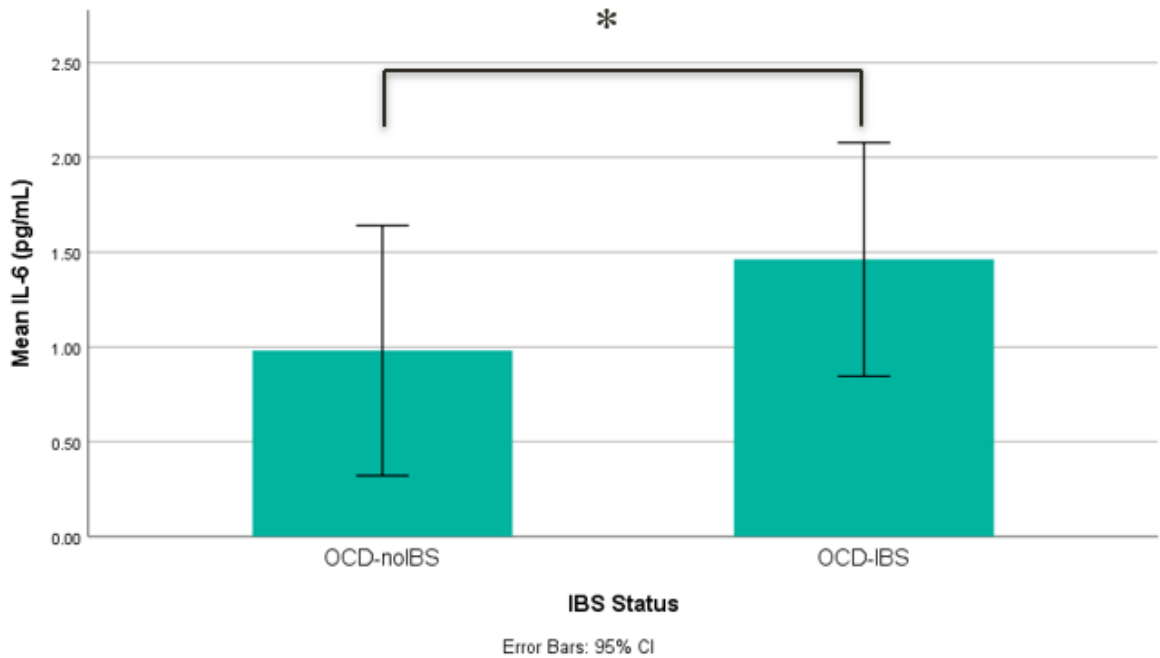
pain, $r=0.310$, $p=0.043$) and depressive symptoms (MADRS, $r=0.325$, $p=0.034$).

However, these differences were no longer significant once multiple comparisons were corrected for ($p<0.025$). Like CRP, individuals with IBS did not demonstrate higher levels of TNF- α ($t(41)=1.295$, $p=0.203$). A one-way ANOVA indicated no differences in TNF- α levels when considering Comorbid GAD ($F(2,40)=2.495$, $p=0.095$) and presence of Tics ($F(2, 40)=1.832$, $p=0.173$).

5.3.3b IL-6

No clinical correlates of IL-6 levels were identified. Mean IL-6 did not differ when examined based on Comorbid GAD [$H(2)=1.524$, $p=0.467$] or presence of Tics [$H(2)=1.926$, $p=0.382$]. Although IL-6 was elevated in participants with IBS, this difference did not reach statistical difference ($U=257$, $p=0.055$). This relationship was further explored in only OCD patients with IBS (OCD-IBS) and OCD patients with no IBS (OCD-noIBS). IL-6 was elevated in OCD-IBS patients [OCD-IBS; mean IL-6= 1.462pg/mL (SD=0.862) versus OCD-noIBS; mean IL-6= 0.981pg/mL (SD=0.982); $U=85.0$, $p=0.035$] (Figure 5.2).

Figure 5.2 Mean IL-6 levels are elevated in OCD-IBS patients compared to those OCD patients without IBS.



5.3.4 Differences in the gut microbiome based on inflammatory markers

Participants were categorized into ‘High’ (n=12, $\geq 2\text{mg/L}$) and ‘Low’ (n=9, $< 2\text{mg/L}$) CRP and compared to controls (n=22). The groups did not differ using any β -diversity metric including Bray-Curtis ($F(2,40)=0.872$, $p=0.654$), Jaccard ($F(2, 40)=0.908$, $p=0.657$), unweighted Unifrac ($F(2,40)=1.088$, $p=0.219$), and weighted Unifrac ($F(2, 40)=0.966$, $p=0.465$)(Figure 5.3 a-d). Although Observed, Chao1, Shannon and Simpson α -diversity metrics did not differ these categories, InvSimpson diversity index approached statistical significance ($H(2)=5.219$, $p=0.074$), with a lower diversity index in those with ‘High’ CRP.

Figure 5.3 Principle analysis coordinate pots demonstrating β -diversity categorized by High' ($\geq 2\text{mg/L}$) and 'Low' ($< 2\text{mg/L}$) CRP levels in OCD patients compared to contorols. OCD patients (High CRP– green; Low CRP – Blue); CONTROLS (red)



Levels of TNF- α , CRP and IL-6 were not correlated with the specific OTUs or any metric of α -diversity ($p>0.05$), as such, the likelihood of any measured microbiome metric acting as a mediator of the relationship between inflammation and OCD severity was limited.

5.3.5 Autoimmune conditions in OCD

Given that greater systemic inflammation was noted in OCD patients, post-hoc analyses were conducted to examine whether OCD patients presented a higher frequency of allergies and also whether more first-degree relatives of OCD patients were diagnosed with autoimmune disorders. A Chi-Square test revealed higher prevalence of allergies in OCD patients (61.9%) than controls (26.1%) ($\chi^2(1)=5.225$, $p=0.022$). More OCD patients also reported first-degree family members with autoimmune conditions than did controls (38.1% vs. 8.9%; $\chi^2(1)=5.064$, $p=0.024$). With reduced α -diversity and increased CRP also noted in patients with Comorbid GAD and those with no tics, frequency of allergies and familial autoimmune conditions were pursued. A Fisher's Exact Test (two-sided) was utilized as $>25\%$ of cells presented values less than 5. Compared to those without GAD, OCD patients with GAD comorbidity did not present with any difference in prevalence of allergies, and in their first-degree relatives, no difference in history of autoimmune disorders was noted. This finding was similar to when extended to OCD patients with comorbid tics compared to those with no tics.

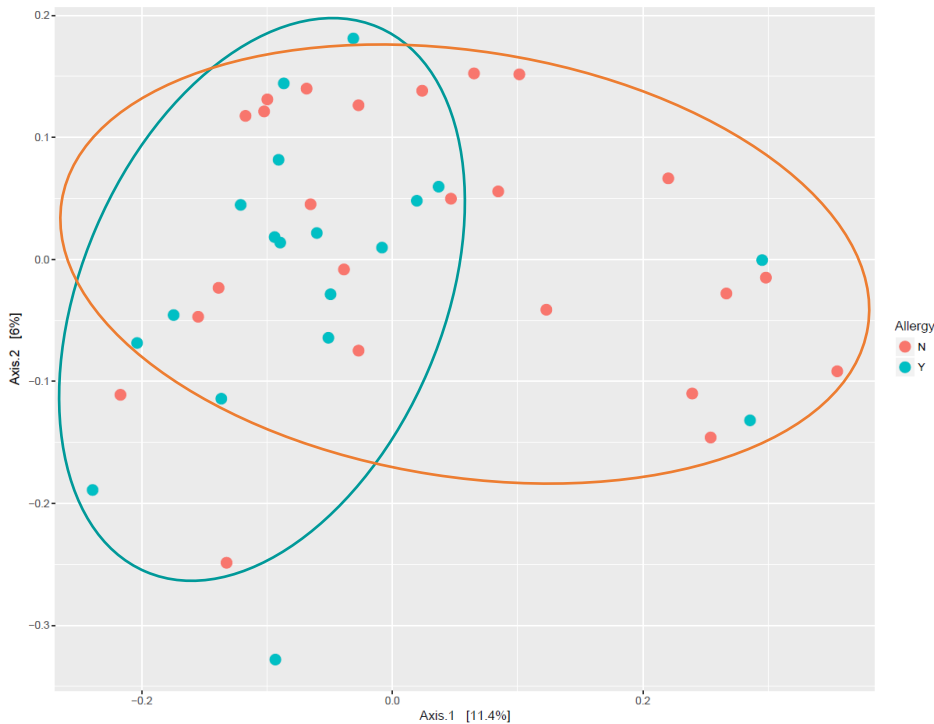
Levels of CRP, IL-6 and TNF- α did not differ significantly based on presence of allergies in OCD patients or autoimmune disorders in their first-degree relatives (Table 5.2).

Table 5.2 Mean levels of circulating cytokines in OCD patients as per presence of self-reported allergies and history of autoimmune conditions among first-degree family members

	Mean plasma CRP (mg/L)(mean±SD)	Mean serum IL-6 (pg/mL)(mean±SD)	Mean serum TNF- α (pg/mL)(mean±SD)
Family history			
Autoimmune			
YES (n= 8)	3.938±3.001	1.417±1.114	0.941±0.349
NO (n=13)	2.846±2.880	1.082±0.831	0.855±0.216
p-value	$U=64.50, p=0.374$	$U=68.00, p=0.268$	$t(19)=0.708, p=0.487$
Allergies			
YES (n=13)	3.454±3.054	1.251±1.123	0.920±0.296
NO (n= 8)	2.950±2.811	1.143±0.580	0.835±0.219
p-value	$U=56.00, p=0.804$	$U=43.00, p=0.547$	$t(19)=0.703, p=0.490$

Presence of allergies did reveal significant clustering using the Unweighted Unifrac metric ($F(1, 41)=1.341, p=0.043$) among all study samples (Figure 5.4). While Weighted Unifrac ($F(1,41)=1.782, p=0.079$) and Bray-Curtis ($F(1, 41)=1.493, p=0.095$), the Jaccard metric did not ($F(1, 41)=1.276, p=0.133$). When organized based on Condition (Allergy Y/N, OCD vs controls) the difference was no longer significant. No differences in α -diversity or OTU distribution were noted. Autoimmune disorders in first-degree relatives were not associated with any microbial differences.

Figure 5.4 Significant clustering based on presence of allergies among OCD patients and healthy controls (Unweighted Unifrac)



5.3.6 Do α -diversity and CRP predict likelihood of OCD?

Given that both α -diversity and CRP differed between OCD patients and controls, a logistic regression was performed to ascertain the effects of the InvSimpson α -diversity metric and CRP levels on the likelihood that patients have OCD. Although it would have been of interest to incorporate the relative abundance of the OTUs identified in the taxon analysis, due to the small sample size, the model was limited to two predictors (Norman & Streiner, 2008). Collinearity diagnostics revealed that multicollinearity was low in the proposed model (eigenvalues not close to 0, condition index <15). The model was statistically significant ($\chi^2(2)= 14.04, p=0.001$) and explained 37.1% of the variance in

OCD. It correctly classified 69.8% of cases and increasing CRP levels (OR=1.577 95% CI: 1.043 – 2.38, p=0.031) and decreasing α -diversity (OR=0.744, 95% CI: 0.528 -1.047, p=0.049) were associated with a higher likelihood of OCD.

5.4 Summary of findings

This chapter evaluated differences between circulating cytokines in patients with OCD compared to controls. While TNF- α and IL-6 did not reveal any differences based on condition, CRP levels were higher in OCD patients than in controls (Table 5.1), suggesting potentially higher systemic inflammation. A value of 2 mg/L has been cited as a clinically relevant cut-off for increased cardiovascular risk (Ridker, 2016), similar values have been used in studies of depression and CRP (Ford & Erlinger, 2004; Chamberlain et al., 2018). Mean CRP levels in this sample exceeded these clinically relevant levels despite no comorbid depression. Differences in levels of IL-6 and TNF- α were not as prominent. Higher systemic inflammation was associated with greater gastrointestinal symptom severity, IBS in OCD patients, comorbid GAD and no tics. A role for an immune-related pathophysiology is further supported by the higher prevalence of allergies in OCD patients and higher rates of autoimmune conditions in their first-degree relatives compared to controls.

CHAPTER 6: GASTROINTESTINAL FUNCTION IN NON-DEPRESSED, UNTREATED OCD PATIENTS VERSUS HEALTHY CONTROLS

Forward to CHAPTER 6: The results of this Chapter target Secondary Objectives 3 and 4. These include, determining the proportion of OCD patients with IBS, as dictated by the Rome III criteria for Irritable Bowel Syndrome and characterizing gastrointestinal function in OCD patients.

6.1 Results

6.1.1 Gastrointestinal function in OCD patients versus Controls

Greater gastrointestinal dysfunction was noted in the OCD group (Table 6.1). Total GSRS was higher in the OCD group than in controls, as were scores for all associated subscales (abdominal pain, reflux, indigestion, and bowel dysfunction) suggesting greater symptom severity across all measured domains. Although the SF-LDQ total score demonstrated a similar trend, this difference did not reach statistical significance (Table 6.1). The SF-LDQ revealed that indigestion (23.8%) and nausea (23.8%) were the most commonly reported problems in the OCD group.

Table 6.1: Gastrointestinal symptom severity in OCD patients versus CONTROLS

Measure	OCD (n=21) (Mean ± SD)	Controls (n=22) (Mean ± SD)	p-value
GSRS total	8.67 ± 6.72	2.32 ± 2.12	$t(20)= 3.644, p=0.002$
GSRS (abdominal)	0.86 ± 0.79	0.14 ± 0.35	$t(20)= -3.627, p=0.002$
GSRS (reflux)	1.62 ± 2.06	0.32 ± 0.65	$t(20)= -2.532, p=0.020$
GSRS (indigestion)	2.43 ± 2.29	0.86 ± 0.94	$t(20)= -2.770, p=0.012$
GSRS (bowel dysfunction)	3.76 ± 3.32	1.00 ± 1.38	$t(20)= 3.071, p=0.006$
SF-LDQ total	5.25 ± 6.24	2.14 ± 2.33	$t(20)= -2.045, p=0.055$

When looking at both groups, greater gastrointestinal symptom severity (as per total GSRS) was related to increasing psychiatric symptom severity. Strong associations ($r > 0.6$) were seen with OCD severity [total DDOCS ($r=0.629$, $p=0.01$) and total OCI-R ($r=0.669$, $p=0.01$)], depression [DASS-D ($r=0.664$, $p=0.01$) and MADRS total ($r=0.731$, $p=0.01$)], and anxiety [DASS-A ($r=0.729$, $p=0.01$) and DASS-S ($r=0.758$, $p=0.01$)]. Gastrointestinal severity did not differ based on pediatric or adult OCD onset (GSRS total: $t(19)=1.088$, $p=0.290$).

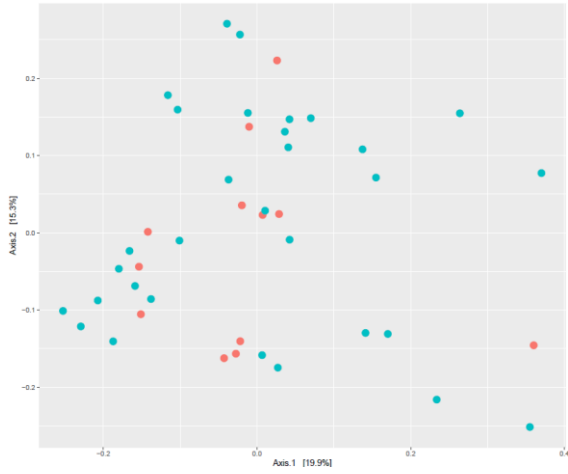
6.1.2 Prevalence of Irritable Bowel Syndrome

In the current sample, prevalence of IBS (as per Rome III criteria) was higher in the OCD group ($n=10$, 47.6%) than in controls ($n=1$, 4.5%) ($\chi^2(1)=10.471$, $p=0.001$). A Cramer's V value of 0.493 suggested a moderate to strong effect ($p=0.001$). Frequency of IBS was equal among males and females ($\chi^2(1)=1.393$, $p=0.238$). In the OCD group, diarrhea-predominant IBS was most common (27.3% of OCD group, 60% of the IBS-OCD group, $n=6$) with only 4 patients in total meeting criteria for either constipation-predominant ($n=2$) or mixed IBS ($n=2$).

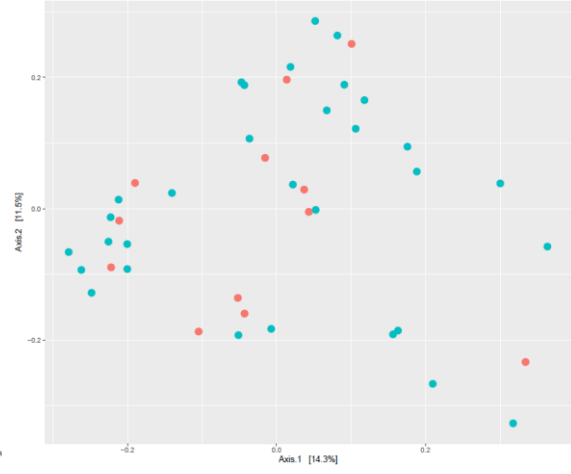
Participants who met criteria for IBS did not present with any differences in the β -diversity metrics (Figure 6.1a-d) including Bray Curtis ($F(1,41)=0.729$, $p=0.767$), Jaccard ($F(1,41)=0.790$, $p=0.805$), Unweighted Unifrac ($F(1,41)=0.936$, $p=0.604$) and Weighted Unifrac ($F(1,41)=0.485$, $p=0.895$).

Figure 6.1. Principle Coordinate Analysis plots illustrating β -diversity of whole sample with and without IBS (orange IBS; blue no IBS)

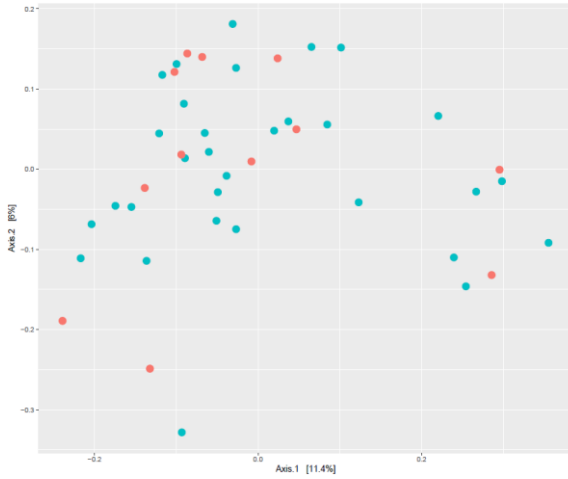
a. Bray Curtis



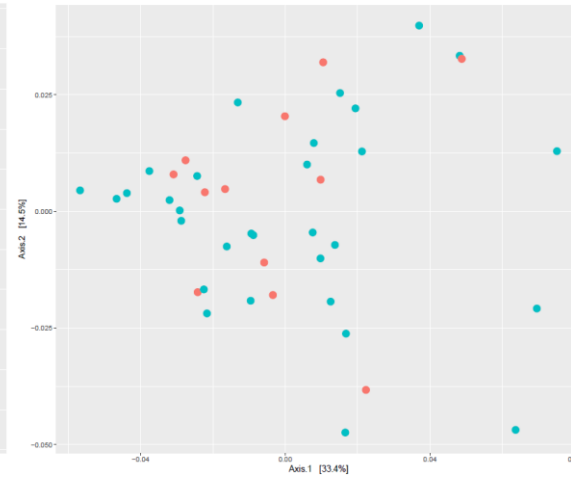
b. Jaccard



c. Unweighted Unifrac



d. Weighted Unifrac



Similarly, the various α -diversity metrics did not differ based on presence of IBS (Observed: $U=166.50$, $p=0.791$; Chao1: $U=180.00$, $p=0.911$; Shannon: $U=181.00$, $p=0.889$; Simpson: $U=175.00$, $p=0.978$; InvSimpson: $U=172.50$, $p=0.989$). Nor did the 3 OTUs identified in the taxon analysis in Chapter 4 (Oscillospira: $U=137.00$, $p=0.350$; Odoribacter: $U=156.00$, $p=0.693$; Anaerostipes: $U=159.50$, $p=0.756$).

6.1.2a Characterization of the OCD with IBS group

Almost half (47.6%) of OCD patients met Rome III criteria for IBS, and in the previous Chapter, increased levels of the pro-inflammatory cytokine IL-6 were demonstrated in OCD-IBS. As such further characterization of these groups was of interest.

Clinical Outcomes

Both groups reported similar psychiatric severity, as suggested by the number of comorbid disorders (mean 3.1 vs 2.7 comorbid disorders). OCD symptom severity did not differ based on IBS criteria (Y-BOCS: 27.60 (SD=2.413) vs 27.18 (SD=5.154), $t(19)=-0.234$, $p=0.818$; OCI-R: 29.80 (SD=5.903) vs 27.18 (SD=12.384), $t(14.609)=-0.627$, $p=0.550$; DDOCS: 21.40 (SD=4.624) vs 22.45 (SD=7.917), $t(19)=0.368$, $p=0.717$). However, these patients did have fewer and likely less severe compulsions (as per the compulsion-specific questions on the Y-BOCS – Items 6-10) [13.40 (SD=1.776) vs 15.45 (SD=2.296; $t(19)=-2.276$, $p=0.035$]; no difference was noted with obsessions [13.50 (SD=2.171) versus 11.73 (SD=3.524), $t(19)=1.207$, $p=0.242$].

Patients with IBS reported higher depressive symptoms (MADRS: 12.60 (SD=1.897) vs 6.91 (SD=2.773), $t(19) = -5.430$, $p < 0.001$; DASS-D: 17.80 (SD=9.355 vs 7.40 (SD=11.118), $t(19) = -2.263$, $p = 0.036$) and gastrointestinal symptoms (Table 6.2). The OCD-IBS group also illustrated higher self-reported anxiety and stress ratings (DASS-A: 14.40 (SD=8.475); DASS-S: 24.20 (SD=7.857)) than other OCD patients (DASS-A: 7.40 (SD=8.746); DASS-S: 16.80 (SD=10.758), however these differences were not statistically significant. GAD comorbidity was high in the overall OCD sample (76.2%, Table 4.2). Interestingly, GAD comorbidity was also high in the OCD-IBS group (80%, $n=8$). Other comorbid conditions in the OCD-IBS group are outlined in Table 6.3.

Table 6.2. Gastrointestinal symptom severity in OCD patients with IBS and those without

GI Measure	OCD-IBS (Mean ± SD)	OCD-noIBS (Mean ± SD)	p-value
GSRs total	12.90 ± 6.154	4.82 ± 4.708	$t = -3.399$, $p = 0.003$
GSRs (abdominal)	1.50 ± 0.527	0.27 ± 0.467	$t = -5.658$, $p < 0.001$
GSRs (reflux)	2.10 ± 1.663	1.18 ± 2.359	$t = -1.021$, $p = 0.320$
GSRs (indigestion)	3.90 ± 2.424	1.09 ± 1.044	$t = -3.389$, $p = 0.005$
GSRs (bowel dysfunction)	5.40 ± 2.836	2.27 ± 3.101	$t = -2.403$, $p = 0.027$
SF-LDQ total	8.10 ± 6.47	2.40 ± 4.719	$t = -2.251$, $p = 0.037$

Table 6.3 Comorbid conditions in the OCD-IBS group

Comorbidity	% (n) of OCD-IBS group
GAD	80 (8)
Past MDD	70 (7)
ADHD	70 (7)
Presence of tics	60 (6)
SAD	40 (4)
BDD	30 (3)

OCD patients with IBS also had a higher BMI [OCD-IBS group; mean BMI = 27.607 (SD=6.309)] compared to those without IBS versus [OCD-noIBS group; mean BMI = 21.833 (SD=2.562), $t(11.663)=-2.699$, $p=0.02$].

Differences in the microbiome

No differences were noted in the relative abundance of *Oscillospira*, *Odoribacter* or *Anaerostipes* ($p>0.05$) amongst OCD patients when examined based on presence of IBS. However, α -diversity (InvSimpson) demonstrated a trend towards higher diversity in patients with IBS ($U=83.00$, $p=0.049$).

6.2 Summary of findings

Microbial dysbiosis has been demonstrated in some functional gastrointestinal disorders. Such conditions, including IBD, have also been associated with high anxiety and mood comorbidity (Mikocka-Walus et al., 2016). As such, this link between gastrointestinal disorders, psychiatric comorbidity and the gut microbiome fueled exploration of gastrointestinal severity in the current study. Despite excluding for IBD, patients with OCD reported greater gastrointestinal symptom severity than controls. This included greater severity of abdominal pain, reflux, indigestion and bowel dysfunction.

Interestingly, almost half of OCD patients also met Rome III criteria for IBS. Consequently, prevalence of IBS was significantly higher in OCD patients than in controls. IBS has often been termed a “disorder of the gut-brain axis” as a physical cause has not been identified. When comparing individuals (OCD patients and controls) with IBS to those who did not meet criteria, no differences were noted in either α - or β -

diversity. However, when comparing OCD-IBS patients to OCD-noIBS patients, higher diversity was demonstrated in the OCD-IBS group, a difference which approached significance. Further, presence of IBS did not dictate the previously detected taxonomical differences. The OCD-IBS group did not report greater OCD symptoms, however depressive and anxiety symptoms were higher in this group than in OCD-noIBS patients. Further comorbid GAD was the most common comorbidity in the OCD-IBS group, paired with the fewer/less severe compulsions may suggest the observed relationship to be explained by GAD rather than OCD. Similarly, these patients presented with higher gastrointestinal symptom severity, BMI and higher levels of IL-6, but not TNF- α or CRP.

CHAPTER 7: DISCUSSION AND CONCLUSIONS

OCD is a debilitating chronic neuropsychiatric illness whose pathophysiology remains unclear. Although the literature supports several potential mechanisms for OCD (outlined in Chapter 1), much of this evidence may also allude to a role for the gut microbiome (discussed in Chapter 2). The gut-brain axis is gaining traction in psychiatric illness and a neuro-endocrino-immunological connection may be implicated. Given that the gut microbiome is a key player in this axis, the primary goal of this thesis was to investigate the involvement of the gut microbiome in OCD. Varying metrics of α - and β -diversity and taxon analysis were used to inform this goal by comparing the stool samples of unmedicated, non-depressed OCD patients and non-psychiatric healthy controls. Next, with systemic inflammation as a potential route of communication of the gut microbiome, levels of circulating cytokines were examined. Finally, the high comorbidity of gastrointestinal problems in disorders where microbial dysbiosis has been implicated led to the exploration of gastrointestinal symptom severity and rates of IBS in OCD. This thesis is the first investigation of the gut microbiome in OCD, and as such, adds significantly to the literature by providing pilot data to potentially guide a new line of research.

7.1 Interpretation of key findings

In Chapter 4, we demonstrated that sample richness and evenness (based on α -diversity) was lower in patients with a primary diagnosis of OCD compared to non-psychiatric age- and sex-matched controls. Three specific OTUs was identified to be lower in OCD

patients. Chapter 5 identified no differences in serum IL-6 or TNF- α levels, however, mean plasma CRP was elevated in OCD patients. Finally, Chapter 6 revealed greater gastrointestinal severity in OCD patients, in addition to a higher prevalence of IBS compared to the controls group and community rates.

7.1.1 Lower α -diversity and relative abundance of *Oscillospira*, *Odoribacter* and *Anaerostipes* in unmedicated, non-depressed OCD patients compared to healthy controls

This is the first study to examine the microbiome profile of patients with OCD. No differences in β -diversity were seen in this study, as demonstrated by the lack of significant clustering of samples noted in PCoA plots. As a measure of “dissimilarity” between samples, this suggested that the samples of OCD patients and controls presented similar profiles. However, α -diversity (as per the InvSimpson and Simpson indices) was lower in OCD patients. Unlike other indices of α -diversity, the InvSimpson and Simpson index both take into account the number of different species present (richness) as well as the relative abundance of each species (evenness). Therefore, as both qualities increase, diversity increases (Lande 1996). The InvSimpson index is also easy to understand conceptually as it describes the number of different species, if they were all evenly abundant or important (Lande 1996). This lower richness/evenness was observed again when the sample was compared based on patients with primary contamination OCD symptoms (ContOCD) and all other OCD patients (OtherOCD). While α -diversity was reduced in both OCD groups, it was only the ContOCD group that statistically differed from controls until correction for multiple comparisons was applied. Similarly, those with

Comorbid GAD and patients without tics also revealed reduced species richness/evenness. This suggested that samples revealed fewer types of bacteria and/or lower relative abundance of bacteria.

The lower α -diversity observed in OCD patients is consistent with the primary dogma of microbiome research. It has been suggested that dysbiosis, or reduced diversity is associated with a diseased state. This ideology extends to previous examinations of the microbiome in psychiatric conditions, with reduced α -diversity noted in ASD (Kang et al., 2017), depression (Jiang et al., 2015), anorexia nervosa (Kleiman et al., 2016) and most recently PANS/PANDAS (Quagliariello et al., 2018). However, the cross-sectional nature of the study limits our ability to examine the cause-and-effect nature of this relationship. The study design controlled for several factors known to impact the presenting microbial profile (e.g. inclusion/exclusion criteria surrounding antibiotic/probiotic or SSRI intake, comorbid autoimmune conditions, etc. (Chapter 3), and inclusion of an FFQ revealed similar diets, suggesting the impact of dietary intake on the microbiome is likely limited. However, in the current study the observed lower diversity may be a consequence of the presenting OCD phenotype as the host's environment can impact the presenting gut microbial profile to an extent. In the current sample over half identified contamination-related OCD symptoms as their most prominent symptoms (ContOCD). These individuals endorsed rituals involving decontaminating their food and/or living spaces, possibly limiting their exposure to bacteria compared to controls. However, since patients were categorized into subtypes based on their primary symptoms (most troubling or time-consuming), we cannot rule out

decontaminating rituals in the OtherOCD group. As such it is possible that the lower species richness observed is a consequence of these behaviours rather than related to the etiology of OCD.

Taxon analysis also revealed lower levels of *Oscillospira*, *Odoribacter* and *Anaerostipes* when comparing OCD patients and controls. *Odoribacter* is of the Bacteroidetes phylum. Both *Oscillospira* and *Anaerostipes* are from the Firmicutes phylum and Clostridia class. This class has been implicated in ASD, with most suggesting higher levels of Clostridia in children with ASDs (Vuong & Hsiao, 2017). Unexpectedly, higher OCD severity was associated with greater mean relative abundance of each OTU, while InvSimpson α -diversity decreased. However, given the high standard error and unequal sample sizes, the reliability of this finding is limited and instead highlights the need for replication in a larger study.

Although these genera have typically been associated with an inflammatory state, recent studies support a role in OCD. For instance, an animal model of OCD using a dopamine agonist (quinpirole) revealed that onset of locomotor sensitivity and compulsive checking (OCD-like behaviours) was associated with changes in OTUs from the *Lachnospiraceae* and *Ruminococcaceae* family (Jung et al., 2018). The differing OTUs identified in this thesis belong to the same taxonomical family. Authors of this study put forth the suggestion that these colonies of gut microbiota are likely related to dopamine activity (Jung et al., 2018) as two studies in patients with Parkinson's disease (Hill-Burns et al., 2017; Keshavarzian et al., 2015) and another using a dopamine agonist in rats (Ning et al., 2017) revealed alterations of OTUs in the same family. Similarly,

Quagliariello et al. (2018) reported that levels of *Bacteroides*, *Odoribacter* and *Oscillospira* were higher in the younger (ages 4-8 years versus age 9) PANS group than in controls. Given the overlap in the PANS/PANDAS and OCD phenotypes, one could expect the results of the current thesis to be inline with these. Quagliariello et al attributed the higher levels of *Odoribacter* and *Oscillospira* to increased inflammation (Quagliariello et al., 2018). However, this was inferred from a few reports correlating inflammatory conditions with *Odoribacter* and *Oscillospira*, despite meta-analytic evidence and several studies supporting the opposing relationship (Konikoff & Gophna, 2016; Gophna, Konikoff & Nielson, 2017; Walters et al., 2014; Morgan et al., 2012). As such, their results may be better explained by inclusion of comorbid depression, as previous studies have reported higher levels of *Oscillospira* in MDD (Jiang et al., 2016; Naserbafrouei et al., 2015). Further, lower levels seen in the current study may also be a compensatory response to the increased levels that may exist in pediatric samples, not distorted by the duration of illness.

7.1.1a *Oscillospira* (OTU13)

Oscillospira (gram-negative, family *Ruminococcaceae*, phylum Firmicutes) is commonly found in the human gut and associated with reduced inflammation and increased leanness (Konikoff & Gophna, 2016; Gophna et al., 2017). A meta-analysis of patients with IBD revealed significant reductions of *Oscillospira* in Crohn's disease (Walters et al., 2014). Similar findings have been reported in pediatric non-alcoholic steatohepatitis, another condition with increased inflammatory tone (Zhu et al., 2013). Previous studies in depression have reported higher levels of *Oscillospira* in patients, but overall levels of

Ruminococcaceae were reduced (Jiang et al., 2015). A mouse model of chronic stress (model of depression) reported decreased *Oscillospira* (Bharwani et al., 2016). Some ASD studies suggest that the *Ruminococcaceae* are increased (Finegold et al., 2002; De Angelis et al., 2013).

Unlike the other lower OTUs in the OCD group, relative abundance of *Oscillospira* decreased as depressive symptoms increased, as per all measures included in this study. Further, those with Past MDD (last episode >5 years ago) also illustrated lower mean relative abundance of *Oscillospira*, which may suggest that alterations in the gut microbiome associated with MDD persist well beyond symptom remission. A negative correlation was also noted with self-reported stress levels (DASS-S), supporting the notion that stress can alter the gut microbiome. Comorbid ADHD was the only other comorbid condition to reveal lower levels of *Oscillospira*. Unlike previous research, BMI was not associated with this genus (Konikoff & Gophna, 2016), nor was it related to levels of inflammatory cytokines studied in this thesis.

7.1.1b *Odoribacter* (OTU92)

Bacteria in the *Odoribacter* genus (family *Porphyromonadaceae*, phylum Bacteroidetes) are commonly found in the healthy gut and lower levels have been noted in IBD (Morgan et al., 2011). Lower levels of these gram-negative have been associated with an inflammatory state, based on plasminogen activator inhibitor-1 levels (Gomez-Arango et al., 2016). Mouse models of depression also revealed higher *Odoribacter* spp. (Bendtsen

et al., 2012) in addition to other genera in the *Porphyromonadaceae* family in humans (Jiang et al., 2015).

Despite previous associations with inflammation, *Odoribacter* did not differ based on CRP, IL-6 or TNF- α levels in the current study. Furthermore, relative abundance was only reduced in patients with comorbid BDD, while levels of *Odoribacter* decreased with increasing MADRS score.

7.1.1c Anaerostipes (OTU137)

Like the other two identified genus, *Anaerostipes* (gram-positive, family *Lachnospiraceae*, phylum Firmicutes) is abundant in the human gut. Decreased *Anaerostipes* have been noted in IBD, a microbial signature that is specific to Crohn's disease in particular (Pascal et al., 2017). A recent study in MDD revealed *Anaerostipes* to be more abundant in females (Chen et al., 2018), however we did not note any gender differences in the current study. While MDD comorbidity was excluded in this study, the relative abundance of *Anaerostipes* decreased as depressive symptoms increased (based on MADRS score). This is similar to findings reported by Naserbafrouei et al. (2014) who reported that the *Lachnospiraceae* family was underrepresented in MDD samples. Murine data also support reductions in genera of the family *Lachnospiraceae* in MDD (Bailey et al., 2011); however other studies in clinical MDD populations suggest increased *Lachnospiraceae* in MDD (Kelly et al., 2016; Jiang et al., 2015). In the current study, decreasing *Anaerostipes* were only associated with clinician-rated depressive symptom severity and self-reported stress levels. Relative abundance was also lower in those with

comorbid ADHD compared to controls but not associated with any marker of inflammation examined in this study.

Butyrate-production

One of the proposed methods by which these bacteria may modulate systemic inflammation is through metabolite production, particularly short chain fatty acids (SCFA). When considering potential modulation of the gut-brain axis, acetate, butyrate and propionate are most prominent (Riviere et al., 2016) and are produced by fermentation of dietary carbohydrates, like fiber. Propionate has demonstrated a marked ability to alter behaviour and intake has been implicated in ASD, albeit at higher doses (MacFabe, 2012). Butyrate is the favoured energy source for colonic epithelial cells, and can influence colonic functioning, but has also been shown to have an anti-inflammatory effect (Russo et al., 2012). Both *Anaerostipes* spp. (Louis & Flint, 2009) and *Odoribacter* spp. are known butyrate-producers (Nagai et al., 2011; Göker et al., 2011) and this may be a putative function of *Oscillospira* spp. (Gophna et al., 2017).

In addition to OCD, patients with Comorbid ADHD and Past MDD presented lower relative abundance of more than one of these butyrate-producing OTUs. There is external evidence to suggest that inflammation may be involved in these neuropsychiatric conditions (Mitchell & Goldstein, 2014; Anand et al., 2017; Dowlati et al., 2010; Miller & Raison, 2016). As an essential metabolite in the human colon, butyrate contributes to the maintenance and integrity of gut

barrier function preventing “leaky gut” syndrome (increased gut permeability) (Riviere et al., 2016). As such, lower levels of butyrate-producing bacteria may be suggestive of lower colonic butyrate and possibly increased intestinal permeability. Increased intestinal permeability is one of the leading hypotheses for microbial dysbiosis in psychiatric conditions as it permits translocation of intestinal contents into systemic circulation resulting in a potential inflammatory reaction. However, given that colonic butyrate levels were not measured in the current study, we can only hypothesize this role.

Butyrate has also revealed therapeutic ability. The sodium salt of butyric acid has also been shown to be anti-inflammatory in microglial cells and has been suggested to be beneficial in neuropathological conditions where inflammation has been observed (Huuskonen et al., 2014). Although it has been difficult to pinpoint the central nervous system consequences of butyrate-mediated effects, butyrate-producing bacteria have been shown to alter the permeability of the blood-brain barrier (Braniste et al., 2014). More specifically, these bacteria were shown to normalize the increased permeability of the blood-brain barrier noted in GF-mice who were compared to specific pathogen free mice with healthy microbiota (Braniste et al. 2014). Diets high in soluble fibre have also demonstrated an ability to attenuate lipopolysaccharide-induced increases in IL-1 β and TNF- α . This “anti-inflammatory effect” is elicited by increasing IL-1 receptor antagonists (inhibitor of IL-1 β) in the brain (Sherry et al., 2010). Further, increases in IL-4 mRNA, a cytokine known to increase IL-1 receptor antagonists,

were also noted (Sherry et al., 2010). Insoluble fibre intake did not reveal similar changes. Given that butyrate is the metabolic product of fiber digestion, these results suggest that the anti-inflammatory effects of a soluble fiber diet may be related to production of butyrate by the gut bacteria. The FFQ utilized in this study only provided an overall measure of dietary fiber, as such the relationship between soluble fiber intake and the respective OTUs could not be examined but would be of interest in future studies.

7.1.2 Plasma CRP but not serum IL-6 or TNF- α levels are elevated in OCD patients

Many studies have examined cytokine differences in OCD producing a very mixed literature. The current study was restricted to cytokines most commonly implicated in neuropsychiatric conditions. However, to fully determine the presence of immune dysregulation in OCD, a wider array of anti- and pro-inflammatory cytokines need to be examined. Further, most microbiome studies in psychiatric populations where inflammation is thought to be a mediating factor do not explore the relationship between observed microbial differences and markers of inflammation. As such, this study is one of the first to explore whether differences in the gut microbiome relate to levels of circulating cytokines.

7.1.2a CRP

The finding of increased CRP (marker of systemic inflammation) in the OCD group and lower levels of butyrate-producing bacteria may further support the role for immunomodulating effects of butyrate. A cut-off of 2 mg/L has been suggestive of

increased cardiovascular risk and used in some studies of depression. Mean CRP in the OCD group exceeded this level despite exclusion of comorbid depression. It was also independent of BMI, age or sex. Overall, the CRP results observed in the current study suggest increased inflammation in OCD.

CRP was not correlated with α -diversity; however, was elevated in groups where lower α -diversity was previously demonstrated (Chapter 4; those with comorbid GAD and no tics). Differentiating OTUs were not related to CRP levels, despite being implicated in inflammatory conditions. As such, this limits our ability to suggest a direct relationship between systemic inflammation and butyrate in OCD. However, both increasing CRP levels and decreasing species richness both predicted likelihood of OCD to a significant degree. To date, only one cross-sectional study has examined levels of CRP levels in OCD patients (Ekinici & Ekinici, 2017). Similar to the current study, CRP was elevated in the OCD group compared to controls. However, the results of this thesis are the first study to relate CRP levels with changes in the gut microbiome and psychiatric symptomatology in this population.

7.1.2b IL-6 and TNF- α

CRP is typically synthesized hepatically in response to IL-6, which would lead to the expectation of elevated IL-6 in the OCD group given the observed CRP differences in the current thesis. Rather, levels of IL-6 were slightly higher in the control group, while TNF- α was slightly higher in the OCD group. However, neither difference was statistically

significant. IL-6 and TNF- α were not correlated with CRP levels in this study, despite their established upstream effects on CRP production (Ridker 2016).

Unlike CRP, there is no approved assay for IL-6 measurement in clinical settings. Analysis is complicated due to circadian variations, short half-life, post-prandial effects, and assay stability (Ridker 2016). A previous study examining plasma levels of IL-6 and TNF- α in addition to a variety of other cytokines reported higher levels of both in drug-naïve, comorbidity-free OCD patients and controls (Rao et al., 2015). Similarly, Konuk et al. (2008) also demonstrated elevated plasma IL-6 and TNF- α of OCD patients compared to controls; but authors could not preclude the effects of potential confounding variables (e.g. comorbid depression). Many studies have also reported no difference or decreased levels of IL-6 and TNF- α in OCD patients compared to controls (Brambilla et al., 1997; Monteleone et al., 1998; Denys et al., 2004; Fluitman et al., 2010). While the current thesis examined serum levels of IL-6 and TNF- α , much of the literature examines plasma levels of the cytokines (Rao et al., 2015; Konuk et al., 2008; Monteleone et al., 1998; Brambilla et al., 1997). Although cytokine levels are thought to be comparable between plasma and serum samples, some have suggested that plasma may be a better choice for studies looking at IL-6 (de Jager et al., 2009). Given the different sample types used in the current study and those published in the literature, direct comparison of was difficult. Regardless, the values seen in this study are not outside of the wide range that has been previously reported but are still low. As such, it is possible that methodological differences may have resulted in the presenting data, in addition to potential limitations discussed below.

Neither IL-6 or TNF- α explained differences in the gut microbiome of OCD patients when compared to controls; however, future larger studies need to examine this relationship to better elucidate any potential relationship.

7.1.2c Autoimmune basis of OCD

Beyond circulating cytokines, recent studies have used family or personal medical history of autoimmune disorders as a proxy for possible immunological dysfunction (Perez-Vigil et al., 2016; Mataix-Cols et al., 2017). However, individuals with autoimmune conditions were excluded in this study to limit the confounding effects of such illnesses on presenting cytokine levels. Given that CRP was elevated, but TNF- α and IL-6 were not, post-hoc analyses examining rates of familial autoimmune conditions and personal allergies between the OCD and control groups were pursued. This comparison was possible as a detailed personal and family medical and psychiatric history was documented in the assessment process. Similar to previous reports (Perez-Vigil et al., 2016; Mataix-Cols et al., 2017), a higher prevalence of autoimmune conditions amongst first-degree relatives of OCD patients was noted. Autoimmune disorders included psoriasis, type I diabetes, rheumatoid arthritis, and systemic lupus erythematosus. No differences in cytokine levels or gut microbiome profile were noted amongst patients with and without first-degree relatives with autoimmune disorders.

Meta-analytic evidence has suggested that rates of OCD are higher in patients with rheumatic fever with Sydenham's chorea, while findings related to other autoimmune diseases have been inconclusive (Perez-Vigil et al., 2016). Further, a

nationwide study revealed that individuals with OCD and Tourette's Disorder were more likely to present with a comorbid autoimmune condition (Mataix-Cols et al., 2017). As observed in the current thesis, autoimmune conditions were also more prevalent in first-degree relatives of OCD patients than controls, suggesting a familial link to autoimmune disorders in general (not limited to Streptococcus-related conditions) (Mataix-Cols et al., 2017). It has also been suggested that mother-specific factors (e.g. placental transmission of antibodies) may also be involved (Mataix-Cols et al., 2017). However, the current study did not possess enough power to examine this association.

More patients in the OCD group also reported mild allergies (typically seasonal) in the OCD group than did controls. No cytokine differences were present in the whole sample of participants with or without allergies, or when limiting this analysis to participants in the OCD group specifically. However, Unweighted Unifrac revealed clustering when all participants were organized based on whether allergies were present. This suggested that regardless of OCD or controls status, the gut microbiome of those with allergies differed than those without allergies. When participant condition (OCD vs control) was taken into consideration the difference was no longer significant, potentially due to the small sample size. Given this signal, this relationship should be further examined in larger studies in the future.

7.1.2d Interpretation

Although levels of the examined inflammatory markers were not directly associated with microbial differences, CRP was elevated in OCD patients compared to controls. Paired

with the high prevalence of autoimmune disorders in first-degree relatives of OCD patients and allergies in the OCD group, these results may support the role for the immune dysregulation in OCD. As such, it may be of interest to examine a wider array of pro- and anti-inflammatory cytokines as was done previously (Rao et al., 2015), in a similar non-depressed, unmedicated OCD group. Aside from potential differences in participants with and without allergies, we were unable to demonstrate any other statistically significant relationship between systemic inflammation and the gut microbiome.

A possible explanation for the negative results may be that the relationship between the gut microbiome and inflammation is more prominent earlier in life. For instance, the inflammation literature in pediatric OCD patients has provided greater insight than in adults with findings consistently suggesting increased inflammation (Simsek et al., 2016; Rodriguez et al., 2017; Sivri, Bilgic & Kilinc, 2018). While the adult microbiome is susceptible to change based on numerous variables, the predominant microbiome profile is determined in early life. This has been best demonstrated by the GF-mouse model where re-establishment of the gut microbiota early in life has been shown to normalize performance on rodent behavioural tests. This normalization does not apply if the microbiota is reconstituted in adulthood (Heijtz et al., 2011; Clarke et al., 2012; Neufeld et al., 2011a; Neufeld et al., 2011b). As such, if the gut microbiome has a role in the pathophysiology of OCD, perhaps changes early on in life are critical. Animal studies reveal the susceptibility of the gut microbiome to stress, and the risk of developing internalizing disorders (including OCD) following early life stress is also well

documented (Kessing, Agerbo & Mortenson, 2003; Nugent et al., 2011). Paired with the more consistent literature suggesting elevated inflammation at this age (reviewed in Mitchell & Goldstein, 2014), perhaps the relationship between gut microbiome and OCD should be explored in youth or pediatric samples.

These findings also suggest that the HPA axis may serve as a fitting target for OCD gut microbiome research. Much of the gut-brain literature linked to psychiatric illness focuses on the HPA axis, which can influence inflammation and also the gut microbiome. Increased basal activity of the HPA axis has been reported in adult OCD as indicated by elevated cortisol levels and increased levels of ACTH and CRH in CSF (Monteleone et al., 1997; Kluge et al., 2007), perhaps this elevation is a consequence to modulation by the gut microbiome. Similar findings have also been reported in pediatric samples (Gustafsson et al., 2008). Some have also suggested that a pro-inflammatory maternal state (increased circulating cytokines) may alter the fetal immune milieu, and that the maternal HPA axis may present an important link to neurodevelopmental differences (Rogers et al., 2016) increasing susceptibility to neuropsychiatric conditions like OCD.

7.1.3 Higher prevalence of IBS and greater gastrointestinal severity in OCD patients

High rates of psychiatric comorbidity have been noted in populations suffering from gastrointestinal disorders. To date, much of this literature has focused on anxiety and mood disorders and ASD, with little focus on OCD. In patients with functional bowel disorders, OCD has been reported as the second most common psychiatric comorbidity

(20%) behind dysthymia (25%) (Fakhraei et al., 2015). In cohorts of OCD patients, gastrointestinal diseases are also quite common (20.5%) (Aguglia et al., 2018) and some have suggested constipation to be an issue specific to female OCD patients (North et al., 1995). With the knowledge of significant gastrointestinal symptoms in ASD fueling the extensive gut microbiome literature in the ASD population, we examined gastrointestinal symptoms in OCD to provide evidence suggesting a similar role.

7.1.3a Gastrointestinal Severity

Overall, the results derived from this study reveal that gastrointestinal severity is higher in OCD. Analogous to the autism literature where the degree of gastrointestinal symptoms (most commonly diarrhea and bloating) is positively correlated with the severity of autism; we noted a strong positive correlation between gastrointestinal symptoms and OCD symptom severity. Further, previous reports have suggested that diarrhea and constipation in children with ASD, was associated primarily with OCD related outcomes (i.e. OCD diagnosis, and parental reports of repetitive and compulsive behaviours) (Peters et al., 2014). In this sample, greatest severity was seen with indigestion and bowel dysfunction symptoms in OCD patients. The role of gut dysbiosis in ASD has also been supported by successful treatment of symptoms with antibiotics (Sandler et al., 2000). Antibiotic treatment (adjunctive minocycline) has shown potential as a treatment in OCD (Rodriguez et al., 2010; Esalatamanesh et al., 2016). This suggests that microbial dysbiosis may be present in OCD as the symptomatic improvement may be related to the drastic alterations in the gut microbiome consequent to antibiotic treatment. This finding

of increased gastrointestinal severity in OCD patients – independent of IBS criteria – is particularly important given the immediate clinical implication as many first-line pharmacological treatments targeting OCD can be associated with significant gastrointestinal side effects. As such, whether patients with pre-existing gastrointestinal problems are more susceptible to these side effects or less compliant with treatment may also be of interest.

7.1.3b Prevalence of IBS

Meta-analytic evidence has revealed global prevalence of IBS to be at 11.2% (95% confidence interval [CI] 9.8–12.8) (Lovell & Ford, 2012), while rates in North America have ranged from 3.0% to 20.1% (Canavan, West & Card, 2014). Previously, two studies have examined prevalence of IBS among OCD patients. The first utilized Rome I criteria, reporting IBS in 35.1% of OCD patients (Masand et al., 2006), while a subsequent report using Rome II criteria reported prevalence of IBS in 16.0% of OCD patients (Gros et al., 2009). In the current sample, 47.6% of OCD patients met Rome III criteria for IBS which may be over 2 times reported rates of IBS in North America. Those with IBS also reported greater gastrointestinal severity than OCD patients who did not endorse IBS specific symptoms. Psychiatric symptom severity also differed between the two groups, as has been suggested in the past in non-OCD samples (Gros et al., 2009). The IBS-OCD group endorsed greater depressive, anxiety and stress ratings than did those without; however, no differences were noted in OCD symptom severity. GAD comorbidity was high in the IBS-OCD group and fewer/less severe compulsions were noted. This may also lead to the suggestion that perhaps the IBS and gastrointestinal symptoms are being

driven by GAD rather than OCD. Given the few patients without comorbid GAD and IBS, the study was not powered enough to delineate these effects.

Although gut microbiome differences have been demonstrated in IBS, we did not note any differences in diversity or OTU distribution in the current sample. Previous studies in adults have observed lower proportions of *Bacteroides* spp. and increased abundance of Firmicutes (Jeffrey et al., 2012; Krogius-Kurikka et al., 2009; Kassinen et al., 2007). However, the differences may be IBS-subtype specific, which may explain the lack of differences in the current study. Levels of IL-6 were higher in OCD patients with IBS than those who did not meet Rome III IBS criteria in this thesis; however, TNF- α and CRP did not. A low-grade inflammatory response has been described in IBS (Jeffrey et al., 2012). Evidence gleaned from a recent systematic review revealed that systemic IL-10 (an anti-inflammatory cytokine) may be decreased in IBS compared to non-IBS controls while IL-6, IL-8, IL-1 β and TNF- α were increased (Martin-Winas & Quigley, 2016). However, findings were not consistent across all studies, differed in methodology and in some instances were limited to certain IBS sub-population (typically diarrhea-predominant IBS) (Martin-Winas & Quigley, 2016). The sample size of the current IBS subset was small, as such a comparison looking at differences amongst IBS subtypes was not possible.

Unlike previous studies examining gastrointestinal problems in similar populations (Gros et al., 2009), a clinician-rated measure was utilized instead of solely relying on self-reported gastrointestinal severity. Further, existing studies have not incorporated a control group, limiting the interpretation of their results. Increased rates of

IBS and gastrointestinal problems in the OCD group further support involvement of the HPA axis. With the HPA axis as the central stress-response system, it is interesting to note that stress (activation of HPA axis) not only worsens symptoms of IBS (Fadgyas-Stanculete, Buga, Popa-Wagner & Dumitrascu (2014), but also symptoms of OCD (Rosso et al., 2012). These two conditions have been associated with hyperactivity of the HPA axis (Dinan et al., 2006). Like in OCD, IBS has been associated with alterations in ACTH, cortisol and catecholamine levels (Dinan et al., 2006). Consequently, interaction between factors like the gut-brain axis, HPA axis and the inflammatory response may play a role in IBS etiology (Fadgyas-Stanculete et al., 2014); but given the results of the current study they may all play a role in OCD as well.

7.2 Limitations of the Study

Limitations of the current study stem from its pilot nature. For instance, the sample size of this study is small, limiting the power of many of the analyses completed. However, it is critical to note that even comparisons that did not attain statistical significance may still be of interest for future larger studies. As a cross-sectional examination, we are unable to comment on the causality of the lower microbial diversity observed in the OCD group. Potential confounding variables were controlled for in the study design, and similarity in diet suggested that the presenting differences were less likely to be reflective of food intake. However, whether the observed reduced diversity is the result of the decontamination-related behaviours or if this profile predisposes individuals to OCD cannot be answered based on the existing study design. If in fact a potential downstream effect of restricted diet or exposure to bacteria, this lower diversity may instead

predispose OCD patients to other microbial dysbiosis associated conditions (i.e. IBD) but a longitudinal or follow-up study design would be required to inform this possibility.

Several sample types can be utilized for gut microbiome analysis, ranging from stool samples (most common) to increasingly invasive options like sub-mucosal biopsies (Fraher, O'Toole & Quigley, 2012). Consequently, the selected sample type may not accurately represent the microbial community throughout the entire gastrointestinal tract. For instance, stool samples are more representative of luminal populations rather than the mucosal groups believed to interact with the host immune system (Fraher et al., 2012). Further, amplification of the 16S rRNA gene is also the current gold standard for microbial community analysis and its use has the advantage of encoding several conserved regions that are exclusive to all bacteria and hypervariable regions that confer specificity to a large number of OTUs (Rosselli et al., 2016). However, it is limited in its ability to classify bacteria mostly down to the genus level rather than identifying specific strains or species of bacteria (Winter, Hart, Charlesworth & Sharpley, 2018). This is an important consideration as there can be major differences between even two strains of the same species. For instance, of two bacterial strains (same species) one can be non-pathogenic while the other a pathogen, as is the case with strains of *Escherichia coli* (Conway & Cohen, 2015). Finally, rarefaction of the microbiome data may also be seen as a limitation as the number of reads per sample was restricted to 13,000 reads in the current study. Although this is a methodologically sound technique, it remains a contentious topic amongst microbiome researchers. McMurdie & Holmes (2013) have reported that rarefying data results in a loss of potentially important information. They

have also suggested that it be avoided in small sample, pilot studies as it may prematurely end a line of research with the increased risk of a Type II error (McMurdie & Holmes, 2013). The study population may also have been victim to selection bias. The gut microbiome has gained a lot of traction in public media, particularly when considering gastrointestinal and mental health. As such, it is possible that the high rates of IBS and gastrointestinal problems may have been influenced by recruitment materials seeking OCD patients for a gut microbiome study.

There are also potential limitations associated with the cytokine analysis conducted in this study. To limit certain confounding variables, individuals with comorbid autoimmune conditions, current depression, concomitant psychotropic or immunomodulating medications were excluded. Samples were all drawn in the morning to limit potential diurnal variability. However, we failed to note any significant differences among IL-6 and TNF- α which may be related to limitations particular to the methodology. Plasma versus serum levels of cytokines are typically thought to be comparable with levels consistent across molecules (de Jager et al., 2009). In some instances, serum IL-6 levels may be lower than plasma, when collected from the same donor (de Jager et al., 2009). There is a possibility of this being an issue in the current study, as levels in both groups were low. Further, previous cytokine studies in OCD patients have examined plasma levels, making a direct comparison with our results difficult. While samples are expected to be stable for up to 3 years at -80 degrees Celsius, storage time in the current study may have compromised the results. To minimize experimental variation, serum samples were stored until the entire cohort was recruited.

However, this led to significant variations in sample storage time with some being frozen for 3 years, while others were stored only for a few weeks. This may have also led to the drastic differences in cytokine results resulting in the large standard deviation of each group (de Jager et al., 2009). Several rounds of freeze-thawing can also affect the stability of cytokines; however, serum samples were frozen in 3 aliquots so that additional assays could be performed without this issue (de Jager et al., 2009).

Despite its limitations, numerous considerations also make this study design particularly robust for a pilot gut microbiome study. This study was informed by limitations of previous microbiome studies, particularly those examining its role in psychiatric conditions. For instance, many studies fail to incorporate an indicator of diet (we utilized an FFQ) despite diet being a strong driving force for alterations in the gut microbiome. While the FFQ is limited by its self-report and retrospective nature, this measure still provides insight on diet where many studies have ignored this confounding variable entirely. Further, inclusion criteria for controls were very strict with the sample endorsing very few to no psychiatric symptoms at all. These individuals also did not present with any physical health issues, unlike controls utilized in other studies (Naserbafrouei et al., 2015). Although our sample was not necessarily drug-naïve, patients were not receiving current treatment, having discontinued any psychotropic medications or psychotherapy at least 3 months prior to sample collection.

7.3 Future Directions and Conclusions

This is the first study to examine the gut microbiome in OCD, and reveal reduced diversity and relative abundance of specific genera. More importantly this study, although a small pilot study, provides valuable information to guide an entirely novel line of research to explore the gut microbiome in OCD. First, confirming these findings in larger longitudinal designs with repeated microbiome analysis could provide more insight on a specific bacterial signature in OCD. Future studies could also explore whether the gut microbiome profile changes with the waxing and waning nature of OCD symptom severity, potentially mediated by life stressors (Rosso et al., 2012). A finding of paired changes between the gut microbiome profile and OCD symptoms would prove favourable to a role for the gut microbiome in OCD. Changes associated with treatment would also be of interest, particularly if a specific microbial signature could characterize patients who respond from those who do not respond to treatment. A positive association could prove pivotal to OCD research given that treatments response rates are moderate at best in OCD patients. Overall, these research questions and study designs could also provide additional evidence regarding the cause-and-effect basis of this relationship. With the heterogeneity seen in OCD symptomatology, it would also be of interest to examine gut microbiome in patients with primary symmetry/ordering or harm avoidance/checking symptoms which the current study was not powered to do. As mentioned earlier, a study examining the gut microbiome of children with OCD, specifically cases not associated with a *streptococcal* infection (PANDAS), may be of particular interest as establishment of the mature microbiome is primarily influenced by prenatal and early life events.

The current study only examined three pro-inflammatory cytokines, as such examining a more diverse assay of cytokines (i.e. Rao et al., 2015) may help replicate previous findings while providing more accurate information on the state of immune dysregulation in medication-free, non-depressed OCD patients. Rao et al. (2015) revealed increased levels of IL-10 (an anti-inflammatory cytokine). This was thought to be a compensatory response to the elevated pro-inflammatory state, suggesting that duration of illness may confound their results, further supporting exploration in pediatric OCD samples. If findings of the current study are replicated furthering the notion of an altered gut microbiome in OCD, intestinal inflammation may be a target of interest rather than continued pursuit of circulating cytokines.

Although gut microbiome research in clinical conditions currently limits our ability to clarify the cause-and-effect relationship between observed microbial differences and disease, the GF-mouse model may be an effective proof-of-principal next step. Zheng et al. (2016) demonstrated the onset of depressive behaviours in mice who received MDD patient fecal microbiota transplant, while those receiving colonized with control samples did not illustrate these behaviours. As such, a translational fecal microbiome transplant study with OCD and control stool samples may further clarify this relationship between the gut microbiome and OCD.

As an area of research still in its infancy, it is premature to propose treatments targeting the gut microbiome in OCD. However, given the moderate levels of response to existing treatments and immense interest in non-medication treatments among patients with mild symptoms, interventions targeting the gut microbiome are not out of the realm

of possibility. Prebiotics (food ingredients that promote the growth or activity of beneficial gut bacteria; e.g. kefir, kimchi, etc.) and probiotics (oral bacteria intake; e.g. pill or powder form) with a focus on butyrate-production would be supported by the results of the current study. It has been suggested that the health benefits of prebiotics and probiotics are related to the interactions with the resident gut microbiota rather than the gut being populated by the consumed bacteria (Scott et al., 2015; Cani & Van Hul, 2015). For instance, prebiotics or probiotics containing *bifidobacterium* may be an effective treatment option. Species of this genus cross-feed and interact with butyrate-producing bacteria and have been shown to promote behavioural improvements (Riviere et al., 2016). Further, pure butyrate by means of tablets or rectal enemas has been used as a therapeutic agent for IBD treatment, a condition where reductions in the same genera have been implicated (Geinhart et al., 2014). If a role for butyrate can be noted in OCD, ideally following evaluation of reduced colonic butyrate concentrations, diets high in soluble fibre may prove beneficial to OCD patients as well (Sherry et al., 2010). Adjunctive minocycline has shown potential in refractory OCD (Rodriguez et al., 2010; Esalatamanesh et al., 2016); however, given reduced richness noted in the current study, it is possible that antibiotics may exacerbate this profile. In addition to probiotics and antibiotics, fecal microbiota transplants may be a future treatment target of interest.

Taken together, the results of this study provide evidence for microbial dysbiosis and inflammation in OCD. Evidence regarding gastrointestinal symptoms and IBS in OCD is also provided, furthering the possible role of the gut microbiome in OCD as this mirrors data in ASD, a condition where the role for the gut microbiome is best developed.

Work presented in this thesis provides a clear direction for future research to guide a new line of OCD research and shape the future of novel therapies for a condition that is known to be refractory in nature and whose pathophysiology remains unclear.

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APPENDIX

Appendix 1. Food groups and Nutrients evaluated by the EPIC Norfolk FFQ

FOOD GROUP CODE	DESCRIPTION	UNITS
900	Alcoholic beverages	g
901	Cereals and cereal products	g
902	Eggs and egg dishes	g
903	Fats and oils	g
904	Fish & fish products	g
905	Fruit	g
906	Meat and meat products	g
907	Milk and milk products	g
908	Non-alcoholic beverages	g
909	Nuts and seeds	g
910	Potatoes ¹	g
911	Soups & sauces	g
912	Sugars; preserves and snacks	g
913	Vegetables	g
NUTRIENT CODE	DESCRIPTION	UNITS
1	Alpha carotene	mcg
2	Alcohol	g
5	Beta carotene	mcg
8	Calcium	mg
9	Carotene - total (carotene equivalents)	mcg
11	Carbohydrate - total	g
12	Cholesterol	mg
13	Chloride	mg
15	Copper	mg
20	Englyst Fibre - Non Starch Polysaccharides (NSP)	g
22	Iron	mg
55	Total folate	mcg
56	Carbohydrate - fructose	g
57	Carbohydrate - galactose	g
58	Carbohydrate - glucose	g
61	Iodine	mcg
63	Potassium	mg
64	Energy_kcal	kcal
65	Energy_kj	kJ
66	Carbohydrate - lactose	g
68	Carbohydrate - maltose	g
69	Magnesium	mg
70	Manganese	mg
72	Sodium	mg
74	Niacin	mg
77	Phosphorus	mg
83	Protein	g
86	Vitamin A - retinol	mcg
88	Vitamin A - retinol equivalents	mcg
89	Vitamin B2 - riboflavin	mg
92	Selenium	mcg
97	Carbohydrate - starch	g
98	Carbohydrate - sucrose	g
99	Vitamin B1 - thiamin	mg
100	Nitrogen	g
101	Carbohydrate - sugars (total)	g
103	Vitamin B12 - cobalamin	mcg
104	Vitamin B6 - pyridoxine	mg
105	Vitamin C - ascorbic acid	mg
106	Vitamin D - ergocalciferol	mcg
107	Vitamin E - alpha tocopherol equivalents	mg
109	Zinc	mg
600	Fat - total	g
601	Monounsaturated fatty acids (MUFA - total)	g
603	Polyunsaturated fatty acids (PUFA - total)	g
607	Saturated fatty acids (SFA - total)	g