Ollscoil na hEireann

National University Ireland

Colaiste na hOllscoile, Corcaigh

University College Cork

The Microbiota-Gut-Brain Axis in Social Anxiety Disorder

Thesis presented by

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under the supervision of

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for the degree of

Doctor of Philosophy

July, 2021

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Contents

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Author Contributions

All of the work conducted in this thesis was performed independently by the author with

the following exceptions:

In Chapter 3, Dr Conall Strain performed the whole genome shotgun microbiota sequencing and Dr Thomaz Bastiaanssen assisted with bioinformatics analysis.

In Chapter 4, Dr Francisca Villalobos-Manriquez assisted with analysis of serum gut permeability markers.

In Chapter 5, Dr Gerard Clarke performed the high-performance liquid chromatography (HPLC) analysis on plasma samples to generate the tryptophan and kynurenine data. Dr Gerard Moloney assisted with enzyme-linked immunosorbent assay (ELISA) of plasma cytokines.

In Chapter 6, Dr Francisca Villalobos-Manriquez assisted with analysis of hair cortisol samples.

In Chapter 7, Dr Fiona Tuohy and Dr Raul Cabrera Rubio performed the 16S RNA microbiota sequencing and Dr Conall Strain performed the gas chromatography for the short-chain-fatty-acid (SCFA) analysis. Dr Thomaz Bastiaanssen assisted with bioinformatics analysis.

Signed

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Mary Butler

Acknowledgments and Dedications

I would like to express my sincere thanks to my supervisors, Prof. Timothy G. Dinan, Dr. Gerard Clarke and Dr. Siobhain M. O'Mahony for their expert guidance, invaluable advice and generous support throughout my doctoral studies. A particular thank you to Ted for encouraging me to delve into the world of research in the first instance, and for so kindly continuing to supervise my thesis despite his retirement from psychiatry. I feel incredibly privileged to have had the opportunity to work under the supervision of such talented, enthusiastic and gracious supervisors. The research experience has taught me afresh the importance of approaching my patients with a sense of curiosity and open-mindedness, and has given me a renewed passion for my clinical psychiatry work.

In addition, I owe a huge thanks to many people, both past and present, from the APC Microbiome Ireland centre, University College Cork. The culture of friendliness and willingness to always provide help made this a truly enjoyable journey. In particular, I would like to thank Dr Caitriona Long-Smith, Dr Gerard Moloney, Dr Kenneth O'Riordan, Dr Thomaz Bastiaanssen, Dr Kirsten Berding Harold, Nathaniel Ritz, Dr Sabrina Morkl and Prof. John Cryan. I would also like to express my gratitude to the many wonderful psychiatrists with whom I have worked over the past number of years for their inspiration and support.

I dedicate this thesis to my wonderful husband, Will, without whose love, kindness and selflessness I would not have been able to complete the work. A special thanks also to my parents who have been tireless supporters throughout the good times and the bad, and to my beautiful twins, Íde and Ruadhán, who have tried to be patient while waiting for the 'PhG' to be finished.

Finally, I would like to thank all the patients with social anxiety disorder who participated in this study. I hope that, through the combined efforts of researchers and clinicians, we can develop a greater understanding of anxiety disorders, and improve the lives of the many people suffering with these conditions.

"Self-consciousness is the enemy of all art, be it acting, writing, painting, or living itself, which is the greatest art of all."

Ray Bradbury, American Author, 1963.

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- 'Cortisol Awakening Response in patients with Social Anxiety Disorder'. Annabel Umeh, **Mary I Butler**, Sabrina Mörkl, Caitriona Long-Smith, Gerard Clarke, John F. Cryan, Timothy G. Dinan. *Winner of the medical student poster presentation at the Winter Conference of the College of Psychiatrists of Ireland Nov 2020. (Presented by Annabel Umeh)*
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Abstract

The past decade has seen huge interest in the role of microbiota-gut-brain (MGB) axis in psychiatric disorders. Significant preclinical efforts have been made to elucidate the role of the gut microbiome in the stress response, and there is an ever-growing body of evidence demonstrating the effect of gut microbiome modulation on behaviour in various animal models of anxiety and depression. Additionally, studies in healthy human volunteers have generated hope that microbiome-based interventions may improve mood and anxiety symptoms. Despite this, the MGB axis remains largely unexplored in patients with clinical anxiety disorders, such as social anxiety disorder (SAD). Indeed, investigation of the neurobiological basis of this and other clinical anxiety conditions is limited, and these disorders remain poorly understood. To this end, we hypothesized that the gut microbiota would be altered in those with SAD, and that gut barrier dysfunction would be evident. We proposed that physiological systems of relevance in MGB communication, including neuroendocrine, immune, and tryptophan-kynurenine pathways would show differences compared to controls.

In this thesis, we demonstrate that the gut microbiome is compositionally and functionally altered in patients with SAD, and that this patient group have compromised intestinal permeability. We demonstrate that SAD is associated with differences in various systems involved in MGB communication. We report elevated kynurenic acid (KYNA) levels and an increased KYNA/Kynurenine ratio in our patient group. Additionally, SAD patients show lower levels of the anti-inflammatory cytokine, interleukin-10, along with various neuroendocrine alterations including lower oxytocin levels and differences in the cortisol awakening response, chronic cortisol concentrations and morning salivary alpha amylase levels. Taken together, our results raise the possibility that the MGB axis may represent an important aetiological node and potential therapeutic target for this early-onset, chronic disorder. Our work supports the need for larger, longitudinal studies to further explore the role of the MGB axis in clinical anxiety disorders.

In addition, we report on a longitudinal study assessing the impact of a dietary change involving the consumption of unpasteurised dairy, on the gut microbiome of healthy volunteers. We demonstrate that intake of unpasteurised dairy is associated with significant increases in *Lactobacillus,* a psychobiotic bacterial genus which is recognised as having anxiolytic and antidepressant effects. This work supports the possibility that dietary change may have therapeutic potential in psychiatric conditions.

Chapter 1: General Introduction

1.1 A Brief History of Social phobia/Social Anxiety Disorder

The concept of social anxiety is not a new one. Indeed, it was an ailment recognised by Hippocrates as evidenced by the following description of a patient by the father of medicine; *'He dare not come into company for fear he should be misused, disgraced, overshoot himself in gestures or speeches, or be sick; he thinks every man observeth him'* (Burton, 1621). The first and second editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM) (APA, 1952, 1968) did not recognise social phobia (SP) although they did allow for a more general category of phobic reactions/phobic neurosis. SP was first proposed as a distinct clinical disorder, differentiated from other phobias, over 50 years ago (Marks and Gelder, 1966) and characterised as an exaggerated fear of scrutiny or evaluation by others leading to distress and/or avoidance when engaging in performance or social interactions. This description was broadly similar to that included in the DSM – 3rd edition (APA, 1980). However, an exclusion criterion for the diagnosis was avoidant personality disorder (APD), a new personality disorder category which encompassed those exhibiting "hypersensitivity to potential rejection, humiliation, or shame; an unwillingness to enter into relationships unless given unusually strong guarantees of uncritical acceptance; social withdrawal in spite of a desire for affection and acceptance; and low self-esteem". This resulted in the diagnosis of SP being limited to those with quite specific, often single, social fears such as performance anxiety and placing patients exhibiting more widespread social fears into a personality disorder category, a factor that was criticized by some psychiatrists on the basis that it would reduce the chances of these patients being offered potentially beneficial pharmacological treatment (Liebowitz *et al.*, 1985). Several years later, the Revised DSM-III (APA, 1987) removed the APD exclusion criterion and added a 'generalised' subtype to the SP classification which allowed for the incorporation of those with extensive social fears. The DSM-IV (APA, 1994) saw the alternative name of social anxiety disorder (SAD) being introduced after clinicians advocated that this term more accurately reflected the extensive symptomatology and associated functional impairment (Liebowitz *et al.*, 2000). SAD became the primary naming convention in the DSM-5 (APA, 2013). A similar direction of change has been seen in the International Statistical Classification of Diseases and Related Health Problems (ICD-10) (WHO, 1992) which currently classifies social phobias (F40.1) under the subgroup, phobic anxiety disorders (F40). However, the ICD-11, which will come into effect on 1st January 2022, has changed the name to SAD, which will now be categorised under a new grouping with anxiety or fear as the primary clinical feature.

1.2 Symptoms and Classification of SAD

The current DSM-V description of SAD is a condition characterised by 'marked fear or anxiety about one or more social situations in which the individual is exposed to possible scrutiny by others; these fears generally extend across a variety of situations including social interactions (e.g., having a conversation, meeting unfamiliar people), being observed (e.g., eating or drinking), and performing in front of others (e.g., giving a speech) (APA, 2013). A 'performance-only' specifier has replaced the 'generalised' specifier, based on evidence that those with performance-only anxiety represented a distinct subgroup and following expert consensus that it was easier to define this limited subtype than to elicit what exactly satisfied the 'generalised' specifier (Heimberg *et al.*, 2014). The diagnostic criteria are broadly similar to that of the tenth and current edition of the ICD and are outlined in Table 1.

Table 1: Diagnostic criteria for SAD, based on current Diagnostic and Statistical Manual for Mental Disorders – 5th edition (DSM-5) and the International Statistical Classification of Diseases and Related Health Problems – tenth edition (ICD-10).

DSM-V Criteria (Social Anxiety Disorder)	**ICD-10 Criteria (Social Phobia)**
A. Marked fear or anxiety about one or more social situations in which the individual is exposed to possible scrutiny by others. Examples include social interactions (e.g., having a conversation), being observed (e.g., eating or drinking) or performing in front of others (e.g., giving a speech) **B.** The individual fears that he or she will act in a way or show anxiety symptoms that will be negatively evaluated by others (i.e., will be humiliating or embarrassing; will lead to rejecting or offend others). **C.** The situations almost always provoke fear or anxiety. **D.** The social situations are avoided or endured with marker fear or anxiety **E.** The fear or anxiety is out of proportion to the actual threat posed by the social situation and the sociocultural context. **F.** The fear, anxiety or avoidance is persistent, typically lasting 6 months or more. **G.** The fear, anxiety or avoidance cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.	**A.** Either (1) or (2): 1. marked fear of being the focus of attention or fear of behaving in a way that will be embarrassing or humiliating 2. marked avoidance of being the focus of attention or situations in which there is fear of behaving in an embarrassing or humiliating way These fears are manifested in social situations, such as eating or speaking in public; encountering known individuals in public; or entering or enduring small group situations, such as parties, meetings and classrooms. **B.** At least two symptoms of anxiety in the feared situation at some time since the onset of the disorder, as defined in criterion B for agoraphobia (Autonomic arousal symptoms (1) Palpitations or pounding heart, or accelerated heart rate. (2) Sweating. (3) Trembling or shaking. (4) Dry mouth (not due to medication or dehydration). Symptoms concerning chest and abdomen (5) Difficulty breathing. (6) Feeling of choking. (7) Chest pain or discomfort. (8) Nausea or abdominal distress (e.g. churning in stomach). Symptoms concerning brain and mind (9) Feeling dizzy, unsteady, faint or light-headed. (10) Feelings that objects are unreal (derealization), or that one's self is distant or "not really here" (depersonalization). (11) Fear of losing control, going crazy, or passing out. (12) Fear of dying. General symptoms (13) Hot flushes or cold chills. (14) Numbness or tingling sensations) and in addition, one of the following symptoms:

H. The fear, anxiety or avoidance is not attributable to the physiological effects of a substance (e.g., a drug of abuse or a medication) or another medical condition. **I.** The fear, anxiety or avoidance is not better explained by the symptoms of another mental disorder, such as panic disorder, body dysmorphic disorder or autism spectrum disorder. **J.** If another medical condition (e.g., Parkinson's disease, obesity, disfigurement from burns or injury) is present, the fear, anxiety or avoidance is clearly unrelated or excessive. **Specify if:** Performance Only	○ Blushing. ○ Fear of vomiting ○ Urgency or fear of micturition or defecation. **C.** Significant emotional distress caused by the symptoms or by the avoidance **D.** Recognition that the symptoms or the avoidance are excessive or unreasonable **E.** Symptoms are restricted to, or predominate in, the feared situation or when thinking about it. **F.** Most commonly used exclusion criteria: Criteria A and B are not caused by delusions, hallucinations or other symptoms of disorders such as organic mental disorders, schizophrenia and related disorders, affective disorders or OCD, and are not secondary to cultural beliefs.

1.3 Epidemiology of SAD:

SAD is one of the most common psychiatric disorders with estimated lifetime prevalence rates of as high as 13% in the United States (Kessler *et al.*, 2012). Similarly high lifetime prevalence rates are seen across a large number of European countries with values ranging from 3.9% to 13.7%, the median being 6.65% (Fehm *et al.*, 2005). Most of the data in relation to the epidemiology of SAD has been obtained from high incomes countries in the West with a lack of such information for developing countries. A recent paper sought to address this disparity and analysed data from almost 150,000 respondents from a variety of low-, middle- and high-income countries as part of the World Mental Health (WMH) Survey Initiative (Stein *et al.*, 2017a). Differences in SAD prevalence were evident, with lowest rates seen in low/lower-middle income countries and in the African and Eastern Mediterranean regions. However, several consistent patterns were apparent across the globe, including the early age of onset, chronicity, functional impairment in multiple domains and significant psychiatric comorbidity, emphasizing the international clinical importance of this condition.

As is the case with other anxiety disorders, SAD appears to be more common in females. Although women are more likely to report greater severity of symptoms, men are more likely to seek treatment (Asher, Asnaani and Aderka, 2017), a feature which may be partly explained by differing gender roles, societal expectations and the resultant functional impairment associated with the condition (Turk *et al.*, 1998). SAD typically begins early in life with a mean age of onset of 14.3 years (Lijster *et al.*, 2017) and onset after the age of 25 appears to be rare (Fehm *et al.*, 2005). SAD tends

to run a chronic, often lifelong, course (Keller, 2003). It is associated with serious functional disability in virtually every aspect of life including education, employment, interpersonal relationships and social activities, and sufferers report a markedly reduced quality of life (Stein and Kean, 2000).

Comorbidity appears to be the rule rather than the exception with lifetime comorbidity rates as high as 92% reported (Faravelli *et al.*, 2000). The most common comorbid disorders include other anxiety conditions, affective disorders and substance abuse, which generally arise subsequent to SAD onset (Chartier, Walker and Stein, 2003). In particular, SAD markedly increases the risk of subsequent depression (Beesdo *et al.*, 2007) which is associated with a poorer prognosis and greater risk of suicide attempts (Stein *et al.*, 2001b). SAD is also recognised as an important comorbidity in schizophrenia (Achim *et al.*, 2011). Although it is unclear whether SAD precedes the onset of schizophrenia, there is evidence that SAD is present in a significant proportion of patients with first-episode psychosis (Michail and Birchwood, 2009) or recent-onset psychotic disorders (Roy *et al.*, 2015), suggesting that the onset of SAD is at least as early as the onset of psychosis. Another disorder with which SAD commonly co-occurs is autism spectrum disorder (ASD), where it is associated with poorer social skills, competence and social motivation (Spain *et al.*, 2018).

1.4 Aetiology of SAD

1.4.1 Genetics

Twin and family studies have revealed that there is a significant heritability component to SAD (Stein *et al.*, 2001a; Stein, Jang and Livesley, 2002; Merikangas *et al.*, 2003; Isomura *et al.*, 2015). The first genome-wide association study carried out in SAD recently confirmed this genetic basis, which appears to be shared with the personality trait of extraversion (Stein *et al.*, 2017b). However, disentangling the effects of genetic predisposition versus shared family environmental influences in the development of SAD is necessary. Factors in the family environment including parental personality, anxiety and behaviours along with parenting style can contribute to the onset of SAD in children (Beidel and Turner, 2007). A meta-analysis sought to address this issue and examined the distinct impact of genetics, along with shared and non-shared environmental factors, on SAD development. The shared environment appeared to have minimal impact while heritability and non-shared environmental aspects exerted significantly more influence, although estimates varied widely across studies with a range of 0.13-0.60 for heritability and 0.31-0.78 for the shared environment (Scaini, Belotti and Ogliari, 2014). This meta-analysis also found that the heritability component is far less in adults than in children with SAD, suggesting that environmental factors are of increasing importance as the disorder progresses with age.

Some attempts to elucidate the specific genes and associated biological pathways involved in SAD have been carried out and focus mainly on neurotransmitters. A genome-wide linkage scan reported evidence for linkage between SAD and Chromosome 16. Since the gene encoding the norepinephrine transporter protein (SLC6A2) maps to this broad region authors suggested that this may be a candidate gene for influencing SAD risk (Gelernter *et al.*, 2004). Due to evidence of dopamine dysfunction in SAD from functional brain imaging studies, another study examined polymorphisms in the dopamine D2, D3 and D4 receptor genes and the dopamine transporter gene. However, they found no link between these genes and SAD diagnosis (Kennedy *et al.*, 2001). In contrast, serotonin (5-HT)-related genotypes have been implicated. Polymorphisms in the 5-HT transporter gene-promotor region (5-HTTLPR) located on chromosome 17, have been associated with blushing in SAD (Domschke *et al.*, 2009) as well as with symptom severity and amygdala responsiveness in patients with the disorder (Furmark *et al.*, 2004; Furmark *et al.*, 2009). Furthermore, the effect of variations in the 5-HTTLPR genotype in SAD may be buffered by environmental factors such as the level of social support, emphasizing the importance of gene-environment interactions on SAD development (Reinelt *et al.*, 2014). The impact of genotypes on responsiveness to treatment has also been investigated in SAD. An association between functional polymorphisms in the 5-HTTLPR gene and responsiveness to SSRI treatment was seen in a small study of SAD patients (Stein, Seedat and Gelernter, 2006) while a much larger study (n = 346 patients) found that two of four single nucleotide polymorphisms within the regulator of G-protein signalling 2 gene (RGS2) predicted remission to sertraline treatment, suggesting that this gene could be a biomarker of the likelihood of benefiting from SSRI medication (Stein *et al.*, 2014). Epigenetic modifications of the oxytocin receptor gene (OXTR) have also been shown to be relevant in SAD. Hypomethylation of the promotor region of OXTR was significantly associated with a diagnosis of SAD, increased scores on SAD symptom severity scales, an elevated cortisol response to the Trier Social Stress Test (TTST), and increased amygdala activity during SAD-relevant word processing (Ziegler *et al.*, 2015).

1.4.2 Neuroimaging

In terms of structural brain differences, patients with SAD have been shown to have significantly reduced amygdalar (13%) and hippocampal (8%) size, with smaller right-sided hippocampal volumes significantly associated with disorder severity (Irle *et al.*, 2010). Findings in relation to cortical thickness have been inconsistent (Brühl *et al.*, 2014). Functional imaging studies, following a variety of stress exposures, strongly implicate hyperactivity of the amygdala and insula (Etkin and Wager, 2007) as well as in the anterior cingulate and prefrontal cortex (Brühl *et al.*, 2014), representing an

exaggeration of the normal fear response pattern. Recent task-related functional studies have also revealed hyperactivation of medial parietal and occipital regions (posterior cingulate, precuneus and cuneus) in SAD along with a reduced connectivity between parietal and limbic and executive network regions (Brühl *et al.*, 2014) Functional neuroimaging has been used to ascertain brain changes before and after psychological and pharmacological treatments, providing a useful way to identify markers differentiating patients who respond to treatment from non-responders (Bandelow *et al.*, 2016).

1.4.3 Behavioural Inhibition

One of the most well-established early risk factors for the development of SAD is the childhood temperamental trait of behavioural inhibition (BI) (Chronis-Tuscano *et al.*, 2009). BI is characterised by a persistent tendency to show extreme fearfulness or avoidance towards situations or people which are unfamiliar (Hirshfeld-Becker *et al.*, 2008). This characteristic emerges early in childhood and has a strong genetic component (Robinson *et al.*, 1992). Behaviourally inhibited children show strong signs of physiological arousal at rest (Fox *et al.*, 2005) as well as altered amygdala responsivity (Pérez-Edgar *et al.*, 2007), characteristics shared by those with SAD. BI is a significant predictor of the later onset of SAD with almost half of inhibited children eventually going on to develop the disorder. Given the many shared features it is reasonable to question whether BI is actually an early manifestation of SAD as opposed to a distinct construct. However, not all inhibited children go onto develop SAD and, despite the overlap in features, there are factors which distinguish between the temperament of BI and the clinical syndrome of SAD, including the degree of functional impairment and the potential for symptom change with psychological intervention (Rapee and Coplan, 2010).

1.4.4 Environmental Risk Factors

Parental psychopathology and parenting style:

As with other anxiety disorders, there is evidence that a wide range of environmental factors may play a role in the development of SAD. A parental history of SAD is strongly associated with the development of SAD in offspring (Stein *et al.*, 1998; Lieb *et al.*, 2000) which may predominantly reflect the genetic component of SAD as discussed previously, but may also be a manifestation of the parent-child interactions and the crucial role this plays in the child's learned social skills (Masia and Morris, 1998). Indeed, associations between parenting style, in particular parental overprotection and rejection, and SAD development have been reported (Arrindell *et al.*, 1989; Parker, 1979; Lieb *et al.*, 2000). Additionally, parental disorders including depression, other anxiety

disorders and alcoholism also increase in the risk of SAD (Kessler, Stein and Berglund, 1998; Lieb *et al.*, 2000; Bandelow *et al.*, 2004). Conversely, birth risk factors including age of mother or father over 35 years at childbirth, premature birth, low birth weight, Caesarean section, perinatal complications or congenital defects do not appear to increase the risk of SAD (Bandelow *et al.*, 2004).

Childhood Trauma:

It is well recognised that exposure to childhood trauma and adversity increase the risk of both anxiety and depressive disorders, likely associated with complex neurobiological changes which predispose to later psychopathology (Heim and Nemeroff, 2001). With regards to SAD, it appears that a history of emotional abuse is more strongly related to the diagnosis than either physical or sexual abuse (Gibb, Chelminski and Zimmerman, 2007), findings which have been replicated (Kuo *et al.*, 2011). However, an increased frequency of childhood sexual abuse in patients has also been reported (Bandelow *et al.*, 2004).

1.4.5 Neurotransmitter Abnormalities

A role for serotonin in the pathogenesis of SAD is supported by a variety of research approaches although the relationship between serotonin and anxiety is complex. The well-recognised therapeutic efficacy of selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitor (SNRIs) suggests that a deficiency in serotonergic transmission may underlie SAD. Patients who were successfully treated with an SSRI and then exposed to a tryptophan depletion challenge, showed a significantly elevated autonomic nervous system (ANS) response to a public speaking task compared to a placebo group, although no increase in anxiety was reported (van Veen *et al.*, 2009). In addition, patients with SAD have been shown to exhibit an exaggerated cortisol response when administered the serotonergic agent fenfluramine, indicative of super-sensitivity of the post-synaptic serotonin receptors and thus suggestive of reduced post-synaptic serotonergic input (Tancer, 1993). However, other studies have suggested that an overactive central serotonergic system may be implicated in SAD. A small single-photon emission computed tomography (SPECT) imaging study reported increased serotonin transporter binding in the thalamus of SAD patients (van der Wee *et al.*, 2008) while a recent study using positron electron tomography (PET) scan data found that SAD patients had increased serotonin synthesis and/or serotonin transporter availability in various brain regions involved in the fear response. Furthermore, the rate of serotonin synthesis in the dorsal amygdala correlated positively with severity of social anxiety symptoms (Frick *et al.*, 2015). Another finding that is consistent with reports of increased central serotonergic activity in SAD is that of downregulated postsynaptic 5-HT_{1A} receptors in the amygdala, anterior cingulate and

dorsal raphe nuclei of these patients (Lanzenberger *et al.*, 2007). The paradoxical effects of serotonin are highlighted by the phenomenon of antidepressant-induced jitteriness/anxiety syndrome, which describes the early worsening of anxiety, agitation and irritability in some patients commencing serotonergic antidepressants (Sinclair *et al.*, 2009), possibly related to 5-HT_2 receptor subtype activation (Jaggar *et al.*, 2019; Vicente and Zangrossi, 2012) . The syndrome has predominantly been reported in depressed patients commencing SSRI treatment (Näslund *et al.*, 2017) but also in patients with panic disorder (Sinclair *et al.*, 2009). It has not been studied specifically in SAD, although there have been a few case reports of the syndrome in these patients published (Jackson and Lydiard, 1990; Muhtz, Agorastos and Kellner, 2009). While the relationship between serotonin and social anxiety is clearly not a straightforward one, it is evident than this neurotransmitter plays a role in SAD.

Dopamine is another neurotransmitter of interest. SAD is more prevalent than one would expect in Parkinson's disease (Richard, 2005) and symptoms of social anxiety increased in patients with Tourette's syndrome following administration of the dopamine antagonist, haloperidol (Mikkelsen, Detlor and Cohen, 1981). Alterations in striatal dopamine transporter binding in SAD have been reported although findings have varied from increased (Tiihonen *et al.*, 1997) to decreased levels (van der Wee *et al.*, 2008) to no difference from healthy controls (Schneier *et al.*, 2009). Alterations in D2 receptor availability in SAD has also been reported (Schneier *et al.*, 2000) but again findings are inconsistent (Schneier *et al.*, 2009). A recent neuroimaging study investigating the co-expression of serotonin and dopamine transporters in SAD reported that the interaction between both transport systems was more important than each carrier individually, with a SAD diagnosis being significantly predicted by the statistical interaction between the serotonin reuptake transporter (SERT) and dopamine transporter (DAT) availability, in the amygdala, putamen, and dorsomedial thalamus (Hjorth *et al.*, 2019).

A role for the catecholamines is clinically implicated in SAD given the common symptoms of autonomic hyperarousal. Several studies have supported a role for central norepinephrine overactivity. Administration of intravenous clonidine, an α2 adrenergic agonist which decreases sympathetic output and stimulates growth hormone (GH) release, resulted in a blunted GH response in patients with SAD, suggestive of reduced postsynaptic adrenergic-2 receptor functioning owing to norepinephrine overactivity (Tancer, Stein and Uhde, 1993). Additionally, patients with SAD undergoing an orthostatic challenge test showed higher plasma levels of norepinephrine than both patients with panic disorder and healthy controls (Stein, Tancer and Uhde, 1992). However, this finding was not replicated in a later study, although SAD subjects did exhibit increased blood pressure responsivity to Valsalva and exaggerated vagal withdrawal in response to isometric

exercise, suggesting impaired parasympathetic (not sympathetic) activity (Stein, Asmundson and Chartier, 1994). Another study investigating the response of patients with SAD to plasma infusion of epinephrine found that there was no observable anxiety response in 10 of 11 patients (Papp *et al.*, 1988). Evidence for the role of norepinephrine in SAD from a pharmacological perspective is limited. Although the SNRI Venlafaxine has been shown to be effective in reducing SAD symptoms (Liebowitz *et al.*, 2005; Rickels, Mangano and Khan, 2004), there is no difference in efficacy between low and high doses, suggesting that the benefit is due to the serotoninergic action rather than the noradrenergic one, which only comes into play at higher doses (Stein *et al.*, 2005). In addition, no difference in efficacy has been found between venlafaxine and the SSRIs, paroxetine (Liebowitz, Gelenberg and Munjack, 2005; Allgulander *et al.*, 2004) or sertraline (Pollack *et al.*, 2014). Furthermore, an RCT using atomoxetine, a pure norepinephrine reuptake inhibitor, found no difference to placebo in treating SAD (Ravindran *et al.*, 2009). Thus, the role of the ANS and norepinephrine in SAD is, at present, incompletely understood.

Given the efficacy of benzodiazepines (Stein and Stein, 2008; Pollack *et al.*, 2014) and other GABAergic treatments such as gabapentin, tiagabine (Dunlop *et al.*, 2007; Urbano *et al.*, 2009) and topiramate (Van Ameringen *et al.*, 2004) in SAD, a role for γ-aminobutyric acid (GABA) is also suggested. GABA is the main inhibitory neurotransmitter in the brain and thought to play a role in the modulation of anxiety responses both in the normal and pathological state (Nuss, 2015). Unfortunately, apart from the pharmacological support, evidence for the role of GABA specifically in SAD is quite limited. A small magnetic resonance spectroscopy (MRS) study has demonstrated lower levels of GABA in the thalamus of people with SAD (Pollack *et al.*, 2008) and variations in the glutamate decarboxylase (GAD)-1 gene, responsible for synthesizing GABA, were seen across a range of patients with anxiety disorders which included SAD (Hettema *et al.*, 2006).

1.5 Treatment Options for SAD

A large body of evidence from randomised controlled trials (RCTs) has allowed the production of robust evidence-based treatment guidelines for SAD and current first-line treatments include SSRIs and cognitive behavioural therapy (CBT) (Baldwin *et al.*, 2014). Several SSRIs (escitalopram, sertraline, paroxetine, fluoxetine and fluvoxamine) have been shown to be well tolerated and effective in the treatment of this disorder (Blanco *et al.*, 2013), although it is worth noting that the data on fluoxetine is inconsistent (Kobak *et al.*, 2002; Davidson *et al.*, 2004). The SNRI venlafaxine has consistently been shown to be effective and can also be considered a first-line therapy (Blanco *et al.*, 2013). Alternative pharmacotherapy options with proven efficacy in SAD include the monoamine

oxidase inhibitor (MAOI), phenelzine, the reversible inhibitor of monoamine oxidase A (RIMA) moclobemide, the benzodiazepines bromazepam and clonazepam, the anticonvulsants gabapentin and pregabalin and the antipsychotic olanzapine (Baldwin *et al.*, 2014). However, these are generally considered second-line treatments due to greater side effect profiles and/or less robust evidence to support their use. Beta-blockers do not appear to be effective in generalised SAD although they can be beneficial in reducing performance anxiety (Blanco *et al.*, 2013). It is unclear how long patients should continue pharmacotherapy. Although data is limited, several studies suggest that discontinuing medication after the initial 12-20 week period of treatment results in high relapse rates and it appears to be advantageous for patients to continue treatment for at least 3-6 months following a response (Blanco *et al.*, 2013)

Individual CBT has shown large effect sizes in the treatment of SAD and is also recommended as a first-line option (Mayo-Wilson *et al.*, 2014). There does not appear to be any significant difference in efficacy between SSRIs and CBT, although the beneficial effects of CBT may be longer-lasting (Canton, Scott and Glue, 2012). There is no evidence that a combination of psychological and pharmacological therapy is more efficacious than either leading monotherapy although the data on combination therapy is limited (Canton, Scott and Glue, 2012). Nonetheless, given the apparent greater benefit of CBT in maintaining symptom improvement, it is recommended to consider CBT after response to drug treatment in patients with a high risk of relapse (Baldwin *et al.*, 2014).

Unfortunately, a significant proportion of patients fail to adequately respond to first-line SSRI or SNRI treatment (Stein and Stein, 2008) and even fewer patients will respond to subsequent treatments. In a large RCT investigating augmentation and switch strategies for refractory SAD (defined as more than two unsuccessful adequate pharmacological treatment trials), only 46% of patients demonstrated a response to treatment while only 21% of all patients achieved remission at the 12-week endpoint (Pollack *et al.*, 2014). These figures highlight the necessity for a greater understanding of the neurobiological basis for the condition and the development of alternative therapeutic strategies. The microbiota-gut-brain (MGB) axis may represent one such potential avenue for investigation.

1.6 The Microbiota-Gut-Brain Axis in Psychiatry: An Overview

The human gastrointestinal tract (GIT) harbours an immense collection of microorganisms termed the gut microbiota. This consists predominantly of bacteria but also includes viruses, protozoa, fungi and archaea. Although more conservative than previously reported, recent estimates place the number of bacteria in the human gut at approximately 3.8×10^{13}, slightly in excess of the total

number of human cells (Sender, Fuchs and Milo, 2016). The collective genome of these bacterial cells, the gut microbiome, vastly exceeds the amount of human DNA present in the body, such that, for every one human gene we have over 100 bacterial genes (Backhed *et al.*, 2005). The presence of the human gut microbiota has long been known but it was presumed that these bacteria were commensal organisms i.e., unharmful but unhelpful for the human host. It is now recognised that this was a gross under-appreciation of what is actually a complex symbiotic interaction which influences many human biological systems. Given the enormous genetic potential of the microbiota it is unsurprising that it appears to play a role in a wide variety of physiological and psychological processes. The MGB axis is currently a hot topic in psychiatry and there is huge interest in the gut microbiome as a potential therapeutic target for mental illness.

The relationship between psychological functioning and physical symptomatology has long been appreciated, although ideas regarding the nature of this relationship have changed dramatically over time. Freudian theory dominated psychiatric thinking in the early 20th century and promoted the concept of 'conversion' or 'hysteria'. Freud hypothesized that unresolved emotional conflicts were 'converted' into physical disorders and could account for many physical conditions (Breuer and Freud, 1895). While psychiatry has moved beyond the case reports and postulations that characterised Freudian psychoanalysis to a more evidence-based medical model of thinking, the concept of the mind-body link in the causation of disease is more relevant than ever. This is particularly true when it comes to the GIT. Irritable bowel syndrome (IBS) is the archetype of functional gastrointestinal disorders, i.e., disorders characterised by the presence of clinical symptoms where no underlying structural or biochemical cause can be found. While the aetiology of IBS is unclear, it is well recognised that rates of psychiatric comorbidity, especially depression and anxiety, are extremely high (Wilmes *et al.*, 2021; Staudacher, Mikocka-Walus and Ford, 2021), suggesting a significant aetiological role for psychological factors. The same is true for many common non-functional gastrointestinal disorders including inflammatory bowel disease (IBD) (Graff, Walker and Bernstein, 2009), coeliac disease (Zingone *et al.*, 2015) and peptic ulceration (Lim *et al.*, 2014). While Freud was adept at dramatic theorizing, he was at least partially correct when suggesting that psychological stress or trauma could produce physical symptoms.

The concept of the 'gut-brain axis' refers to the bidirectional biochemical signalling that takes place between the GIT and the central nervous system (CNS) (Cryan *et al.*, 2019). This is a complex and dynamic system, capable of continuously transmitting, interpreting and responding to information. Within this vast communication matrix lies the gut microbiome, which we now recognise as playing a vital role. The mechanisms by which our gut bacteria communicate with, and influence, the CNS are gradually being uncovered and span neural, endocrine and immune systems. There is a striking

overlap between those pathways influenced by the microbiome and those involved in mental illness (Table 2). These pathways of communication between the microbiome, gut and brain are outlined in greater detail in the next section.

Table 2: Microbiota-Gut-Brain Axis communication pathways and their relevance to the pathogenesis of psychiatric disorders

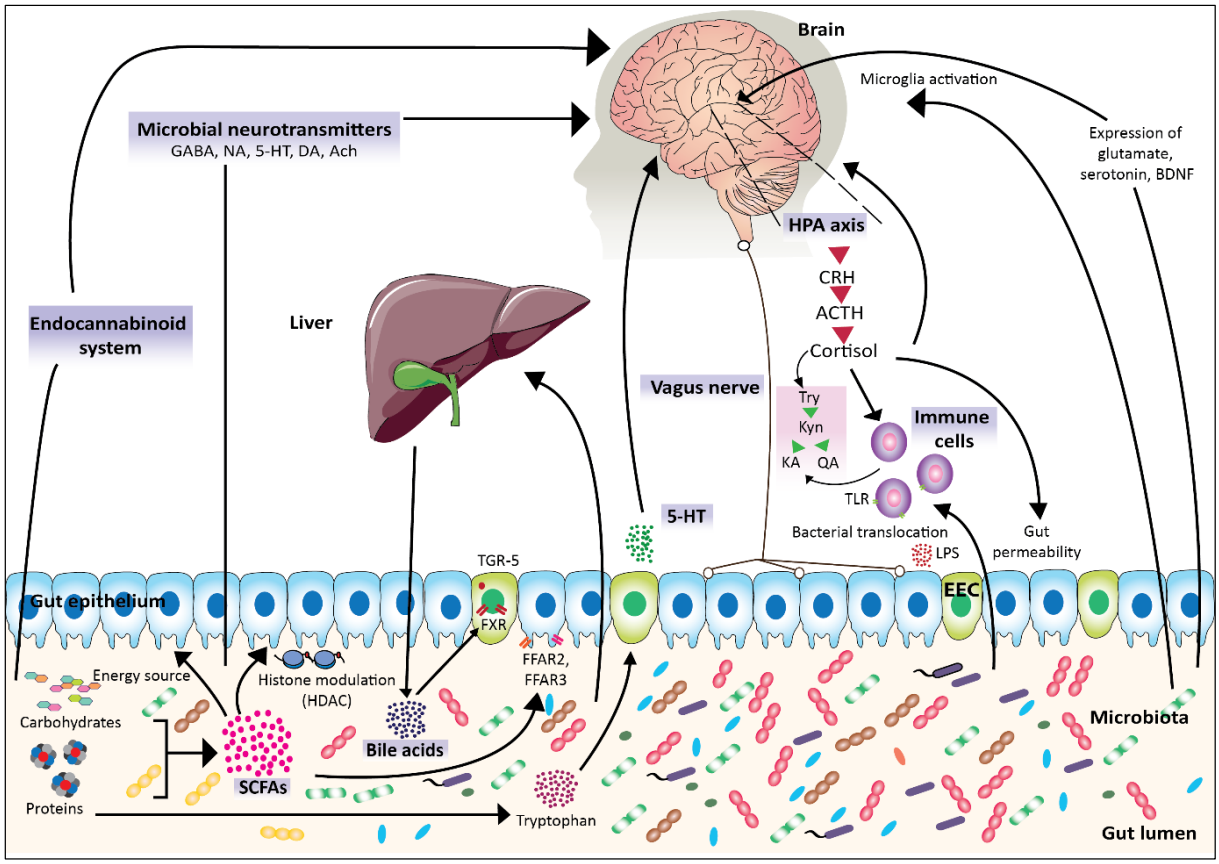
Microbiome-Gut-Brain Axis; Pathways of Communication	**Link with Psychiatric Disorders**
Immune modulation: The gut microbiome plays a major role in induction and development of the immune system (Belkaid and Hand, 2014). Gut dysbiosis is linked to abnormal production of inflammatory cytokines (Schirmer *et al.*, 2016). Translocation of microbes from the gut into the bloodstream is generally prevented by the tightly-adherent intestinal epithelium. However, stress has been shown to disrupt barrier integrity and a 'leaky gut' could allow movement of microbes out of the gut, thus stimulating an inflammatory response (Foster, Rinaman and Cryan, 2017)	Many psychiatric conditions are associated with chronic low-grade inflammation and raised pro-inflammatory cytokines, the source of which is unknown (Bauer and Teixeira, 2018). Gut microbiota disturbances may represent one possible mechanism linking chronic stress, a 'leaky gut', cytokine production and neuropsychiatric disorders such as depression (Kelly *et al.*, 2015).
Stress responsivity: The hypothalamic-pituitary-adrenal (HPA) axis mediates the stress response through a cascade of interactions culminating in the production of cortisol. There is substantial evidence that the gut microbiome is a key regulator of this stress pathway (Rea, Dinan and Cryan, 2016). Several probiotics (Takada *et al.*, 2016; Allen *et al.*, 2016) and prebiotics (Schmidt *et al.*, 2015) have been shown to reduce cortisol stress responses in healthy humans	Early life adverse events and chronic stress are major risk factors for depression, anxiety and other psychiatric disorders (Agid, Kohn and Lerer, 2000). HPA axis dysfunction is a feature of many psychiatric illnesses, in particular, mood and anxiety disorders (Keller *et al.*, 2006)
Production of neuroactive substances: Gut bacteria can actually directly produce neurotransmitters used in the human body including GABA, serotonin, norepinephrine, acetylcholine and dopamine (Roshchina, 2010). In addition, they produce short-chain-fatty-acids (SCFAs) such as butyrate, which is thought to be of importance in brain health (Bourassa *et al.*, 2016).	GABA, serotonin, norepinephrine, acetylcholine and dopamine are, of course, of major interest in all psychiatric disorders. However, the quantities produced by bacteria are relatively small and unlikely to influence human neurotransmission directly to any great extent. Butyrate has been shown to demonstrate antidepressant (Wei *et al.*, 2014; Valvassori *et al.*, 2015; Yamawaki *et al.*, 2018) and antimanic (Resende *et al.*, 2013) effects in animal models. It has also been shown to be beneficial in preclinical studies of Huntington's (Ferrante *et al.*, 2003), Parkinson's (Sharma, Taliyan and Singh, 2015) and Alzheimer's (Govindarajan *et al.*, 2011) disease.
Tryptophan and Serotonin Metabolism: Tryptophan is an essential amino acid. Although most commonly known for its role as the precursor for serotonin, the majority is actually metabolised via an alternative route, the kynurenine pathway. This pathway results in the production of neuroactive compounds such as kynurenine, kynurenic acid (KYNA) and quinolinic acid (QA). The gut microbiota appears to control host tryptophan metabolism along this kynurenine pathway, thus increasing the production of neuroactive KYNA and QA, while simultaneously reducing the amount of	Serotonin is perhaps the most studied of all neurotransmitters when it comes to psychiatric illness, particularly in relation to anxiety and depressive disorders. However, the kynurenine pathway, may well be just as important in the pathogenesis of depression. (Myint *et al.*, 2007). Kynurenine and QA appear to be depressiogenic while KYNA has neuroprotective properties. An imbalance in these metabolites may be associated with depression (Maes *et al.*, 2011).

tryptophan available for serotonin synthesis (O'Mahony *et al.*, 2015).	
Given the widespread use of antidepressant medications and the emerging evidence of their antimicrobial action, there is a growing concern about the possible contribution of such medications to antibiotic resistance (Macedo *et al.*, 2017). To date, research on the effects of psychotropic medications on the microbiome has been primarily limited to the preclinical domain and human studies are required. Probiotic or prebiotic trials in clinical populations have either excluded patients taking medications or have been unable to account for the medication effect.	**Effect of Psychotropic medications on the Microbiome:** In keeping with the bidirectional nature of the MGB axis, recent studies have demonstrated the ability of psychotropic medications to alter the microbiome composition. Various non-antibiotic medications exert an effect on the gut microbiome (Maier *et al.*, 2018). In particular, atypical antipsychotics (Davey *et al.*, 2013) and serotonin-specific reuptake inhibitor (SSRI) antidepressants (Cussotto *et al.*, 2018) appear to alter host microbiota composition.

1.7 The Microbiota-Gut-Brain Axis: Mechanisms of Communication

Over the past 15 years there has been a huge proliferation of preclinical studies attempting to elucidate the mechanisms by which the gut microbiome communicates with, and influences, the brain. Although we tend to view this work as 21st century matter, an appreciation that gut microbes could alter brain chemistry was actually noted as far back as 1986. In a study designed to assess the influence of nephrectomy (removal of a kidney) on the brain, researchers found that germ-free mice had lower baseline levels of histamine in the hypothalamus than conventional controls (R. Hegstrand and Jean Hine, 1986). The implications of this finding were not fully appreciated at the time and the paper remains relatively unknown. We can more accurately realise its importance today. The current widespread use of germ-free (GF) animals (i.e., animals that are delivered surgically and raised in a sterile environment with no microbial exposure) along with animals whose microbiome is altered by exposure to probiotics, prebiotics, pathogenic bacterial infections, antibiotics and faecal microbiome transplantation (FMT), has allowed microbiome researchers to uncover the complex and varied pathways that are involved in the MGB axis (Figure 1).

Figure 1: Routes of communication between gut microbes and the brain: The microbiota-gut-brain axis is a complex, bi-directional system involving a variety of pathways through which the gut microbiota and brain communicate. Key players in this gut-brain signalling system include the vagus nerve, the HPA-axis, the immune system and tryptophan metabolism, directed towards either serotonin production or the alternative kynurenine pathway. Additionally, gut microbiota can influence the brain by the production of various neuroactive metabolites including microbial neurotransmitters and SCFAs. Emerging evidence in relation to the impact of gut microbiota on the endocannabinoid system, bile acid metabolism and microglia activation also suggest that these systems may be of relevance to gut-brain signalling. (Butler, Cryan and Dinan, 2019)



(Ach = acetylcholine; ACTH = adrenocorticotrophic hormone; BDNF = brain derived neurotrophic factor; CRH = corticotropin releasing hormone; DA = dopamine; EEC = entero-endocrine cell; FFAR = free fatty acid receptor; FXR = farnesoid X receptor; GABA = gamma-Aminobutyric acid; HDAC = histone deacetylase; HPA = hypothalamic-pituitary-adrenal axis; KA = kynurenic acid, Kyn = kynurenine, SCFAs = short chain fatty acids; 5-HT = 5-hydroxytryptamine; NA=norepinephrine; QA = quinolinic acid, TLR = toll-like receptor. Try = tryptophan)

1.7.1: The Microbiome and Stress Response

The hypothalamic-pituitary-adrenal (HPA) axis is best known for its role in mediating the stress response. Stress, defined as a state where homeostasis is under or perceived to be under threat, activates the axis by stimulating the production of corticotrophin-releasing hormone (CRH) by the hypothalamus, resulting in subsequent downstream production of adrenocorticotropic hormone (ACTH) by the pituitary and ultimately, cortisol by the adrenal glands. HPA axis dysfunction has been implicated in the pathophysiology of a variety of psychiatric disorders, in particular, depression (Holsboer, 2000) and anxiety disorders (Tafet and Nemeroff, 2020)

An individual's 'stress responsiveness' is determined by a combination of gene-environment interactions. Early-life stress is a significant environmental variable which impairs HPA axis activity, contributing to future maladaptation of the stress-response and increasing the risk of mental health difficulties in adulthood (Maniam, Antoniadis and Morris, 2014). The gut microbiome has a profound effect on HPA axis development, as demonstrated by a milestone study in 2004 (Sudo *et al.*, 2004). Researchers discovered that GF mice exhibited higher ACTH and corticosterone release following a mild restraint stress in comparison to their control counterparts. This exaggerated stress response was partially reversed by colonisation with faeces from the control mice and completely reversed by giving the mice a single bacterial strain, *Bifidobacterium infantis*. However, the reversal was time-dependent and only occurred if the bacterial reconstitution took place at an early stage, indicating that there is a critical time period in early life during which colonization of the GIT must occur in order for normal HPA axis development. Another study used maternal separation as an early life stressor, a paradigm which has proven to be of value as a model of depression in animals (O'Mahony *et al.*, 2011).This team found increases in plasma corticosterone in the stressed rat pups along with an alteration of the faecal microbiota when compared with the control group (O'Mahony *et al.*, 2009). Thus, it appears that exposure to stress not only impacts the HPA axis but also modifies microbiome composition, a finding which has been replicated in several subsequent studies (Bangsgaard Bendtsen *et al.*, 2012; Bailey *et al.*, 2011).

One mechanism by which the microbiome may influence the HPA axis is via alteration of central gene expression in areas of the brain such as the amygdala, hippocampus and prefrontal cortex, regions known to play a role in learning, memory, mood and anxiety. Both glutamate and serotonin influence hypothalamic CRH release and alterations in expression of these neurotransmitter receptors may thus alter HPA function in GF animals. Another protein of interest is brain-derived-growth-factor (BDNF), a key neurotrophin involved in neuronal plasticity which is thought to play an important role in the aetiology of depression (Castren, Voikar and Rantamaki, 2007). Microbiome-mediated changes in BDNF expression, along with glutamate and serotonin receptor levels, have been confirmed in rodent studies, albeit inconsistencies are evident. Sudo et al (Sudo *et al.*, 2004) reported a decrease in BDNF along with decreased expression of the glutamate NMDA receptor in the hippocampus and cortex of male GF mice. On the other hand, Neufeld et al (2011) reported an increase in hippocampal BDNF mRNA in female mice while Clarke et al (2012) reported decreases in hippocampal BDNF mRNA levels and distinct changes in the hippocampal serotoninergic system. Such incongruities in animal studies obviously raise concerns in relation to applicability to the human system with important considerations around the impact of sex and strain of animal on results

obtained. Nevertheless, it is clear that the development and function of the HPA axis is affected by the compositional and functional status of our gut microbiome.

1.7.2: The Microbiome and Immunity

The immune system is a complex and fascinating system extending its influence to virtually all disease processes. Although far from being fully understood, psychology is beginning to appreciate immune dysfunction as an important underlying pathophysiological factor in mental illness. An aetiological role for immune dysfunction in depression was first considered in response to the study of cytokine-induced 'sickness behaviour'. Following peripheral infection, innate immune cells release signalling proteins called cytokines which act on the brain to cause symptoms such as loss of appetite, irritability, low mood, loss of motivation, social withdrawal, fatigue and impaired attention. This constellation of symptoms is termed 'sickness behaviour' and is an adaptive response, encouraging the sick person to retreat, conserve energy and fight off the infection. The symptoms of sickness behaviour bear a striking resemblance to the clinical picture that characterises depression. It has consistently been shown that depression is associated with a low-grade elevation in inflammatory markers (O'Brien, Scott and Dinan, 2004) and, in fact, depression can actually be induced by administration of cytokines (Udina *et al.*, 2012). While the evidence pertaining to anxiety disorders is sparse and less convincing, there does appear to be immune dysfunction in some patients with anxiety disorders (Vogelzangs *et al.*, 2013) and targeting inflammation has been suggested as a potential therapeutic target for anxiety (Michopoulos *et al.*, 2017).

The gut microbiome is a long-overlooked modulator of immune function. In the first instance the microbiome plays a vital role in the early development of the immune system (Hooper, Littman and Macpherson, 2012). Immune cells possess transmembrane pattern-recognition receptors called toll-like receptors (TLRs) which recognise characteristic molecular patterns on bacterial cells. When these TLRs are activated, they initiate a cascade ultimately resulting in cytokine production. This process continues and the immune system matures by constantly responding to different structural components of bacterial cells. A process that is initially located at the gut intestinal epithelium eventually extends throughout the body. The result is not only the production of a wide variety of cytokines but also the development of lymphocytes and guidance of antigen-specific acquired immunity (Akira and Takeda, 2004). Without the microbiota, certain TLRs are not fully expressed in the gut (O'Hara and Shanahan, 2006) and it appears that certain host-specific bacterial species need to be present for complete immune development (Chung *et al.*, 2012).

The source of the low-grade inflammation seen in depression and other related psychological conditions is as yet unknown and an interesting hypothesis involves the gut microbiome and alterations in gastrointestinal permeability (the 'leaky gut'). The intestinal epithelium, a single cell layer with an overlying mucus sheet, plays a vital role in maintaining a selectively permeable barrier between the gut lumen and the rest of the body. The gut microbiome, in close proximity to the gut epithelium, appears to regulate the homeostasis of the epithelial barrier, and also influence colonic mucus secretion (Pearson and Brownlee, 2010). This theory supposes that increased permeability of the gut barrier, resulting in translocation of gut bacteria, or bacterial components such as lipopolysaccharides (LPS), normally safely confined to the gut lumen, could trigger a systemic inflammatory response. The hypothesis is certainly supported by the finding that raised LPS, or corresponding immunoglobulin levels have been reported in depression (Maes, Kubera and Leunis, 2008), autism (Emanuele *et al.*, 2010) and Alzheimer's disease (Zhang *et al.*, 2009). Chronic stress is a significant risk factor for depression and anxiety disorders and it is well recognised that stress, through the production of cortisol and catecholamines, increases intestinal permeability, thus providing another plausible link in the leaky gut – inflammation – psychopathology chain. It is noteworthy that probiotic bacteria such as *Bifidobacterium infantis 35624*, which reduce depressive behaviours in a rat model of depression, have also been shown to attenuate the pro-inflammatory state seen in these animals (Desbonnet *et al.*, 2010). The same probiotic normalises the pro-inflammatory state in patients with IBS as well as significantly reducing gastrointestinal symptomatology (O'Mahony *et al.*, 2005).

While our discussion regarding the impact of the microbiome on the immune system has focussed predominantly on the peripheral inflammatory response, it is worth noting an exciting 2015 study which revealed that the microbiome can directly influence brain microglia. Erny et al (Erny *et al.*, 2015) revealed that GF-mice display widespread defects in the maturation and function of microglia, resulting in deficient innate immune responses. Furthermore, they demonstrated that a full repertoire of gut bacteria is needed for microglia development and a limited microbiome results in defective microglia. A key signalling pathway mediating the influence of the microbiome on microglia development was found to be the production of bacterial short-chain-fatty-acids.

1.7.3: The Microbiome and Short-Chain Fatty Acids

A major function of our gut bacteria is the digestion of carbohydrates and proteins which we consume in our diet. Principal metabolites of this digestive process are short-chain-fatty-acids (SCFAs), namely acetic acid, propionic acid, and n-butyric acid. Bacteria in the colon are particularly

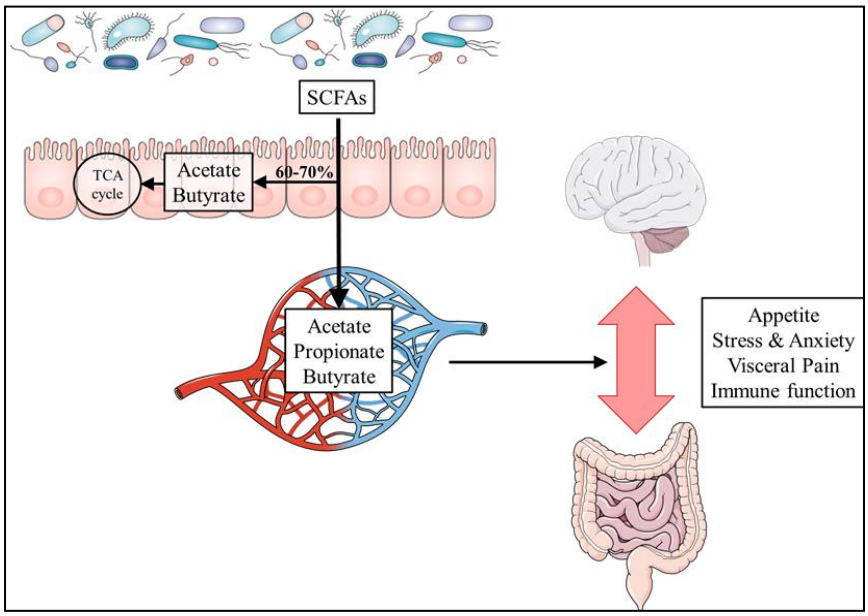
adept at producing SCFAs from resistant starches and fibres which are not digested in the upper part of the GIT. At a local level SCFAs serve many functions. They are the major energy source for intestinal epithelial cells (Topping and Clifton, 2001), regulate energy homeostasis (Ichimura *et al.*, 2009) and directly influence the release of GI signalling molecules such as peptide YY (Holzer and Farzi, 2014) and serotonin (Yano *et al.*, 2015) Their role, however, is not just a local one and we now realise that these molecules travel throughout the body and exert an effect at several distal organs (Clarke *et al.*, 2014) (Figure 2).

An important site of action appears to be the liver, given that a large proportion of circulating SCFAs are taken up here as they transit through the hepatic portal system (Cummings *et al.*, 1987). Following hepatic uptake, peripheral blood concentrations are low but the widespread presence of SCFA transporters and receptors suggest that these compounds must play a role outside the GIT. SCFAs are transported across cell membranes by monocarboxylate transporters (MCTs) which are found, not only at the gut, but at various other sites including the kidney and brain (Ganapathy *et al.*, 2008). Three specific SCFA receptors have been identified, free fatty acid receptors (FFAR), FFAR2 and FFAR3 (Bolognini *et al.*, 2016) and hydroxycarboxylic acid receptor 2, (HCAR2) (Singh *et al.*, 2014) FFAR2 and FFAR3 are widely expressed on leukocytes supporting a role in immune regulation (Kim, Park and Kim, 2014). Propionate has been shown to affect intracellular calcium concentrations in neutrophils, further supporting an immune signalling role (Naccache *et al.*, 1988). In addition, SCFA receptors are found in adipose tissue and are thought to be regulators of host adipocyte function and plasma lipid levels (Ge *et al.*, 2008). There is no doubt that SCFAs could indirectly affect the brain by their influence on metabolism and immunity. However, the major question remains as to whether or not they can exert a direct influence the CNS. The presence of specific SCFA receptors within the brain would support a direct role. FFAR3 (Bonini, Anderson and Steiner, 1997) and HCAR2 (Fu *et al.*, 2015) are both expressed in rat brain tissue. However, human studies investigating the presence of SCFAs in the brain or cerebrospinal fluid are awaited.

Once it gains access to a cell, butyrate demonstrates an exciting ability to influence gene transcription, no small feat for a small, single molecule produced by bacteria. It accomplishes this by inhibiting histone deacetylation (HDAC) (Boffa *et al.*, 1978). Histone acetylation is an epigenetic modification which increases the propensity for gene transcription. Acetyl groups may be removed by histone deacetylases (HDACs) resulting in reduced transcription. Butyrate acts by inhibiting these HDACs and thus can influence the expression of a large number of genes and many different pathophysiological pathways. The potential of such a molecule cannot be overestimated and this is reflected in the widespread research of butyrate across many disorders, neuropsychiatric and otherwise. Butyrate demonstrates antidepressant effects preclinically (Schroeder *et al.*, 2007) and in

an autism mouse model it has been shown to attenuate social deficits by transcriptional modification (Kratsman et al., 2016). It improves neurodegeneration and extends the life-span in a mouse-model of Huntington's disease (Ferrante *et al.*, 2003), and has been shown to be similarly beneficial in preclinical studies of Parkinson's (Sharma, Taliyan and Singh, 2015) and Alzheimer's disease (Govindarajan *et al.*, 2011). SCFAs also appear to have anxiolytic effects and can alleviate the heightened stress-responsiveness seen in mice exposed to a chronic psychosocial stressor (van de Wouw *et al.*, 2018). The therapeutic possibilities seem endless but it is early days in butyrate and SCFA research.

Figure 2: Local and systemic effects of SCFAs: SCFAs, produced by gut bacteria from the metabolism of carbohydrates and proteins, include acetate, propionate and butyrate. At a local level, SCFAs provide energy for gut epithelial cells and regulate energy homeostasis. Additionally, following absorption into the bloodstream, they can travel distally to influence a variety of gut-brain processes including appetite regulation, the stress response, anxiety, visceral pain and immune function.



(SCFA: Short-chain-fatty-acid, TCA cycle: Tricarboxylic acid cycle)

1.7.4: The Microbiome and Neurotransmitter Production

Remarkably, our gut bacteria have the capability of directly producing neurotransmitters and neuromodulators which are an exact match to those produced by our human cells and widely used throughout the human body (Lyte, 2011). *Lactobacillus spp.* and *Bifidobacterium spp.* produce gamma-aminobutyric acid (GABA); *Escherichia spp., Bacillus spp. and Saccharomyces spp*. produce noradrenalin; *Candida spp., Streptococcus spp., Escherichia spp. and Enterococcus spp*. produce serotonin; *Bacillus spp.* produce dopamine; and *Lactobacillus spp*. produce acetylcholine (Roshchina,

2010). Of course, the quantities produced by bacteria are relatively small and often demonstrated in vitro only and therefore their potential to exert a significant impact on human neurotransmission is questionable. However, these microbial neuroactive compounds do represent a potential mechanism whereby our microbes could directly interact with human cells and even small amounts may impact important processes governing mood, anxiety and cognition.

Let us take GABA as an example. GABA is the main inhibitory neurotransmitter in the brain (Roberts and Frankel, 1950). It mediates its effects through two major classes of receptors—the ionotropic GABA¬A and the G-protein coupled GABAB receptors. The GABAergic system is implicated in the pathogenesis of depression and anxiety (Cryan and Kaupmann, 2005) and these receptors are important and effective pharmacological targets for anti-anxiety agents (e.g. benzodiazepines acting on GABA¬A receptors). Certain strains of *Lactobacillus* and *Bifidobacteria*, derived from the human intestine, can produce GABA from monosodium glutamate in vitro and many foodstuffs containing probiotic bacteria e.g. Japanses funa-sushi and Chinese traditional paocai, contain levels of GABA in significant quantities. Thus, GABA, either produced by intestinal bacteria from dietary glutamate or ingested in fermented foodstuffs, may be present in sufficient quantities intestinally to influence local receptor-mediated immune and neural systems. It is not just peripheral GABA concentrations that are impacted by gut bacteria. While the mechanism is unclear, gut bacteria can actually influence GABA concentrations in the brain. The probiotic, *Lactobacillus rhamnosus*, which reduces anxiety and depressive behaviours in mice, also modulates GABA receptor expression in various brain regions, including the cortex, hippocampus and amygdala (Bravo *et al.*, 2011; Janik *et al.*, 2016).

1.7.5: The Microbiome and Vagus Nerve

The vagus nerve is the major parasympathetic nerve in the body and plays a key role in regulating several organ functions including heart rate, bronchial constriction and gut motility. Stimulation of this parasympathetic pathway has an anti-inflammatory effect, resulting in reduced pro-inflammatory cytokine production and attenuation of the systemic inflammatory response (Borovikova *et al.*, 2000) Vagal nerve stimulation has been to shown to be beneficial in treatment-resistant depression (Sackeim *et al.*, 2001) and treatment-resistant anxiety disorders (George *et al.*, 2008) as well as exerting an analgesic effect (Kirchner *et al.*, 2000).

The vagus nerve is an important signalling pathway in the gut-brain axis and appears to respond to, and relay information about, changes in gut bacteria (Fülling, Dinan and Cryan, 2019). Vagal afferent nerve fibres are distributed throughout the intestinal wall but are precluded from direct contact with

the microbiota by the intestinal epithelial barrier (Bonaz, Bazin and Pellissier, 2018). Thus, they respond to bacterial signals indirectly following exposure to bacterial neuro-metabolites such as neurotransmitters or SCFAs, and through interaction with gut enteroendocrine cells (Raybould, 2010). Key insights into the role of the vagus nerve in gut-brain signalling have been provided using animal models of GIT infection. A research team from the University of Virginia, USA (Gaykema, Goehler and Lyte, 2004; Goehler *et al.*, 2008) sought to establish the anatomical basis for the anxiety-like behaviours observed in rodents infected with *Campylobacter jejuni*, a food-borne pathogen. Using FOS immunocytochemistry, they demonstrated that infection with *Campylobacter jejuni* resulted in increased activation in several brain areas that process visceral/autonomic information, including those that are typically activated following vagal stimulation. Another team highlighted the role of the vagus nerve using rats who had undergone vagotomy i.e. surgical cutting of the vagus nerve. Rats infected with *Salmonella Typhimurium* displayed functional activation of various brain regions and altered T-cell counts. These responses were significantly reduced in rats who had previously been vagotomised (Wang *et al.*, 2002). Probiotic studies have also used the vagotomy technique. A study mentioned previously, which demonstrated the positive effect of *Lactobacillus rhamnosus* (JB-1) on anxiety behaviours and central GABA receptor levels, reported that neither the behavioural nor neurochemical effects of the probiotic was evident in vagotomised mice (Bravo *et al.*, 2011).

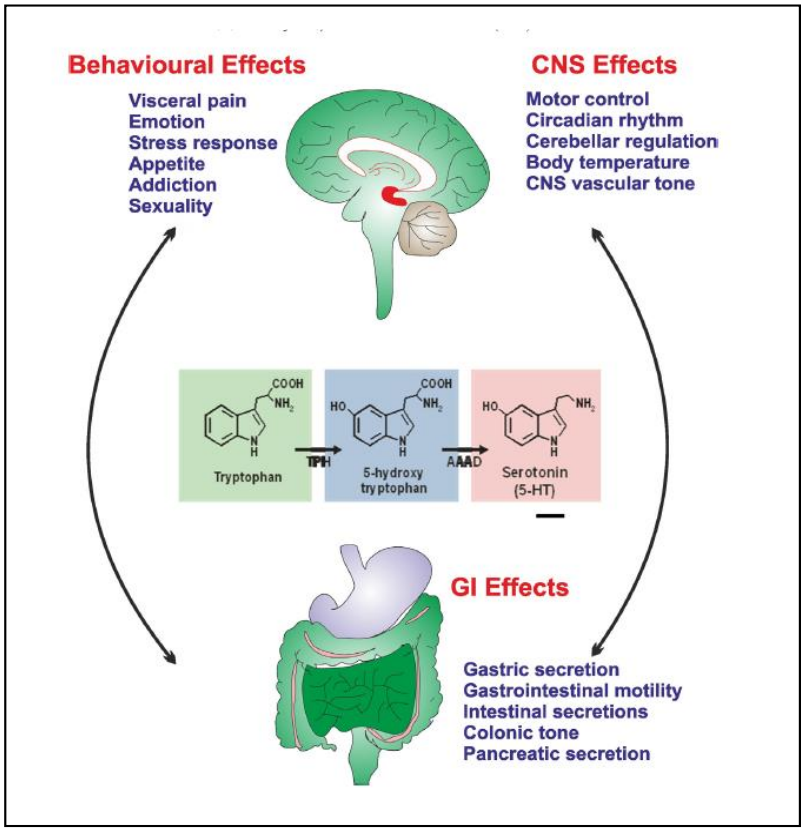
1.7.6: The Microbiome, Serotonin and the Kynurenine Pathway

Serotonin (5-hydroxytryptamine, 5-HT) is a metabolite of the essential amino acid, tryptophan and is one of the most widely-studied of all neurotransmitters. It plays a key role in anxiety and mood regulation as well as influencing many other bodily functions including gastrointestinal function, blood pressure regulation, platelet aggregation and bladder control (Figure 3). Serotonin-specific reuptake inhibitors (SSRIs) are the most commonly used class of antidepressant and anxiolytic and act to increase the availability of serotonin at the synapse. Interestingly, SSRIs have also proven to be of benefit in gastrointestinal disorders such as IBS (Tack *et al.*, 2006) and IBD (Macer, Prady and Mikocka-Walus, 2017). The overlap in serotonergic dysfunction between psychiatric and gastrointestinal disorders has prompted a keen interest in the role of the gut microbiome in tryptophan and serotonin metabolism.

We tend to think of serotonin as a predominantly CNS-based molecule and indeed, this is where most attention on the neurotransmitter has been focussed. However, more than 90% of the body's serotonin is actually synthesized in the gut, predominantly by specialized endocrine cells called

enterochromaffin cells (ECs). The metabolic pathway involves the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme, tryptophan hydroxylase (TPH). 5-HTP is a short-lived intermediate product and quickly converted to 5-HT (Berger, Gray and Roth, 2009). A forerunner in the investigation of this area was a metabolomics study in 2009 which revealed that conventionally colonised mice had 2.8 times greater plasma serotonin levels in comparison to GF mice (Wikoff *et al.*, 2009). The mechanism behind this was subsequently revealed to be a microbiome-driven increase in transcription of the rate-limiting enzyme tph1 (Yano *et al.*, 2015). The elevated levels of TPH permitted increased 5-HT synthesis in ECs, thus resulting in increased peripheral serotonin levels. Furthermore, this team demonstrated the specific microbes capable of promoting serotonin production to be spore-forming bacteria, primarily from the *Clostridium* genus.

Figure 3: The brain-gut axis and serotonergic metabolism. (Figure taken from (O'Mahony *et al.*, 2015)



Following absorption from the gut, tryptophan enters the circulation and travels to the brain where it is metabolised is several ways. A small amount is used for protein synthesis. Some is used for serotonin production and the synthetic cascade is identical to that in the gut as detailed above. The remainder, accounting for the vast majority of tryptophan in the brain, is metabolised via an alternative route, the kynurenine pathway. We have been aware of this pathway for many decades

but it is only recently being appreciated that dysfunction may have important consequences for CNS function (Ruddick *et al.*, 2006) and indeed play a role in disorders such as Alzheimer's disease (Bonda *et al.*, 2010) and depression (Réus *et al.*, 2015). The enzymes, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-diogenase (TDO) catalyse the initial rate-limiting metabolic step of the kynurenine pathway and lead to the production of kynurenine. TDO is influenced significantly by glucocorticoid induction whereas IDO levels are regulated by cytokines (Badawy, 2017). Kynurenine can be metabolised via a number of different routes and ultimately results in the production of neuroactive compounds such as kynurenic acid and quinolinic acid. Kynurenic acid is thought to be a neuroprotective substance and acts as an NMDA antagonist (Albuquerque and Schwarcz, 2013). Quinolinic acid, conversely, is an NMDA agonist and is neurotoxic (Schwarcz and Pellicciari, 2002).

The kynurenine pathway offers a unifying theory in the pathogenesis of depression integrating immune activation and HPA axis dysfunction with abnormal neurotransmission of serotonin and glutamate (Maes *et al.*, 2011). Immune activation, possible mediated by a 'leaky gut' as discussed previously, induces IDO. Chronic stress, HPA axis activation and the ensuing glucocorticoid production increases TDO concentrations. The result is a dysfunctional kynurenine pathway with consequent abnormalities in serotonin and glutamate neurotransmission. Interestingly, although plasma tryptophan and kynurenine levels are not reduced in depressed patients, they do have lower kynurenic acid concentrations suggesting the metabolism of kynurenine is preferentially directed into the neurotoxic quinolinic pathway (Myint *et al.*, 2007). A similar aetiological role for the immune-kynurenine pathway in anxiety disorders has been considered although studies are sparse (Kim and Jeon, 2018).

How does this relate to the microbiota? Interestingly, the gut microbiome appears to directly influence tryptophan break-down at several points along the metabolic pathway. Although GF mice exhibit lower plasma serotonin levels than conventionally colonised mice, they have 40% greater plasma tryptophan levels (Wikoff *et al.*, 2009). Clarke et al. (2013) expanded on these findings in a study using GF mice to explore the influence of the gut microbiome on serotonergic neurotransmission in the hippocampus, an area of the brain well known to play a role in stress, anxiety and depression. Researchers found that male GF mice exhibit a significant elevation in hippocampal concentration of serotonin. Concentrations of tryptophan were also increased in the plasma of GF mice, along with a reduced plasma kynurenine:tryptophan ratio, suggesting that the microbiota alters central serotonergic neurotransmission via an influence on tryptophan metabolic pathways. Of note, these changes were only present in male mice, demonstrating a sex-specificity not seen in studies exploring immunological and neuroendocrine effects of the microbiome.

1.7.7: The Microbiome and Bile Acids

A recent and exciting frontier in microbiome research is the exploration of cross-talk between bile acids and gut bacteria, and the subsequent impact on host metabolism. Bile acids (BAs), which are metabolised by gut bacteria, have major regulatory and signalling roles. They are of increasing interest as potential aetiological agents in a range of disease states including obesity, metabolic syndrome, IBD and GIT cancers (for review see (Staley *et al.*, 2017). This interest has extended in recent years to several neuropsychiatric conditions including depression (Jia *et al.*, 2016), autism (Golubeva *et al.*, 2017) and neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Ackerman and Gerhard, 2016). However, studies in the area are sparse and we are only beginning to appreciate a possible role for the gut microbiome to impact the brain through BA transformation.

The liver synthesizes BAs as a product of cholesterol metabolism. Primary BAs in humans are cholic acid (CA) and chenodeoxycholic acid (CDCA), which are conjugated by liver cells with one of two amino acids, glycine or taurine, prior to excretion into bile. The conjugated BAs are stored in the gallbladder and released into the duodenum following a meal, where they aid in the digestion and absorption of lipids. Gut bacteria de-conjugate the BAs, which in their unconjugated form can subsequently be transformed into secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA). Deconjugation of BAs is an ability shared by most gut bacteria. The production of secondary BAs is a more exclusive function, and carried out by only a small number of anaerobic bacteria, predominantly of the *Clostridium* and *Eubacterium* genera (Ridlon, Kang and Hylemon, 2006). Approximately 95% of BAs are absorbed in the terminal ileum and returned to the liver via the portal system, with the remainder being eliminated in the faeces. The interplay between BAs and the microbes which metabolise them is complex. While BAs are dependent on gut bacteria for transformation, BA pool size and composition appear to be major regulators of gut microbial structure. Secondary BAs such as DCA have potent antimicrobial activity (Begley, Gahan and Hill, 2005) and BA pool size is greatly reduced in conventional animals in comparison to their GF counterparts (Sayin *et al.*, 2013)

While the major function of BAs is cholesterol clearance, they are now recognised as playing a major role in glucose homeostasis, an action which obviously has far-reaching effects throughout the body. BAs regulate glucose metabolism through a direct action on two receptors, a nuclear farnesoid X receptor (FXR) and a cytoplasmic G protein coupled receptor, TGR5 (Gonzalez-Regueiro *et al.*, 2017). They stimulate the release of fibroblast growth factor-19 (FGF19) from the ileum which, independent of insulin, plays an important role in mediating an appropriate postprandial response

by the liver (Kir *et al.*, 2011). A research team from New York has recently expanded on this to demonstrate that FGF19 can regulate glucose metabolism via a direct action on hypothalamic neurons, thus defining a new node in the gut-brain axis which influences glucose homeostasis (Liu *et al.*, 2018).

The gut microbiome may influence CNS disease states through BA alterations. A recent study using an animal model of autism, demonstrated that a reduction in the abundance of particular bile-metabolizing bacteria was associated with marked GI dysfunction and increased autistic-like behavioural scores (Golubeva *et al.*, 2017). Authors described a significant reduction in the relative abundance of *Blautia* species, a member of the *Clostridium* genera and one of a minority of bacteria capable of producing secondary BAs. Reduced numbers of secondary BAs, known for their potent antimicrobial activity, could lead to intestinal bacterial overgrowth and a disruption of the epithelial barrier or 'leaky gut', which, as discussed previously, has been linked to the pro-inflammatory state seen in depression and ASD. Another recent study has linked depression and BA metabolism using a metabonomics approach, which studies changes in metabolic products in response to complex stimuli causing disease (Jia *et al.*, 2016). Researchers exposed rats to chronic unpredictable mild stress (CUMS), a validated animal model of depression. They subsequently measured a series of metabolic pathways in the liver and found primary BA biosynthesis to be one of the pathways playing a key role in the development of CUMS-induced depression. Our evolving understanding of the role of microbiome-BA interplay as an important component of the gut-brain axis will hopefully yield further insights into brain health.

1.7.8: The Microbiome and Endocannabinoid System

Cannabis has long been used by humans seeking relief from a variety of symptoms including anxiety, nausea and chronic pain. The main psychoactive constituent of cannabis, Δ9 -tetrahydrocannabinol (THC) exerts the majority of its effects via activation of two cannabinoid receptors, CB1 and CB2. These receptors, along with their naturally-occurring endogenous ligands, N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) make up the endocannabinoid system (ECS). CB1 is highly abundant in the CNS while CB2 is found predominantly in the gut, expressed on neurons, epithelial and immune cells. Our knowledge of this system has increased hugely in recent years and we now appreciate it as an important component of the gut-brain axis, with emerging evidence that it interacts with the gut microbiome.

The ECS is involved in a wide variety of physiological processes mediating gut-brain signalling. An important remit is the regulation of energy metabolism, hunger signalling and food intake, and it has

been the focus of much interest in relation to the pathophysiology of obesity and metabolic disorders such as Type 2 diabetes (Gatta-Cherifi and Cota, 2015). It also plays an important role in gastrointestinal homeostasis and exerts an influence on gut motility, gut permeability, gastric secretions and inflammation (DiPatrizio, 2016). The gut microbiome appears to influence the ECS in a bidirectional capacity. A Belgian group have published a series of papers exploring the interaction between the gut microbiome and ECS in relation to obesity. Using probiotics, antibiotics and GF-mice, they have demonstrated that the gut microbiota modulates ECS tone. This in turn regulates gut permeability and plasma LPS levels, which subsequently affects adipose tissue metabolism (Muccioli *et al.*, 2010). Another study demonstrated that a *Lactobacillus* probiotic could induce expression of cannabinoid receptors in rodent intestine and mediate an analgesic effect (Rousseaux *et al.*, 2007). Everard et al (2013) used the probiotic *Akkermansia muciniphila* and revealed that it also had the ability to increase intestinal levels of endocannabinoids, reinforcing gut barrier function while improving the metabolic profile of obese mice.

It is not uncommon for individuals suffering with anxiety to reportedly 'self-medicate' with cannabis. While there is currently no sound evidence base for the therapeutic use of cannabis in psychiatry, the ECS does appear to be an important regulator of the stress response, as evidenced by both animal and human studies. Using a variety of stress models, rodent studies have demonstrated that stress results in reduced AEA and increased 2-AG levels. The low levels of AEA result in HPA-axis activation and increased anxiety behaviours while the increased 2-AG appears to modulate HPA adaptation responses as well as altering pain perception, memory and synaptic plasticity (Morena *et al.*, 2016). Concentrations of these endogenous cannabinoid ligands have been shown to be altered by stress exposure in healthy humans (Dlugos *et al.*, 2012) and in patients with major depression (Hill *et al.*, 2008). In addition, chronic stress causes downregulation or loss of CB1 receptors and such impairment in receptor signalling is thought to increase an individual's susceptibility to stress-related pathology (Hill and Patel, 2013). More recently, evidence for endocannabinoid signalling as a mechanistic link between the gut microbiota and chronic stress in an animal model of depression was shown (Chevalier *et al.*, 2020) and a human longitudinal study using twin data expanded on this by demonstrating that gut-microbial diversity might contribute to anhedonia/amotivation via the endocannabinoid system (Minichino *et al.*, 2021).

A major challenge in harnessing the therapeutic potential of endocannabinoids is the widespread nature of their influence and this was highlighted recently by the failure of a new pharmacological agent, Rimonabant which acted as a CB1 receptor antagonist. This anti-obesity drug was approved in Europe in 2006 but withdrawn from the market 2 years later due to serious concerns about psychiatric side effects, including depression, anxiety and increased suicidal risk (Sam, Salem and

Ghatei, 2011). Without doubt, there is potential therapeutic value in the ECS for a variety of metabolic and psychiatric disorders, but safely channelling this capability requires a far greater understanding of its mechanisms.

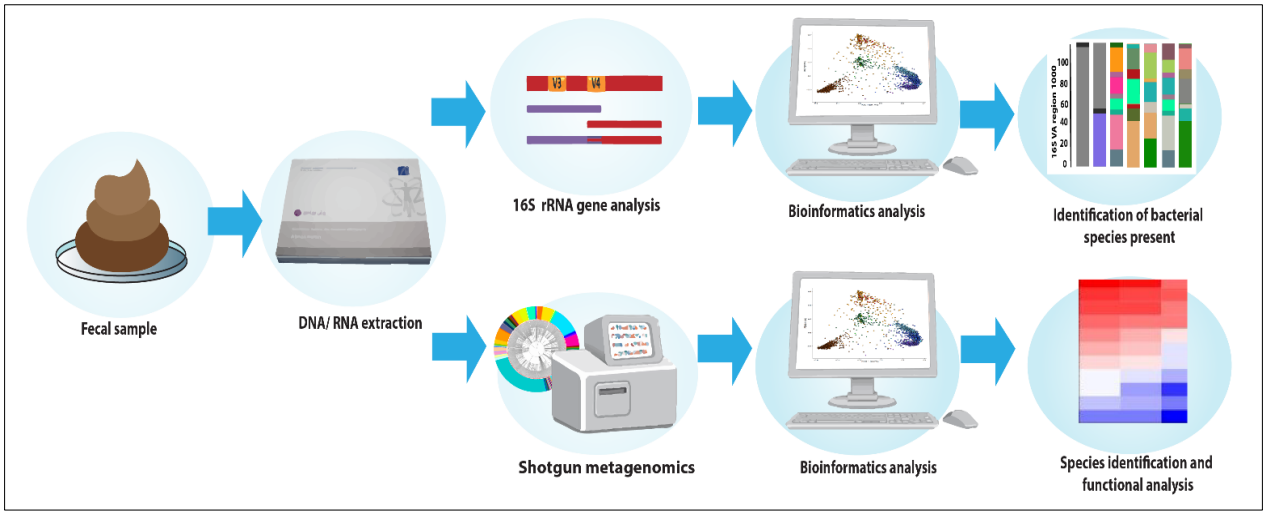
1.8 Techniques for Analysing the Gut Microbiome

Historically, bacteria could only be investigated by culture techniques which involved plating samples on appropriate media and identifying the resultant bacterial growth (Lagier *et al.*, 2015). The problem with this method was that many microorganisms were not suitable for culture and thus, were unable to be identified. The advent of 'metagenomics', a culture-independent system which allows for direct analysis of the genetic material in a sample, has meant that it has become possible to identify all the microorganisms present (Hugenholtz, Goebel and Pace, 1998).

Once a faecal sample has been collected it undergoes processing to extract the DNA and RNA (Figure 4). The resulting genetic material can be analysed in two ways. The first, and most commonly employed technique is 16S rRNA gene analysis. The 16S-ribosomal-RNA (rRNA) gene is a highly-conserved gene present in almost all bacteria. The extracted RNA undergoes polymerase chain reaction (PCR) processing which, using pre-made16S rRNA primers, identifies and amplifies these genes. The resultant genes are then sequenced allowing identification of the different bacteria present in the sample. (Srinivasan *et al.*, 2015). The second, more expensive, method is 'shotgun metagenomics', also called 'whole genome shotgun sequencing'. This is a technique whereby all the extracted DNA in the sample is sequenced, as opposed to only one target gene. It not only identifies which bacteria are present in a sample but also enables an assessment of their function from analysis of all the genes they contain. It is more expensive than 16S-rRNA sequencing but very useful for functional, along with compositional, microbiome analysis (Claesson, Clooney and O'Toole, 2017).

While traditional DNA sequencing was an extremely slow and expensive process, high-throughput 'next generation sequencing' (NGS) technology has revolutionised the microbiome field by allowing billions of DNA strands to be sequenced in parallel, making genome analysis faster, cheaper and more accessible (Heather and Chain, 2016). Following sequencing, huge data-sets are generated and can be analysed using specialised bioinformatics packages. The DNA sequence reads are clustered with similar reads into 'operational taxonomic units' (OTUs), each of which signifies a specific bacterial genera or species.

Figure 4: Analysis of the gut microbiome from a faecal sample can be done in two ways. The more basic method is using 16S-ribosomal-RNA analysis which identifies all the bacterial genera and species present in the sample. Shotgun metagenomics is a more complex and expensive process but provides information on the functional capacity of the microbiome along with bacterial identification (Butler *et al.*, 2019).



1.9 Impact of the Microbiome on Anxiety and Depressive Behaviours in Animal Models

There is wealth of preclinical evidence demonstrating the effect of gut microbiome modulation on behaviour in various animal models of anxiety and depression. Animal models provide an accessible avenue for exploring different microbiome states e.g., GF conditions, antibiotic-depleted microbiota, alteration of specific bacteria through probiotic and prebiotic supplementation, exposure to pathogenic bacterial infections and the use of FMT. A variety of animal models of relevance to psychiatric symptoms are in use. Commonly used animal models of depression include the chronic unpredictable mild stress (CUMS) model (Katz, Roth and Carroll, 1981; Willner, 1991), an early life stress paradigm such as maternal separation (O'Mahony *et al.*, 2011), the social defeat model (Hollis and Kabbaj, 2014) and the chronic restraint stress paradigm (Kim and Han, 2006). Animal models of anxiety also exist, although given the complex and heterogenous nature of anxiety disorders, these models only simulate certain specific elements and not the whole range of anxiety disorder symptoms (Freudenberg *et al.*, 2018). Animal models of anxiety are of two main types. The first involves assessing an animal's conditioned responses to particular stressful and/or unpleasant stimuli (e.g., exposure to electric foot shock). Animal models of both PTSD (Whitaker, Gilpin and Edwards, 2014) and SAD (Toth, Neumann and Slattery, 2012) have been developed using such fear-conditioned responses. The second method involves ethological paradigms and the investigation of an animal's natural reaction (e.g. flight, avoidance and freezing) to stress stimuli. A variety of such ethological or unconditioned tests exist and include the commonly-used elevated plus maze (EPM),

the light-dark box (LDB) and the open field test (OFT) (Bourin *et al.*, 2007). Although often referred to in the literature as animal 'models', this latter group are more accurately described as 'tests' of anxiety as they do not represent a phenotype specifically relevant to a clinical anxiety disorder but rather a behavioural fear-inducing paradigm that allows quantification of the fear response (Cryan and Sweeney, 2011). Another helpful and common approach to the investigation of anxiety in animals, is the use of animal models which have a genetically-determined anxious profile and thus, increased sensitivity to fear-inducing behavioural challenges (Finn, Rutledge-Gorman and Crabbe, 2003).

A role for the microbiome in modulating behaviour, with regards to anxiety and depressive symptoms, has consistently been demonstrated using the aforementioned animal models and behavioural tests. GF mice show reduced anxiety responses to common anxiety tests such as the EPM and LDB, which is somewhat puzzling given that this behavioural response is in contrast to the exaggerated HPA-axis stress response seen in such animals (Cryan and Dinan, 2012). Rodents infected with pathogenic bacteria such as *Trichuris muris* (Bercik *et al.*, 2010) and *Citrobacter rodentium* (Lyte *et al.*, 2006) show increased anxiety behaviours. Conversely, probiotic supplementation can reduce anxiety as well as depressive-like behaviours in animal models, as has been demonstrated by studies using a variety of different probiotics including *Lactobacillus rhamnosus* (JB-1) (Bravo *et al.*, 2011; Bharwani *et al.*, 2017), *Bifidobacterium longum* NCC3001 (Bercik *et al.*, 2010; Bercik *et al.*, 2011), *Bifidobacterium infantis* (Desbonnet *et al.*, 2008; Desbonnet *et al.*, 2010), *Faecalibacterium prausnitzii* (ATCC 27766) (Hao *et al.*, 2019), *Bifidobacterium longum* 1714, *Bifidobacterium breve* 1205 (Savignac *et al.*, 2014), *Lactobacillus plantarum* MTCC 9510 (Dhaliwal *et al.*, 2018), *Lactobacillus plantarum* PS128 (Liu *et al.*, 2016), *Lactobacillus helveticus* NS8 (Liang *et al.*, 2015) as well as several multispecies probiotics containing combinations of various *Lactobacillus* and *Bifidobacterium* species (Abildgaard *et al.*, 2017; Liang *et al.*, 2017; Messaoudi *et al.*, 2011). FMT, which involves a more drastic change in microbiota profile, has also been used to demonstrate the impact of a change in the microbiome on behaviour in animal models. Rodents that receive an FMT from depressed patients (Kelly *et al.*, 2016; Zheng *et al.*, 2016; Liu *et al.*, 2020), or from donor animal models of depression (Li *et al.*, 2019; Lv *et al.*, 2019; Yang *et al.*, 2019; Jiang *et al.*, 2020; Siopi *et al.*, 2020), demonstrate increased anxiety and/or depressive-like behaviours. Conversely, when FMT from healthy or resilient animals is given to rodent models of depression or anxiety, improvements in anxiety and depressive-like behaviours are seen, indicating a protective effect by the microbiota (Langgartner *et al.*, 2018; Schmidt *et al.*, 2020; Yang *et al.*, 2019).

1.10 Current State of Knowledge of the Gut Microbiome in Mood and Anxiety Disorders

There is no doubt that the gut microbiome influences brain function and the vast array of preclinical studies provide us with insights into the mechanisms by which this may be occurring. However, the major question for psychiatrists is whether the science actually translates to the clinic or remains an academic pursuit. The concept of the MGB axis is an exciting one but does it actually mean anything in the management of mental illness in our patients? Unfortunately, there is a marked disparity between the swell of preclinical studies in recent years and the relative paucity of translational research. However, although the human data is certainly lagging behind the laboratory discoveries, application of microbiome-based hypotheses are gradually being tested in clinical populations. Human studies have followed one of two approaches. The first has been a case-control design, profiling the microbiome in patient populations and, by comparison with healthy controls, seeking to establish specific microbiome configurations associated with disease states. The major challenge with this method is that there is large interindividual variability in microbiome composition, not to mention the many confounding factors including diet and medication use. The second approach has been interventional, using various techniques to manipulate the microbiome of both healthy and clinical populations, and assess the outcome on mood, anxiety and other psychological functions. The most common intervention has been the use of probiotics, but prebiotics, antibiotics and dietary change also feature in the literature. In addition, there are some exciting clinical trials using FMT in psychiatric conditions underway.

1.10.1 Anxiety and Related Disorders – Microbiome Composition

There is a striking paucity of studies profiling the gut microbiome in patients with primary anxiety disorders. Ironically, one of the most common psychological outcomes in probiotic interventional studies in both preclinical models and healthy human volunteers is an anxiety or stress measure. Of course, anxiety and stress, along with mood, are dimensional concepts and applicable to all human subjects. Nonetheless, an obvious area for future research should involve profiling the microbiome in patients who cross that line between ‘normal’ and ‘pathological’ and have been diagnosed with a specific anxiety condition.

The only anxiety disorder which has received attention in the microbiome arena to date, has been generalised anxiety disorder (GAD). Three recent studies investigating the gut microbiome composition in GAD have been undertaken, two in Chinese populations and one in the United States. Some consistent findings were found between the former 2 studies. The first (Jiang *et al.*, 2018) undertook a cross-sectional analysis of 40 patients with GAD and 36 controls. They reported several

genus-level differences with higher abundances of *Bacteroidetes, Ruminococcus gnavus* and *Fusobacterium* and lower abundances of *Faecalibacterium, Eubacterium rectale, Sutterella, Lachnospira,* and *Butyricicoccus* in GAD patients. They also conducted a subgroup analysis of 12 treatment-naive patients and 17 controls which additionally revealed higher abundances of *Escherichia-Shigella* and *Lactobacillus* in the GAD group. Furthermore, the unmedicated GAD group had reduced *Lachnospira, Roseburia* and *Subdoligranulum* in addition to reduced *Faecalibacterium* and *Eubacterium rectale.* The second study from China (Chen *et al.*, 2019) involving 36 patients with GAD and 24 healthy controls demonstrated some consistency with the first. They also found that *Escherichia-Shigella* and *Bacteroides* abundances were enriched in the GAD group and that these genera were positively correlated with anxiety severity. Another consistent finding was that of reduced *Eubacterium spp* and *Subdoligranulum* in GAD patients. Moreover, both these Chinese studies reported decreased microbial diversity and richness in patients with GAD. The third study investigating GAD (Mason *et al.*, 2020) had different patient groups. Most patients in this study had comorbid depression and anxiety (n=38) or depression only (n=14) with the GAD-only group involving just 8 patients which were all female. This study reported reduced *Bacteroide*s in the comorbid group in comparison to the depression group, thus suggesting that reduced *Bacteroides* was more associated with anxiety, a contrasting finding to the other two GAD studies. Also contrary to the other studies was the finding of no difference in microbial diversity between the psychiatric and control groups. However, the small sample size and female bias limit the interpretation of the findings. Unfortunately, in all 3 studies there was minimal attention paid to the effect of confounding host variables on microbiome composition which can increase the risk of false positive results. No information on diet or alcohol intake was collected in two studies (Jiang *et al.*, 2018; Mason *et al.*, 2020) although these variables have been shown to have a major impact on human gut microbiome variance (Vujkovic-Cvijin *et al.*, 2020). In addition, none of the studies collected any information on stool quality, another hugely impactful confounding factor (Vujkovic-Cvijin *et al.*, 2020; Falony *et al.*, 2016). Unfortunately, there have been no other compositional studies in people with other primary anxiety disorders such as SAD, specific phobias, separation anxiety disorder, selective mutism, panic disorder or agoraphobia.

Post-traumatic stress disorder (PTSD), while more correctly classified as a trauma-related as opposed to primary anxiety disorder, is worth mentioning here. It has been the subject of one small case-control study where authors analysed the microbiome profile of 18 individuals suffering from PTSD and compared it to that of 12 subjects who, despite exposure to trauma, did not develop PTSD. Although overall diversity measures were similar, the relative abundances of Actinobacteria,

Lentisphaerae, and Verrucomicrobia phyla were decreased in PTSD subjects and able to distinguish PTSD from controls with a high degree of accuracy (Hemmings *et al.*, 2017).

1.10.2: Anxiety and Related Disorders - Microbiome Interventional Studies

To date there has only been one microbiome interventional study in a clinical anxiety population, the cohort in this case comprising 48 unmedicated patients with GAD (Eskandarzadeh *et al.*, 2021). Participants were randomly assigned to two groups, receiving either a multispecies probiotic (*Bifidobacterium longom, Bifidobacterium bifidum, Bifidobacterium lactis and Lactobacillus acidophilus)* or placebo in addition to 25 mg sertraline for 8 weeks. The probiotic + sertraline combination was superior to sertraline alone in decreasing anxiety symptoms, although it did not impact quality of life.

As with mood, most interventional trials exploring the effects of probiotics on anxiety and stress have been conducted in healthy populations and results are varied. A large Japanese study involving over 200 healthy participants reported a slight improvement in anxiety levels following 12-week consumption of *Lactobacillus gasseri* and *Bifidobacterium longum* (Nishihira J. *et al.*, 2014). A similar probiotic combination (*Lactobacillus helveticus and Bifidobacterium longum*) also demonstrated a slight benefit on anxiety scores (Messaoudi *et al.*, 2011). Another trial in healthy male participants revealed that four-week consumption of *Bifidobacterium longum* resulted in a variety of psychological benefits including reduced self-reported anxiety and cortisol levels in response to an acute stressor, reduced daily perceived stress and subtle improvements in visuospatial memory with enhanced frontal midline mobility on electroencephalography (Allen et al., 2016). However, not all studies have been so positive. The probiotic *Lactobacillus casei* failed to impact anxiety measures in two separate studies (Benton, Williams and Brown, 2007; Reale *et al.*, 2012) and *Lactobacillus rhamnosus*, a probiotic which had appeared very promising in preclinical work, demonstrated no benefit over placebo in modifying a variety of biochemical and psychological stress-related measures in healthy subjects (Kelly *et al.*, 2017).

University students, subject to regular intermittent stress in the form of examinations, make for a useful healthy population in which to assess the effect of probiotics on anxiety and stress. Several interventional studies have used this population group. Takada et al. (2016) assessed the effects of *Lactobacillus casei strain Shirota* (LcS) on gut-brain interactions in healthy Japanese medical students undergoing examination stress. As expected, academic stress resulted in increased salivary cortisol and an increase in physical symptomatology. Both of these stress-related responses were significantly suppressed in the LcS group. A similar study in Spain explored the effect of a fermented

milk product containing *Lactobacillus casei, Lactobaciullus delbrueckii subsp. Bulgaricus and Streptococcus salivarius subsp. Thermophilus.* While the probiotic did modulate the immune response of the stressed students, there was no improvement in anxiety scores (Marcos *et al.*, 2004). Probiotic supplementation with *Bifidobacterium longum* 1714 in healthy male students under conditions of exam stress did not impact stress or cognitive measures but did improve some sleep indices (Moloney *et al.*, 2021)

It is worth mentioning those studies which have assessed anxiety outcomes in patients with various medical comorbidities. Several studies, using multispecies probiotics, including various combinations of *Lactobacillus, Streptococcus* and *Bifidobacterium* strains, reported no significant impact on anxiety symptoms in patients with IBS (Simren *et al.*, 2010; Dapoigny *et al.*, 2012; Whorwell *et al.*, 2006; Han *et al.*, 2017). However, a more positive finding was reported in patients with chronic fatigue syndrome (CFS), a disorder of unknown aetiology which, like IBS, is commonly associated with anxiety and low mood. Authors described a significant improvement in anxiety scores in 39 patients with CFS following consumption of *Lactobacillus casei subsp Shirota* for 8 weeks (Rao *et al.*, 2009). Yang et al (2016) investigated the ability of *Clostridium butyricum* to reduce presurgical anxiety in patients prior to surgery for laryngeal cancer. Those in the probiotic group reported less anxiety along with reduced serum CRF levels, supporting a role for probiotics in ameliorating stress in pre-surgical cancer patients.

Prebiotics have also been investigated in relation to anxiety. Prebiotic are non-digestible carbohydrates which are selectively fermented by the bacteria in the large intestine. They can be used to target particular gut bacteria and selectively enhance their growth within the intestine. A research group in Oxford found a significant impact of prebiotics on stress responses in healthy participants (Schmidt *et al.*, 2015). Volunteers received one of two prebiotics (fructooligosaccharides, FOS, or Bimuno®-galactooligosaccharides, B-GOS) or a placebo (maltodextrin) daily for 3 weeks. Those in the BGOS group showed significantly reduced waking cortisol responses as well as reduced attention and reactivity to negative emotions. Another study involving the administration of a specific dietary fibre, polydextrose (PDX) to healthy females reported that, while the supplement did not impact mood, anxiety or the acute stress response, it did result in improvements in certain aspects of cognitive performance (Berding *et al.*, 2021).

A recent meta-analysis has investigated the use of probiotics and prebiotics in reducing anxiety symptoms (Liu, Walsh and Sheehan, 2019). Following the analysis of 22 RCTs, authors reported that probiotics yielded small but significant results in anxiety reduction while prebiotics did not appear to be beneficial.

1.10.3 Depression – Microbiome Composition

Several case-control studies have reported altered gut microbiota composition in patients with depression. The first, published in 2014 (Naseribafrouei *et al.*, 2014), found that the order Bacteroidales was overrepresented, while the family *Lachnospiraceae*, was reduced in patients with depression. Differences in a variety of OTUs assigned to the family *Lachnospiraceae,* between depressed patients and controls, were also reported in another study (Zheng *et al.*, 2016). At lower taxonomic levels, the genus *Alistipes* was overrepresented in depressed patients, a consistent finding in two studies (Jiang *et al.*, 2015; Naseribafrouei *et al.*, 2014). It is noteworthy that *Alistipes* has also been shown to be increased in chronic fatigue syndrome (Fremont *et al.*, 2013) and IBS (Saulnier *et al.*, 2011). A pronounced reduction in the *Prevotellaceae* family and subsequently in the *Prevotella* genus also appears to be associated with depression (Jiang *et al.*, 2015; Kelly *et al.*, 2016) although a later study reported increased *Prevotella* in MDD patients (Lin *et al.*, 2017). Unfortunately, such inconsistent findings are not unusual. While one study (Naseribafrouei *et al.*, 2014) reported no significant difference in species richness between depressed patients and controls, another study (Kelly *et al.*, 2016) demonstrated that depression is associated with decreased gut microbiota richness and diversity. Thus, although case-control studies have confirmed a differential microbiome profile in MDD, there does not appear to be an identifiable 'depression' signature and some findings are contradictory. This may be partly explained by the fact that microbiome composition shows major interindividual variability and these MDD studies were small, ranging from only 34 to 60 subjects in patient groups. A Belgian group has attempted to address the issue recently by a large-scale population study which used data from the Flemish Gut Flora Project to investigate the relationships between microbiome composition and quality of life and depression (diagnosed by a general-practitioner) in 1045 people. They found that two bacterial genera, *Coprococcus* and *Dialister*, were depleted in patients with depression irrespective of antidepressant treatment, and that butyrate-producing *Faecalibacterium* and *Coprococcus* bacteria were consistently associated with higher quality of life measures (Valles-Colomer *et al.*, 2019). Interestingly, *Faecalibacterium* is also decreased in patients with bipolar disorder (Evans *et al.*, 2017) A role for the microbiome in MDD is further supported by the striking observation that when mice are colonised with the microbiome from a depressed patient, through the process of FMT, they begin to exhibit depressive-like symptoms (Kelly *et al.*, 2016; Zheng *et al.*, 2016).

1.10.4: Depression - Microbiome Interventional Studies

The majority of interventional studies exploring the effects of probiotics on mood have been performed in healthy subjects and predominantly investigate various *Lactobacillus* and *Bifidobacterium* strains. The earliest was published in 2007 and assessed the effect of *Lactobacillus*

casei. It was a large study involving 132 healthy adults and although no overall effect of the probiotic was found, post-hoc analysis revealed that those with the lowest baseline mood score did show significant improvements following probiotic supplementation (Benton, Williams and Brown, 2007). This suggests that probiotics may be of benefit to those experiencing low mood while not offering any potential value to healthy adults with normal baseline mood states. Several subsequent probiotic studies revealed mixed results. A combination of *Lactobacillus helveticus* and *Bifidobacterium longum* resulted in subtle improvements in mood (Messaoudi *et al.*, 2011). Another multispecies probiotic (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus casei, Lactobacillus salivarius, and Lactococcus lactis*) demonstrated an ability to significantly reduce overall cognitive reactivity to sad mood, thought to represent 'vulnerability to depression', in healthy adults (Steenbergen *et al.*, 2015). An interesting imaging study involving healthy women found no benefit on mood scores following ingestion of a fermented milk product containing *Bifidobacterium animalis subsp Lactis, Streptococcus thermophiles, Lactobacillus bulgaricus, and Lactococcus lactis subsp Lactis*. However, functional MRI revealed alterations in the activity of brain regions controlling central processing of emotion and sensation (Tillisch *et al.*, 2013).

There have only been a handful of interventional studies in patients with a diagnosis of depression (Table 1). A polybiotic, containing *Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium bifidum*, significantly reduced depressive symptoms in patients with MDD (Akkasheh *et al.*, 2016). Unfortunately, it was unclear whether the probiotic was a sole or adjunctive treatment, thus making it difficult to draw conclusions about its antidepressant potential. A more recent randomised controlled trial (RCT) specifically excluded patients who were taking psychotropic medication. Consumption of a probiotic preparation (*Lactobacillus helveticus and Bifidobacterium longum*) demonstrated no benefit in terms of improving mood or moderating inflammatory or other biomarkers (Romijn *et al.*, 2017). A third trial reported significant benefits of *Bifidobacterium longum NCC3001* in alleviating self-reported mild-to moderate depressive symptoms. Although patients were not taking antidepressant medication, the study sample consisted of patients with IBS, an obvious confounder when looking at probiotic potential (Pinto-Sanchez *et al.*, 2017).

Several systematic reviews and meta-analyses have been carried out in recent years to investigate the effect of probiotics on mood and, for the most-part, confirm the beneficial effects of certain probiotics (Pirbaglou *et al.*, 2016; Huang, Wang and Hu, 2016; Wallace and Milev, 2017; Ng *et al.*, 2018; Liu, Walsh and Sheehan, 2019). However, several caveats are worth noting. Probiotics appear to be of limited efficacy in those with normal baseline mood and a beneficial effect is predominantly seen in those exhibiting depressive symptoms (Ng *et al.*, 2018). In addition, the antidepressant

effects of probiotics seem to be limited to younger adults and not evident in those over the age of 65 years (Huang, Wang and Hu, 2016). Another area of concern is the major inter-study discrepancies in relation to probiotic dosing and duration of treatment, which has reduced the comparability of current clinical trials. Likewise, the use of different bacterial species and strains poses a similar challenge. While those probiotics which appear to have anti-depressant effects are predominantly of the *Bifidobacterium* and *Lactobacillus* genera, there are many different species and strains within these genera and properties are not generalisable.

1.11 Primary hypothesis and aims of thesis

I hypothesise that the gut microbiota is compositionally and functionally different in those with SAD, and that physiological systems of relevance in MGB communication, including neuroendocrine, immune, and tryptophan-kynurenine pathways, along with gut barrier function, will show alterations in this patient group. I hypothesize that altering the microbiome through dietary change may represent a potential therapeutic option for anxiety. These hypotheses will be tested via the following objectives:

1. To determine the composition, richness, diversity and functionality of the gut microbiota in patients with SAD compared to healthy controls.
2. To investigate gut barrier function in patients with SAD compared to healthy controls.
3. To investigate a variety of physiological pathways involved in MGB communication, including neuroendocrine, immune and tryptophan-kynurenine pathway markers in patients with SAD compared to healthy controls.
4. To determine the effect of a dietary change involving the consumption of unpasteurised dairy products on the human gut microbiome, and its potential impact on anxiety levels in healthy participants.

Chapter 2: The Social Anxiety Disorder Phenotype

2.1 Abstract:

Background: Many studies exploring the microbiota-gut-brain (MGB) axis in relation to stress, anxiety or depression have involved healthy volunteers and measure non-specific general anxiety or mood outcomes. Studies of the gut microbiome in patients with clinically diagnosed anxiety disorders are very limited. The use of patients with specific anxiety conditions may allow for more accurate appraisal of the links between the gut microbiome and different anxiety phenotypes. We sought to investigate the microbiota-gut-brain (MGB) axis in a group of carefully-selected patients with SAD, a specific and well-defined anxiety condition. This chapter describes the recruitment and assessment of patients, as well as the clinical phenotype of SAD.

Methods: Patients with a pre-existing diagnosis of SAD were recruited through local general practitioners, psychologists, psychiatry clinics and SAD support groups, and the diagnosis confirmed by an experienced psychiatrist using the MINI International Neuropsychiatric Interview. The patient phenotype was characterised using a wide variety of psychological scales along with detailed demographic data.

Results: Thirty-two patients with SAD and thirty-six healthy controls were recruited. Patient and control groups were matched for age, gender, race, years of education, alcohol and smoking status. Patients with SAD had higher mean BMI levels along with lower exercise levels and poorer sleep quality. Two-thirds of patients had a history of past depression and just over one-third had a secondary comorbid anxiety disorder. Significant differences were seen between patients and controls in relation to general anxiety and stress scales, autistic traits, personality characteristics, empathy scores and history of childhood trauma.

Conclusions: Our patient sample comprises subjects with a distinct clinical anxiety disorder, whose SAD phenotype is well characterized by comprehensive demographic and psychological data. The patient profiles are consistent with the existing literature on SAD. Selecting patients with a well-defined clinical anxiety disorder is vital for studies on the MGB axis.

2.2 Introduction

There is a striking lack of microbiome studies in in patients with a specific clinically-diagnosed anxiety disorder, despite huge interest in the relationship between the gut microbiome and stress-related and anxiety states (Kelly *et al.*, 2015). The term 'anxiety' is used widely and represents a heterogenous and variable concept. For some, it is used to convey general mental symptoms of excessive worry or rumination, whereas for others, it represents a more physical condition involving a wide range of autonomic symptoms such as palpitations, tremor or sweating (Murphy and Leighton, 2009). Moreover, the cultural context can greatly influence the concept of anxiety symptoms (Marques *et al.*, 2011) and a uniform definition of anxiety is difficult (Coutinho *et al.*, 2010). Even amongst different medical disciplines, differences in the understanding of anxiety-related conditions exist (Hanel *et al.*, 2009).

For psychiatrists, the term 'anxiety' is a relatively vague term which could potentially refer to a large variety of different symptoms and a greater accuracy is conveyed by specifically talking about various anxiety disorders, if present. The need for diagnostic specificity is highlighted by the DSM-5 classification of anxiety disorders, which currently includes nine different diagnostic categories including separation anxiety disorder, selective mutism, specific phobia, SAD, panic disorder, agoraphobia, generalized anxiety disorder (GAD), substance/medication-induced anxiety disorder, and anxiety disorder due to another medical condition (APA, 2013). This comprehensive list exists despite the reclassification of obsessive-compulsive disorder (OCD) (included in the obsessive-compulsive and related disorders), acute stress disorder, and posttraumatic stress disorder (PTSD) (included in the trauma and stress-related disorders) which, although closely related, are no longer considered anxiety disorders as they were in the previous version of the DSM (APA, 1994).

The vast majority of human studies investigating the microbiome-anxiety connection, including probiotic or prebiotic interventional studies, have involved healthy volunteers, with non-specific anxiety outcomes measured using various broad-based psychological scales (Liu, Walsh and Sheehan, 2019). Whilst these have merit as proof-of-principle studies and allow the generation of more precise hypotheses, defining more specific anxiety symptom outcomes through the selection of distinct diagnostic groups, may result in far less heterogenous and inconsistent results. Of course, diagnostic categories also have their limitations, and indeed there is an argument for a move away from categorical towards dimensional thinking in psychiatry research (Cuthbert, 2015). However, current anxiety disorder classifications do permit more accurate and exact characterisation of different anxiety states, and would thus allow for more well-defined associations between the microbiome and different anxiety phenotypes to be uncovered.

This thesis sought to investigate the MGB axis in a group of carefully-selected patients with a specific and well-defined anxiety condition. Volunteers were required to have a pre-existing diagnosis of SAD which had been made by a psychiatrist, general practitioner or psychologist and which was confirmed through a structured clinical interview by an experienced psychiatrist. Significant efforts were made to further characterise the group using a wide variety of psychological scales measuring social anxiety symptoms, stress, general anxiety and depressive symptoms, along with childhood trauma, empathy, personality and autistic traits. Additionally, detailed information was collected on demographic factors of relevance to the microbiome, including stool quality, alcohol intake and comprehensive dietary information, data which has often been neglected in previous studies of the microbiome in psychiatric conditions. This chapter outlines the methods for recruitment and assessment of patients along with their demographic and clinical profile.

2.3 Methods

Participants:

Patients with a diagnosis of SAD were recruited through local general practitioners, psychologists and outpatient psychiatric clinics in the North and South Lee Mental Health Services, Cork, Ireland. The study was also advertised through local and national SAD support groups and by an online website www.sadgut.ie. Eligibility was limited to men and women aged between 18-65 years with an existing diagnosis of SAD. Exclusion criteria included any significant acute or chronic medical illness; the presence of any condition or medication which the investigator believed would interfere with the objectives of the study or confound the interpretation of the study including anticonvulsants, centrally acting corticosteroids, opioid pain relievers, laxatives, enemas, antibiotics, anti-coagulants, over-the counter non-steroidal anti-inflammatories (NSAIDS); the use of probiotics, prebiotics or antibiotics in the previous 4 weeks; females who were pregnant or breastfeeding; subjects who were vegan or adhering to a strict specific diet. Specific psychiatric exclusion criteria included a lifetime diagnosis of psychotic disorder, mental retardation, bipolar disorder, dementia or autism spectrum disorder (ASD) and a current diagnosis of major depressive disorder (MDD), eating disorder, alcohol or substance abuse or dependence; A past history of depression was permitted, as was the presence of a comorbid anxiety disorder, provided the clinician was satisfied that SAD was the primary diagnosis. SAD participants were permitted to continue taking their regular psychotropic medication. Healthy controls were recruited through email and print advertising in University College Cork. Controls were required to have no past or current psychiatric diagnosis along with the other general

exclusion criteria outlined for the SAD participants. Participants taking hormonal contraception were excluded for oxytocin analysis but permitted for other investigations.

Procedures:

All study procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Study number APC085) and the study was conducted in accordance with the ICH Guidelines on Good Clinical Practice, and the declaration of Helsinki. All participants provided written informed consent.

All participants were interviewed by an experienced psychiatrist using the MINI International Neuropsychiatric Interview (Version 7.0) (Sheehan *et al.*, 1998) to confirm the diagnosis of SAD based on DSM-5 criteria and assess for any relevant comorbidities. No patient met the criteria for the 'performance only' specifier and all experienced social anxiety symptoms across a range of situations.

Social anxiety symptoms were assessed using the self-rated Liebowitz Social Anxiety Scale-Self Report, a 24-item scale which was initially developed as a clinician-rated instrument (Oakman *et al.*, 2003; Heimberg *et al.*, 1999) but later shown to have excellent psychometric properties as a self-report scale (Fresco *et al.*, 2001; Baker *et al.*, 2002; Oakman *et al.*, 2003). A battery of other psychological scales was also administered including the Perceived Stress Scale (PSS) (Cohen, Kamarck and Mermelstein, 1983), the State-Trait Anxiety Inventory (STAI) (Spielberger CD, 1983), the Hospital Anxiety and Depression Scale (HADS) (Snaith, 2003), the Childhood Trauma Questionnaire (CTQ) (Bernstein *et al.*, 1994), the Davis Interpersonal Reactivity Index (IRI) (Davis, 1983), and the Autism Quotient (AQ) (Baron-Cohen *et al.*, 2001).

To quantify nutrient intake, participants completed the self-administered 152-item SLAN-06 (Survey of Lifestyle, Attitudes and Nutrition in Ireland) food frequency questionnaire (FFQ) which is adapted from the EPIC Norfolk questionnaire (Riboli and Kaaks, 1997) and validated to be used in an Irish population (Harrington J, 2008). Participants were asked to estimate the frequency with which they consumed a specified portion size of each of the foods listed over the preceding year. The FFQs were analysed for nutrient intake using the FETA software (Mulligan *et al.*, 2014). Stool consistency was assessed using the Bristol Stool Chart (BSC) (Lewis and Heaton, 1997). Exercise levels were measured using the International Physical Activity Questionnaire (self-administered short form) (Craig *et al.*, 2003) and sleep, using the Pittsburgh Sleep Quality Index (Buysse *et al.*, 1989).

Patient's weight and height were measured, and body mass index (BMI) scores calculated.

Statistics

All data was analysed using SPSS 25 (IBM, Armonk, NY, USA). Visual inspection of box plots was used to identify outliers and consideration was given to removal of those lying more than three times the interquartile range (IQR) below the first quartile or above the third quartile. Missing values were excluded from analysis. Normality of data was assessed by visual inspection of histograms along with examination of skewness and the Shapiro-Wilk statistic. Independent t-tests were used to compare means between groups where assumptions were met. Welch corrections were used where there was violation of homogeneity of variances. Where parametric assumptions were violated, Mann-Whitney U tests were applied. Categorical data were analysed using the Chi-squared or Fisher's Exact test. Data are presented as mean ± SD unless stated otherwise.

2.4 Results

Thirty-two patients with SAD and thirty-six healthy controls were included in the study. Demographic characteristics and clinical profiles are shown in Table 1. There were no significant differences between patients and controls in relation to age, gender, race, years of education, alcohol or smoking status. Patients with SAD had higher mean BMI levels along with lower exercise levels. Sleep quality was poorer in the patient group.

Regarding clinical profile, three-quarters of SAD patients reported a history of depression and just over one-third had a secondary comorbid anxiety disorder. 65.6% of SAD patients were prescribed psychotropic medication. SAD patients reported significantly higher rates of previous suicide attempts and suicidal ideation in the previous month, compared to the control group.

SAD patients showed elevated scores across the PSS, STAI and HADS scales as well as on the AQ scale. There were significant differences between the SAD and control groups in relation to several personality categories and empathy dimensions. With regards to a history of childhood trauma, the patient group had higher total scores on the CTQ, as well as higher reported levels of emotional and sexual abuse, and emotional and physical neglect.

Table 1: Demographic Characteristics and Psychological Scales

	SAD (n=32)	**Controls (n=36)**	**p-value**
Age (years); mean (SD)	35.66 (11.92)	32.5 (12.46)	0.29
Gender; % female (n)	46.9 (15)	55.6 (20)	0.48
Race; % Caucasion (n)	100 (32)	94.4 (32)	0.44
Years of Education; mean (SD)	17.41 (4.27)	17.85 (4.02)	0.66
BMI (units); mean (SD)	27.7 (5.1)	24.1 (5.09)	0.001 *
Alcohol (units per week); mean (SD)	5.47 (7.2)	4.55 (5.6)	0.56
Smoking status; % smokers (n)	12.5 (4)	8.3 (3)	0.57
Exercise (IPAQ score); mean (SD)	3143.89 (3769.33)	4206.67 (2650.09)	0.013 *
Exercise (IPAQ category); % (n)			
• Low	25.8 (8)	5.9 (2)	0.039*
• Moderate	35.5 (11)	29.4 (10)	
• High	38.7 (12)	64.7 (22)	
Sleep Quality (PSQI score); mean (SD)	8.00 (4.31)	4.72 (3.58)	0.002 *
OCP; % taking (n)	6.2 (2)	22.2 (8)	0.09
Comorbidity: % (n)			
Past history of MDD	75 (24)	N/A	
Other anxiety disorder (current)	37.5 (12)	N/A	
• Agoraphobia	25 (8)	N/A	
• GAD	6.3 (2)	N/A	
• Panic Disorder	3.1 (1)	N/A	
• Multiple	3.1 (1)	N/A	
History of suicide attempt; % yes (n)	28.1 (9)	2.7 (1)	0.004**
Suicidal ideation in previous month; % yes (n)	21.9 (7)	0 (0)	0.003**
Psychotropic medication % (n)			
• No medication	34.4 (11)	100 (36)	
• Taking medication	65.6 (21)	0 (0)	
○ SSRI	46.9 (15)	N/A	
○ SNRI	9.4 (3)	N/A	
○ Other regular anxiolytic	21.9 (7)	N/A	
○ PRN Beta-blocker	12.5 (4)	N/A	
○ PRN Benzodiazepine	6.3 (2)	N/A	
Social anxiety scale scores			
LSAS, mean (SD)			
○ Fear Subscale	44.19 (12.46)	N/A	
○ Avoidance subscale	37.63 (13.6)		
○ Social interaction subscale	38.41 (13.6)		
○ Performance subscale	43.13 (14.16)		
○ Total	81.84 (25.42)		
FQ, mean (SD)	42 (14.97)	N/A	
FNE, mean (SD)	49.81 (8.65)	N/A	
Psychological scale scores			
• PSS, mean (SD)	22.29 (7.69)	9.41 (5.22)	<0.0005 *
• STAI-S, mean (SD)	46.73 (12.32)	26.0 (6.03)	<0.0005 *
• STAI-T, mean (SD)	57.45 (11.78)	29.48 (6.46)	<0.0005 *
• HADS-D, mean (SD)	6.69 (3.76)	1.15 (1.48)	<0.0005 *
• HADS-A, mean (SD)	10.30 (4.51)	3.09 (3.03)	<0.0005 *
• HADS-T, mean (SD)	16.72 (7.42)	4.12 (4.07)	<0.0005 *
Other Scales			
• AQ, mean (SD)	21.79 (7.08)	12.97 (6.30)	<0.0005 *
• TIPI; mean (SD)			
○ Openness	4.32 (1.13)	5.57 (1.25)	<0.0005 *
○ Agreeableness	4.9 (1.19)	5.24 (1.04)	0.23
○ Conscientiousness	4.6 (1.45)	5.71 (1.12)	0.003 *

○ Extraversion	2.92 (1.63)	5.24 (1.21)	<0.0005 *
○ Emotional Stability	2.87 (1.38)	5.61 (0.92)	<0.0005 *
• Davis IRI; mean (SD)			
○ Perspective taking	25.43 (5.06)	23.12 (6.20)	0.11
○ Fantasy	21.40 (5.79)	19.33 (5.66)	0.16
○ Empathic Concern	28.33 (4.86)	24.34 (4.99)	0.002 *
○ Personal Distress	24.14 (5.33)	11.82 (4.89)	<0.0005*
• Childhood trauma questionnaire			
○ Emotional Abuse	11.72 (5.58)	6.76 (2.02)	<0.0005*
○ Physical Abuse	7.23 (4.29)	5.94 (1.94)	0.40
○ Sexual Abuse	6.03 (3.52)	5.24 (1.39)	0.043*
○ Emotional Neglect	13.34 (5.78)	7.03 (2.62)	<0.0005*
○ Physical Neglect	7.29 (2.68)	5.44 (1.02)	0.001*
○ Total	43.96 (12.07)	30.03 (6.48)	<0.0005*

(AQ: Autism Quotient, BMI: Body Mass Index, CTQ: Childhood Trauma Questionnaire, GAD: Generalised Anxiety Disorder, HADS: Hospital Anxiety and Depression Scale; HADS-A: HADS-Anxiety, HADS-D: HADS-Depression, HADS-T: HADS-Total, IRI: Interpersonal Reactivity Index, LSAS: Liebowitz Social Anxiety Scale, PSS: Perceived Stress Scale, MDD: Major Depressive Disorder, SAD: Social Anxiety Disorder, SD: Standard Deviation, SNRI: Serotonin and Norepinephrine Reuptake Inhibitor, SSRI: Selective Serotonin Reuptake Inhibitor, STAI: State-Trait Anxiety Inventory, STAI-S: STAI-State, STAI-T: STAI-Trait)

2.5 Discussion

Demographic and psychological profiles of our SAD group are consistent with existing knowledge in relation to the phenotype of this common anxiety disorder. It is a condition which is, more often than not, associated with comorbid mood and anxiety disorders (Chartier, Walker and Stein, 2003), and this is reflected by the high rates of such comorbidities in our patient group. Even after adjusting for comorbid anxiety and mood disorders, SAD is associated with high rates of suicidal ideation (Sareen *et al.*, 2005), again consistent with our findings of significantly elevated levels of suicidal ideation, as well as previous suicide attempts.

In this study, we assessed the 'Big-Five' personality dimensions using a brief ten-item tool (Gosling, Rentfrow and Swann Jr, 2003). Consistent with other studies (Costache *et al.*, 2020; Kaplan *et al.*, 2015), we found marked case-control differences across all personality dimensions, with the exception of agreeableness. Participants with SAD predictably demonstrated significantly lower scores on emotional stability (reflecting high neuroticism) and on extraversion, along with lower scores on both openness and conscientiousness dimensions. Recently, researchers have attempted to elucidate subtypes of SAD based on variations in the 'Big Five' personality dimensions (Costache *et al.*, 2020). Patients with SAD typically exhibit low extraversion and high neuroticism with weaker associations seen in the other dimensions (Bienvenu *et al.*, 2001; Bienvenu *et al.*, 2004; Kotov *et al.*, 2010; Costache *et al.*, 2020). However, an atypical subtype of SAD characterised by a high novelty-seeking, risk-taking, impulsive pattern of behaviour has also been described (Kashdan and Hofmann,

2008; Kashdan *et al.*, 2009; Mörtberg *et al.*, 2014). It is becoming increasingly evident that significant personality differences exist in people with SAD, emphasizing the multidimensional nature of the disorder and the need for a variety of treatment strategies (Costache *et al.*, 2020).

Our patient group also demonstrated elevated AQ scores. The AQ is a 50-item, self-administered questionnaire which was developed to quantify autistic traits in individuals with normal intelligence. The total score reflects 5 domains; social skills, communication, imagination, attention to detail and attention switching. Over 80% of those with ASD have scores greater than 32 out of a total score of 50 while randomly selected controls score an average of 16.4 (Baron-Cohen *et al.*, 2001). In our study, SAD patients had significantly higher scores than controls with a mean score of 21.79, considered to be an intermediate score by those who developed the tool. This is consistent with a previous study which demonstrated that patients with SAD had AQ scores lying between those with ASD and the general population scores, reflecting the overlap in symptoms between the two disorders with regards to social interaction (Hoekstra *et al.*, 2008). SAD patients also had significantly higher scores on certain subscales of the IRI, a tool used for multidimensional assessment of empathy (Davis, 1983). This is somewhat counter-intuitive considering that one would generally associate autistic traits with reduced empathy (Song *et al.*, 2019). However, it is reflective of the fact that the AQ does not incorporate an empathy assessment and the higher AQ scores in SAD reflect differences in other domains including social skills and communication. It also emphasizes the distinct nature of SAD in comparison to ASD, although it is well accepted that there is some overlap in symptoms (Spain *et al.*, 2018; Spain, Zıvralı Yarar and Happé, 2020; Kleberg *et al.*, 2017). The IRI consists of four seven-item subscales each measuring a different component of empathy; Perspective Taking (PT) refers to the tendency to spontaneously adopt the psychological viewpoint of others; the Fantasy Scale (FS) assesses a person's ability to imaginatively transpose themselves into the feelings and behaviours of fictional characters when watching movies or reading books; the 'Empathic Concern' (EC) subscale evaluates the propensity to experience 'other-oriented' feelings of sympathy and compassion towards another while the 'Personal Distress' (PD) scale appraises 'self-oriented' feelings of distress and unease at the observed discomfort of another (Davis, 1983). The PD and EC subscales are thought to be representative of affective as opposed to cognitive empathy, and have previously been shown to be elevated in patients with SAD (Tibi-Elhanany and Shamay-Tsoory, 2011). However, these increased empathic abilities of patients with SAD appear to be selective and only evident on encountering negative emotions, with a converse finding of impaired positive affective empathy (Morrison *et al.*, 2016). The PT and FS scales measure cognitive empathy, the ability to accurately perceive and understand the emotions of another, which was equivalent between our two groups. This is in line with previous findings of normal theory of mind and cognitive

empathy in SAD (Pepper *et al.*, 2018), although interestingly SAD patients' self-rate their cognitive empathy poorly (Pepper *et al.*, 2019).

It is well recognised that exposure to childhood trauma and adversity increase the risk of both anxiety and depressive disorders, likely associated with complex neurobiological changes which predispose to later psychopathology (Heim and Nemeroff, 2001). With regards to SAD, it appears that a history of emotional abuse is more strongly related to the diagnosis than either physical or sexual abuse (Gibb, Chelminski and Zimmerman, 2007), findings which have been replicated (Kuo *et al.*, 2011). However, an increased frequency of childhood sexual abuse in patients has also been reported (Bandelow *et al.*, 2004). Our findings are again consistent with the literature. Our SAD patients had significantly higher total scores on the CTQ compared to controls. The strongest differences between the groups were seen in relation to emotional abuse and emotional neglect, although they also reported higher levels of sexual abuse and physical neglect.

Significant differences in lifestyle factors were seen in the SAD group, including reduced exercise and poorer sleep quality, along with elevated BMI levels. No study has specifically looked at lifestyle factors in SAD. A meta-analysis confirmed that there is a positive association between anxiety and obesity (Gariepy, Nitka and Schmitz, 2010). With respect to SAD specifically, overweight and obese people have high prevalence rates of social anxiety symptoms (Mirijello *et al.*, 2015; Ostrovsky *et al.*, 2013) and SAD (Barry, Pietrzak and Petry, 2008; Kalarchian *et al.*, 2007). Interestingly, the magnitude of the relationship between obesity, inflammation and metabolic function is 1.5 times stronger among those with more social anxiety symptoms, possibly reflecting the cumulative stressful effects of experiencing weight-related bias as an obese person who is also socially anxious (Jaremka and Pacanowski, 2019). Obese people who experience clinically-significant social anxiety specifically in relation to weight do not differ from those with more pervasive SAD in terms of severity, social and overall functional impairment and could benefit from treatment of their particular weight-related social anxiety (Dalrymple *et al.*, 2011). Elevated BMI levels are in keeping with reduced exercise in the SAD group. Physical exercise has been shown to be a protective factor in anxiety disorders (Zimmermann *et al.*, 2020). There is a reduced prevalence of anxiety disorders in those who engage in physical exercise (ten Have, de Graaf and Monshouwer, 2011), particularly if this exercise is regular (Stroehle *et al.*, 2007) or associated with sports participation (Hiles *et al.*, 2017). Although no studies on the impact of exercise specifically in SAD have been carried out, data from the National Comorbidity Study in the US showed that regular exercise was associated with a lower prevalence of social phobia (OR = 0.65 (0.53, 0.8)) which persisted after adjusting for differences in sociodemographic characteristics, self-reported physical disorders, and comorbid mental disorders (Goodwin, 2003). Physical exercise is also associated with a more favourable course and higher

recovery rate from anxiety disorders (ten Have, de Graaf and Monshouwer, 2011; Boschloo *et al.*, 2014) and appears to be viable potential treatment option for clinical anxiety conditions (Aylett, Small and Bower, 2018).

Our SAD patient group reported significantly poorer sleep quality in comparison to controls. The association between sleep and anxiety disorders is well documented (Mellman, 2006) and SAD, along with GAD in particular are strongly associated with poor sleep quality (Ramsawh *et al.*, 2009). Poor sleep quality can diminish the effects of cognitive behavioural therapy for SAD (Zalta *et al.*, 2013) highlighting the importance of addressing this aspect of the disorder early in treatment. Mindfulness-based stress reduction appears to be effective in improving sleep quality in these patients (Horenstein *et al.*, 2019).

There was no difference in smoking or alcohol consumption between our two groups. However, it is important to note that an exclusion criterion for the study was alcohol or substance abuse or dependence. There is a high rate of comorbid alcohol use disorder in SAD (Schneier *et al.*, 2010) which is not reflected in our study. SAD has previously been shown to be associated with higher rates of nicotine dependence (Sonntag *et al.*, 2000), one reason being that patients may smoke to help cope with anxiety symptoms during social situations (Watson *et al.*, 2012). However, overall prevalence rates of smoking in Ireland continue to drop yearly (Ireland, 2019), which may be reflected in the low rates of smoking in both patients and controls.

A limitation with respect to the demographic data is that we did not collect details on relationship status or co-habitation situation. Given the significant difficulties of SAD patients with respect to interpersonal interactions and relationships, this would have provided important information in relation to functional status. Additionally, such data about social relationships is of relevance to a variety of the biological markers discussed in later chapters, particularly gut microbiota composition (Dill-McFarland *et al.*, 2019) and oxytocin levels (Monin *et al.*, 2019; Schneiderman *et al.*, 2012).

Overall, our patient sample comprised subjects with a distinct clinical anxiety disorder, whose detailed demographic and clinical characteristics are consistent with existing literature outlining the SAD phenotype. Having a well-defined, representative clinical anxiety disorder sample is vital for studies on the MGB axis, particularly given that the vast majority of human studies investigating associations between the microbiome and anxiety have been in healthy volunteers with broad non-specific anxiety measures, thus limiting more accurate exploration of potential links between the gut microbiome and specific anxiety dimensions.

Chapter 3: The Gut Microbiome in Social Anxiety Disorder

3.1 Abstract

Background: The microbiota-gut-brain (MGB) axis plays a role in anxiety and the stress response, and is of increasing interest in neuropsychiatric conditions. The gut microbiome shows alterations in a variety of psychiatric disorders including depression, generalised anxiety disorder (GAD), autism spectrum disorder (ASD) and schizophrenia. The gut microbiome has not previously been investigated in social anxiety disorder (SAD).

Methods: Using whole-genome shotgun analysis of 49 faecal samples (31 cases and 18 sex- and age-matched controls), we analysed compositional and functional differences in the gut microbiome of patients with SAD in comparison to healthy controls.

Results: Overall microbiota composition, as measured by beta-diversity, was found to be different between the SAD and control groups. Several taxonomic differences were seen at a genus- and species-level. The relative abundance of genera, *Anaeromassillibacillus* and *Gordonibacter* were elevated in SAD, while *Parasuterella* was enriched in healthy controls. At a species-level, A*naeromassilibacillus sp An250* was found to be more abundant in SAD patients while *Parasutterella excrementihominis* was higher in controls. No differences were seen in alpha diversity. In relation to functional differences, the gut metabolic module, 'Aspartate Degradation I' was elevated in SAD patients.

Conclusions: The gut microbiome of patients with SAD differs in composition and function to that of healthy controls. Larger, longitudinal studies are warranted to validate our results and explore the clinical implications of these microbiota changes.

3.2 Introduction

Despite a growing interest in the role of the gut microbiome in the neurobiology of the stress response (Dinan and Cryan, 2012) and social behaviour (Sarkar *et al.*, 2020), the gut microbiota has not previously been investigated in patients with SAD. Indeed, apart from a few small studies in generalised anxiety disorder (GAD) (Jiang *et al.*, 2018; Chen *et al.*, 2019; Mason *et al.*, 2020), gut microbiome composition or function has remained unexplored in patients with clinical anxiety disorders, including SAD, panic disorder or agoraphobia. This is despite an abundance of preclinical studies demonstrating that anxiety-like behaviours are altered in animal models following a variety of microbiota manipulations (Cryan *et al.*, 2019). Here we report on compositional and functional differences in the gut microbiota of patients with SAD using whole-genome shotgun analysis of 49 faecal samples (31 cases and 18 controls). The functional differences, based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues, are explored using the recently described gut-brain module (GBM) (Valles-Colomer *et al.*, 2019) and gut metabolic module (GMM) (Vieira-Silva *et al.*, 2016) analysis.

3.3 Methods

Participants and Procedures:

Patients and healthy controls were recruited and assessed as outlined in Chapter 2.

To quantify nutrient intake, participants completed the self-administered 152-item SLAN-06 (Survey of Lifestyle, Attitudes and Nutrition in Ireland) food frequency questionnaire (FFQ) which is adapted from the EPIC Norfolk questionnaire (Riboli and Kaaks, 1997) and validated to be used in an Irish population (Harrington J, 2008). Participants were asked to estimate the frequency with which they consumed a specified portion size of each of the foods listed over the preceding year. The FFQs were analysed for nutrient intake using the FETA software (Mulligan *et al.*, 2014). Stool consistency was assessed using the Bristol Stool Chart (BSC) (Lewis and Heaton, 1997).

Biological/Faecal Samples

Freshly voided faecal samples were collected from study participants into plastic containers containing an AnaeroGen sachet (Oxoid AGS AnaeroGen Compact, Fischer Scientific, Dublin) to generate anaerobic conditions within the container. Participants were instructed to collect the faecal sample at home as close to the study visit as possible, ideally on the morning of the study visit and not more than 12 hours before the study visit. They were asked to keep the sample containers in a

refrigerator at 4°C until delivery to the study site. A cool pack was used to transport the sample to the study site, where it was immediately stored at − 80° for later analysis.

Microbiome Sample Preparation and Whole Genome Shotgun Sequencing

Total bacterial metagenomics DNA was extracted using the QIAmp Fast DNA Stool Mini kit (Qiagen, UK) with a modified protocol combined with repeated bead beating method (Zhongtang Yu & Mark Morrison 2018). Briefly, 1 ml of lysis buffer (500mM NaCl, 50mM Tris-HCl pH8.0, 50mM EDTA and 4% sodium dodecyl sulphate) was added to the stool sample in the bead beating tube. The samples were homogenized using a mini beadbeater (BioSpec) and incubated at 70°C for 15 minutes (for cell lysis) followed by centrifugation at 4°C. The supernatant was removed and the bead beating step was repeated. Ammonium acetate (Sigma Aldrich, Ireland) was added to the pooled supernatant and incubated on ice. Following a centrifugation step the supernatant was transferred to Eppendorf tubes containing iso-propanol. The following day, DNA was pelleted and washed with 70% ethanol and dissolved in Tris-EDTA. The DNA was then RNAse and proteinase-K treated and purified according to the manufacturer's instructions (QIAmp Fast DNA Stool Mini kit; Qiagen, UK). The DNA was quantified using Qubit and stored at -30°C.

Whole genome shotgun sequencing was performed using Nextera XT kit. Library prep was done following the Nextera XT DNA Library Preparation Guide from Illumina. Quality of the library was evaluated using the Agilent High Sensitivity DNA chip and running it on the Bioanalyzer and the DNA was quantified using Qubit DNA High sensitivity kit read on a qubit fluorometer 3.0. The samples were pooled and sequencing was carried out on the NextSeq500 using a 300 cycle High Output v2 kit.

Taxanomic and Functional Analysis

We performed quality checks on raw sequences from all faecal samples using FastQC (Andrews, 2010). Shotgun metagenomic sequencing data were then processed through analysis workflow that utilizes Huttenhower Biobakery pipeline (McIver *et al.*, 2018), including Kneaddata (Huttenhower Lab, [cited 2017 Dec 19]), MetaPhlAn3 (Truong *et al.*, 2015) and HUMAnN3 (Franzosa *et al.*, 2018) to obtain species, genes and pathways abundance matrix. Briefly, quality filtering and host genome decontamination (human) was performed using Trimmomatic (Bolger, Lohse and Usadel, 2014) and Bowtie2 (Langmead and Salzberg, 2012) via Kneaddata wrapper program with following parameters: ILLUMINACLIP:/NexteraPE-PE.fa:2:30:10, SLIDINGWINDOW:5:25, MINLEN:60, LEADING:3, TRAILING:3. Taxonomic and functional profiling of the microbial community was performed using MetaPhlan3 and HUMAnN3 using default parameter. Next, gene abundance matrix was further

collapsed by KEGG Orthology (KO) term and Gene Ontology (GO) term mapping via "humann_regroup_table" function provided within HUMANn3.

Further data-handling was undertaken in R (version 4.03) using the Rstudio GUI (version 1.4.1103). In all microbiome analysis with the exception of alpha diversity, taxa with a prevalence of < 5% of samples at the genus level were excluded from analysis as ratios are invariant to sub-setting and this study employs compositional data analysis techniques (Gloor *et al.*, 2017; Aitchison, 1982). Principal component analysis was performed on centred log-ratio transformed (clr) values using the ALDEx2 library (Fernandes *et al.*, 2014). The number of permutations was always set to 1000. Beta diversity was computed in terms of Aitchison distance, or Euclidean distance between clr-transformed data. Alpha diversity was computed using the iNEXT library (Hsieh, Ma and Chao, 2016). KEGG orthologues were used as features to compute functional alpha diversity. GBMs and GMMs were calculated from HUMANn3 output using the R version of the Gomixer tool (Valles-Colomer *et al.*, 2019). Stacked barplots were generated by normalising counts to 1, generating proportions. Genera that were never detected at a 10% relative abundance or higher were aggregated and defined as rare taxa for the purposes of the stacked barplots. Differential abundance of both microbes and functional modules were calculated using implementations of the ALDEx2 library. Effect sizes were also computed using the method described in the ALDEx2 manual, as the median of the ratio of the between group difference and the larger of the variance within groups. A p-value of <0.05 was deemed significant in all cases. To correct for multiple testing in tests involving microbiome features, the Benjamini-Hochberg (BH) post-hoc was performed with a q-value of 0.1 used as a cut-off for species and 0.2 for functional modules. Plotting was done using the *ggplot2* (Wickham, 2011)and *patchwork* (Pedersen, 2019) libraries in R. Custom R scripts and functions are available online at https://github.com/thomazbastiaanssen/Tjazi). A linear modelling approach was used to test for a group effect on taxonomic and functional differences, whilst adjusting for covariates including age, sex, BMI, exercise and diet. To assess associations between microbial features and biological markers/psychological scales within the SAD group, we likewise used a linear modelling approach.

Statistical Analysis of Metadata

All metadata were analysed using SPSS 25 (IBM, Armonk, NY, USA). Visual inspection of box plots was used to identify outliers and consideration was given to removal of those lying more than three times the interquartile range (IQR) below the first quartile or above the third quartile. Missing values were excluded from analysis. Normality of data was assessed by visual inspection of histograms along with examination of skewness and the Shapiro-Wilk statistic. Differences in demographic data, food frequency questionnaires (FFQ) and Liebowitz social anxiety symptom scale (LSAS) scores

between the SAD and control group were assessed using Chi-squared or Fisher's Exact test for categorical variables, and independent t-tests or non-parametric Mann-Whitney U tests for continuous variables. Data are presented as mean ± SD unless stated otherwise.

3.4 Results

Demographics

Thirty-one patients with SAD and eighteen healthy controls were included in the study. There were no significant differences between patients and controls in relation to age, sex, race, years of education, birth delivery mode, alcohol consumption, smoking status, or stool consistency (Table 1). Individuals in the SAD group had higher BMI scores compared to controls ($t(46)=2.65$, $p=0.01$). SAD patients had significantly lower exercise levels than controls, based on mean IPAQ scores ($t(45)=-2.125$, $p=0.04$), the difference, when looking at exercise categories, being at the level of a trend ($X^2(2)=5.822$, $p=0.054$). Almost three-quarters (74.2%) of SAD patients had a past history of MDD and 35.5% had a comorbid secondary anxiety disorder. Just over two-thirds (67.7%) of patients were taking psychotropic medication. The majority of these patients were prescribed an SSRI (48.4%) (8 taking Escitalopram, 2 taking Vortioxetine, 2 taking Sertraline, 1 taking Citalopram, 1 taking Paroxetine, 1 taking Fluoxetine) or SNRI (9.7%) (3 taking Venlafaxine) with 22.6% (n=7) taking an alternative regular psychotropic medication (1 patient taking Pregabalin, 1 taking Agomelatine, 1 taking Bupropion, 2 taking Trazadone and 2 taking low-dose (50mg) Quetiapine).

Table 1: Demographic Characteristics and Psychological Scales

	SAD (n=31)	**Controls (n=18)**	**p-value**
Age (years); mean (SD)	36.0 (11.96)	41.7 (10.79)	0.10
Gender; % female (n)	48.4 (15)	66.7 (12)	0.25
Race; % Caucasian (n)	100 (31)	94.4 (17)	0.37
Years of Education; mean (SD)	17.32 (4.32)	19.25 (4.89)	0.16
Delivery mode at birth • Vaginal • Caesarean section • Unknown	 74.2 (23) 6.5 (2) 19.3 (6)	 83.3 (15) 5.6 (1) 11.1 (2)	 0.74
BMI (kg/m^2); mean (SD)	28.02 (5.0)	24.22 (4.49)	0.01 *
Alcohol (units per week); mean (SD)	5.52 (7.4)	2.63 (3.1)	0.78
Alcohol categories; % (n) • 0-3 units/week • 4-9 units/week • $\geq$ 10 units/week	 58.1 (18) 16.1 (5) 25.8 (8)	 72.2 (13) 22.2 (4) 5.6 (1)	 0.21
Smoking status; % smokers (n)	12.9 (4)	5.6 (1)	0.64
Exercise (IPAQ score); mean (SD)	3143.89 (3769.34)	5816.17 (4650.98)	0.04 *
Exercise (IPAQ category); % (n) • Low • Moderate • High	 25.8 (8) 35.5 (11) 38.7 (12)	 5.6 (1) 16.7 (3) 66.7 (12)	 0.054

Bristol Stool Chart; % (n) • Score 1 • Score 2 • Score 3 • Score 4 • Score 5 • Score 6	 0 (0) 22.6 (7) 32.3 (10) 29.0 (9) 6.4 (2) 9.7 (3)	 0 (0) 11.1 (2) 38.9 (7) 38.9 (7) 11.1 (2) 0 (0)	 0.50
Comorbidity: % (n)			
Past history of MDD	74.2 (23)	0 (0)	<0.0005 *
Other anxiety disorder (total) • Agoraphobia • GAD • Panic Disorder • Multiple	35.5 (11) 22.6 (7) 6.5 (2) 3.2 (1) 3.2 (1)	0 (0)	<0.0005 *

Psychotropic medication % (n)
• No medication
• Taking medication
  ○ SSRI
  ○ SNRI
  ○ Other regular anxiolytic
  ○ As required Beta-blocker
  ○ As required Benzodiazepine
	
	32.3 (10)
	67.7 (21)
	48.4 (15)
	9.7 (3)
	22.6 (7)
	12.9 (4)
	6.4 (2)
		
		100 (18)
		0 (0)
			
			<0.0005 *

Social anxiety scale scores			
LSAS; mean (SD) • Fear Subscale • Avoidance subscale • Social interaction subscale • Performance subscale • Total	 44.94 (11.91) 38.16 (13.47) 39.13 (13.19) 43.68 (14.04) 83.13 (24.77)	 10.67 (10.31) 8.33 (10.54) 9.2 (10.38) 9.67 (9.71) 19 (19.61)	 <0.0005 * <0.0005 * <0.0005 * <0.0005 * <0.0005 *

(BMI: Body Mass Index, GAD: Generalised Anxiety Disorder, IPAQ: International Physical Activity Questionnaire, LSAS: Liebowitz Social Anxiety Scale, MDD: Major Depressive Disorder, PSQI: Pittsburgh Sleep Quality Index, SAD: Social Anxiety Disorder, SD: Standard Deviation, SNRI: Serotonin and Norepinephrine Reuptake Inhibitor, SSRI: Selective Serotonin Reuptake Inhibitor)

Dietary intake

Based on FFQ analysis, the main difference in nutrient intake seen between the SAD patients and controls was in relation to carbohydrates (Table 2). SAD patients had greater intake of total carbohydrates (U=154, z=-1.983, p=0.047), which appeared to be driven by higher total sugar intake (U=140, z=-2.31, p=0.021) as other carbohydrate groups, starch and fibre, were equivalent in patients and controls. No other differences in nutrient groups, vitamins or minerals were seen between patients and controls.

Table 2: Dietary intake (median ± IQR) obtained from analysis of food frequency questionnaires.

Nutrient	Recommended daily intake*	Control	SAD	p-value
Kilocalories	2,000 – 2,400 (males; depending on activity level)	1493 (1011)	2255 (1771)	0.12
Protein (g)	10-35% of total energy	69 (41) (18.5%)	90 (58) (16%)	0.17
Fat (g)	20-35% of total calories	60 (35) (36%)	67 (78) (27%)	0.66
Carbohydrate (g)	45-65% of total calories	171 (123) (46%)	258 (276) (46%)	0.047 *
Monounsaturated fatty acids (g)	>12% of total energy	25 (14) (15%)	26 (28) (10%)	0.45
Polyunsaturated fatty acids (g)	>6% of total energy	12 (11) (7%)	13 (15) (5%)	0.91
Saturated fatty acids (g)	<10% of total energy	20 (15) (12%)	21 (27) (8%)	0.52
Cholesterol (mg)	300 mg	225 (170)	251 (153)	0.58
Total sugar (g)	<10% of total energy	80 (70) (21%)	118 (87) (21%)	0.02 *
Starch (g)		107 (79)	159 (171)	0.08
Fibre (g)	>25g	16 (20)	21 (21)	0.60
Vitamin A (µg)	800 µg	291 (686)	189 (245)	0.48
Thiamine (mg)	1.1 mg	1.6 (1.1)	2 (1.8)	0.12
Riboflavin (mg)	1.4 mg	1.3 (1)	1.6 (2.8)	0.17
Niacin (mg)	16 mg	22.9 (15.7)	31.5 (25.8)	0.10
Vitamin B6 (mg)	1.4 mg	2.3 (1.3)	2.9 (3)	0.17
Vitamin B12 (µg)	2.5 µg	5.5 (4.7)	5.2 (5.3)	0.60
Folate (µg)	200 µg	277 (266)	350 (445)	0.60
Vitamin C (mg)	80 mg	90 (125)	120 (121)	0.61
Vitamin D (µg)	5 µg	2.7 (3.4)	2.8 (4.2)	0.41
Vitamin E (mg)	12 mg	9.8 (9.7)	12.8 (13.3)	0.60
Phosphorous (mg)	700 mg	1020 (681)	1310 (1402)	0.38
Calcium (mg)	1000 mg	464 (319)	547 (438)	0.78
Iron (mg)	7 mg	12 (8)	14 (20)	0.48
Selenium (µg)	55 µg	53 (38)	66 (55)	0.76

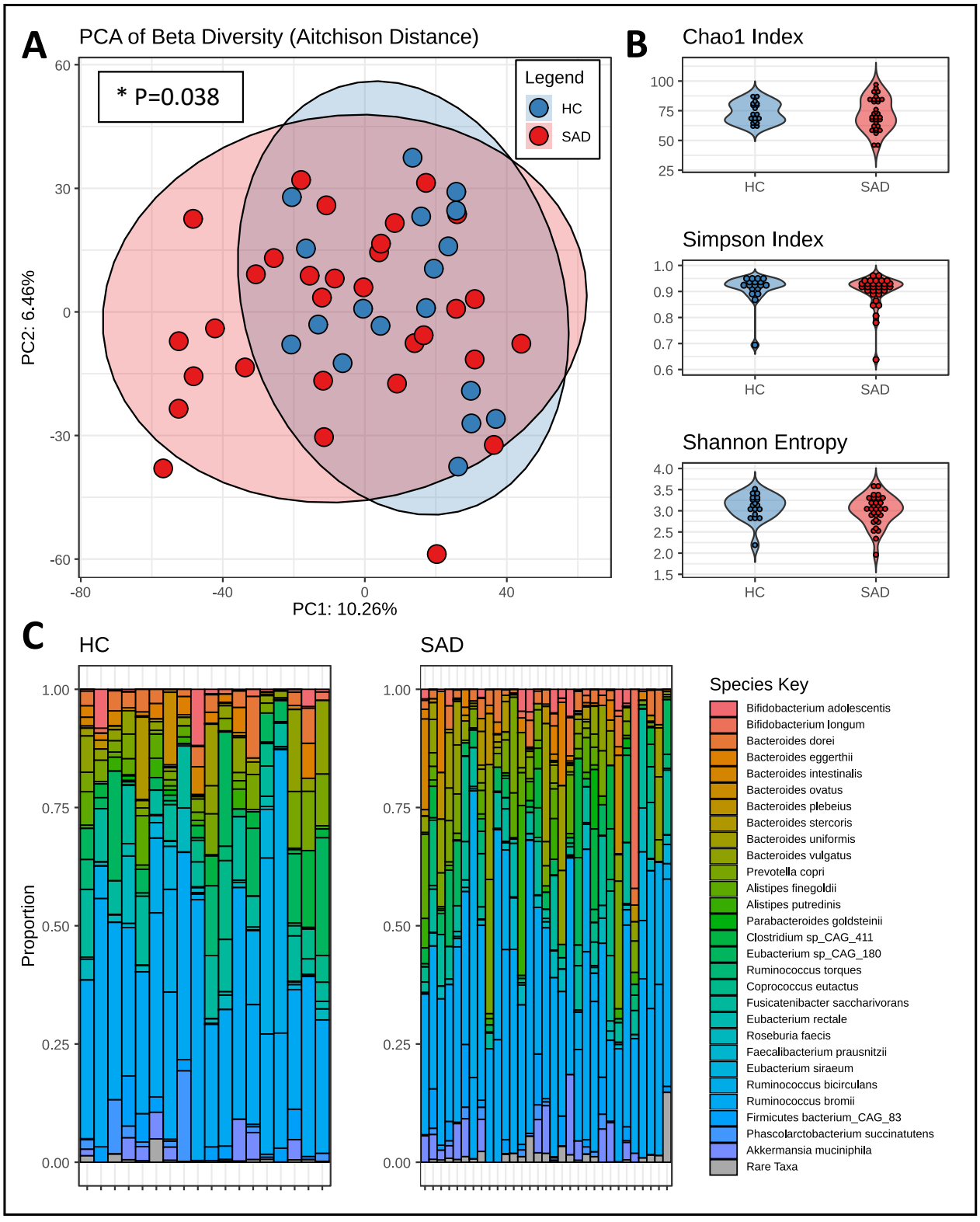
Zinc (mg)	10 mg	7.8 (4.5)	10.4 (8)	0.32
Sodium (mg)	1600 mg	2599 (1597)	2877 (2293)	0.33
Potassium (mg)	2000 mg	3043 (1742)	3422 (3731)	0.61
Magnesium (mg)	375 mg	302 (190)	321 (296)	0.50
Copper (mg)	1 mg	1.1 (0.9)	1.3 (1.4)	0.52
Chloride (mg)	800 mg	3713 (2413)	4338 (3609)	0.29
Manganese (mg)	2 mg	3.6 (2.5)	3.2 (3.1)	0.68
Iodine (µg)	15 µg	101 (59)	117 (79)	0.70

Compositional differences in the gut microbiota of SAD patients

The gut microbiota of patients with SAD differed from those of healthy controls in terms of overall composition as well as in relation to specific genus- and species-level differentially abundant features. Beta diversity was found to be different between the two groups as measured by PERMANOVA ($p = 0.038$, $R^2 = 0.028$) using the compositionally appropriate Aitchison distance metric (Figure 1A). No differences were found between groups in alpha diversity, based on the Chao1, Shannon or Simpson indices (Figure 1B).

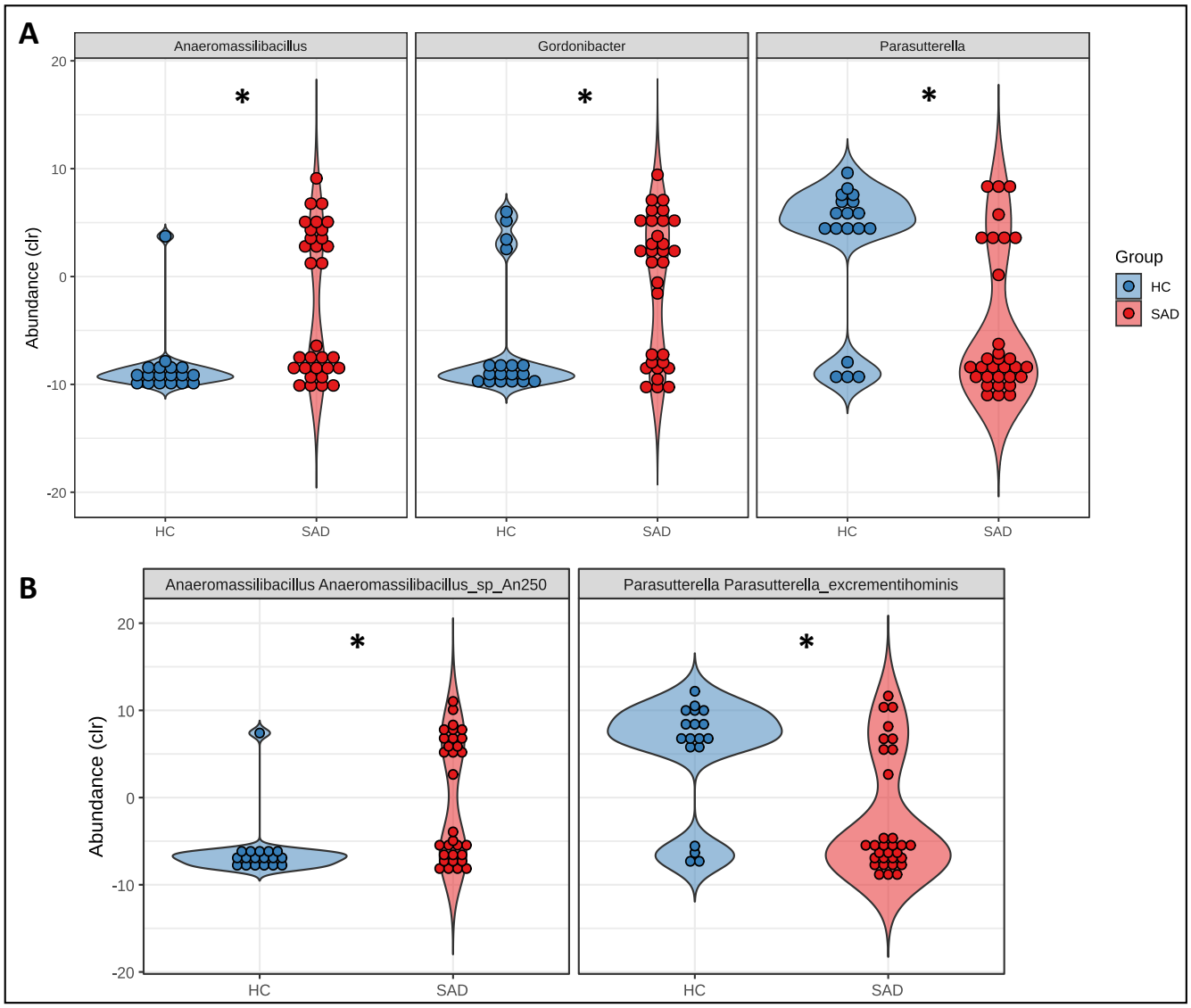
A total of 73 genera and 159 species were identified (Figure 1C). Of these, three genera and two species were found to show significant differences in relative abundance after false discovery rate (FDR) correction using the Benjamini-Hochberg procedure. At a genus level, A*naeromassilibacillus* and *Gordonibacter* were enriched in SAD while *Parasutterella* was more abundant in the control samples (Figure 2A). At a species level, A*naeromassilibacillus sp An250* was found to be more abundant in SAD patients (padj = 0.024, effect size = -1.036). Specifically, this species was present in 48.4% (15/31) of the SAD samples but only found in 5.6% (1/18) of the control samples. Conversely, the species, *Parasutterella excrementihominis* was found to be enriched in healthy controls (padj = 0.042, effect size = 1.120) (Figure 2B). After adjusting for age, sex, BMI, exercise and dietary differences (total carbohydrates), these genus- and species-level relative abundance differences remained significant. We found no differences in the relative abundance of any taxa between unmedicated SAD patients and those taking psychotropic medication (Supplementary data 1).

Figure 1: 1A: Beta diversity between SAD and healthy control groups, as measured by Aitchison Distance. **1B:** Alpha-diversity between SAD and healthy controls, as measured by Chao1, Simpson and Shannon indices. **1C:** Relative abundance of species-level taxa for each participant. Each column represents one participant. Genera that were never detected at a 10% relative abundance or higher are aggregated and defined as rare taxa for the purposes of the stacked barplots.



(HC: Healthy Control, SAD: Social Anxiety Disorder)

Figure 2: 2A: Genus-level differences in relative abundance between SAD and controls seen in three genera; *Anaeromassillibacillus* and *Gordonibacter* are enriched in SAD while *Parasutterella* is enriched in healthy controls. **2B:** Species-level differences in relative abundance between SAD and controls; *Anaeromassilibacillus sp An250* is increased in SAD while *Parasuterella excrementihominis* is enriched in healthy controls.



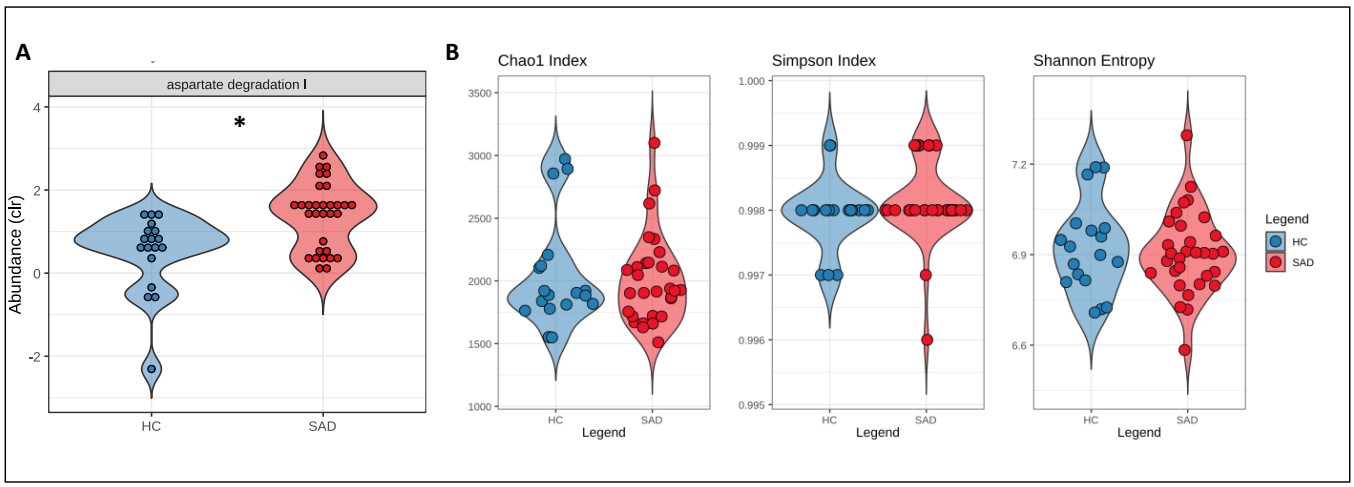
(Clr: centred log-ratio transformed, HC: Healthy Control, SAD: Social Anxiety Disorder)

Functional differences in the gut microbiota of SAD patients

No differences were found in functional diversity between the two groups (Figure 3B). We identified 69 of the 103 GMMs curated in the current database (Vieira-Silva *et al.*, 2016) and 26 of the 56 GBMs characterised by Valles-Colomer (Valles-Colomer *et al.*, 2019). No GBMs reached our threshold for significance after FDR. However, one GMM, Aspartate Degradation I, describing the capacity of the microbiome to degrade aspartate by the enzyme aspartate aminotransferase (AspAT), was found to be significantly more abundant in patients with SAD (padj = 0.150, effect size

= -1.032) (Figure 3A). This functional difference remained between the two groups after adjusting for age, sex, BMI, exercise and dietary differences (total carbohydrates).

Figure 3: Functional differences between SAD and controls. **3A** One gut metabolic module, Aspartate Degradation I, was found to be increased in SAD patients. **3B:** Functional diversity, between SAD and healthy controls, as measured by Chao1, Simpson and Shannon indices. No differences between the groups.



(Clr: centred log-ratio transformed, HC: Healthy Control, SAD: Social Anxiety Disorder)

We then set out to investigate whether any microbial taxa or functional modules were associated with any of the other measured characteristics of the SAD patients including immune, kynurenine, neuroendocrine and intestinal permeability markers as well as LSAS scores. After controlling the FDR, we did not detect any such associations (data not shown).

3.5 Discussion

This study demonstrates, for the first time, that the gut microbiome is compositionally and functionally altered in people with SAD compared with healthy controls. Firstly, beta diversity, an indicator of overall microbiome composition, was significantly different between the two groups. The relative abundance of three genera, A*naeromassilibacillus, Gordonibacter* and *Parasutterella*, and two corresponding species, A*naeromassilibacillus sp An250* and *Parasutterella excrementihominis* differed significantly between SAD patients and controls. Additionally, functional differences were evident with the microbiome of SAD patients enriched for the gut metabolic pathway, aspartate degradation I.

Strikingly, we found *Anaeromassilibacillus sp An250* to be present in almost half of our SAD group but in only one healthy control. *Anaeromassilibacillus* is a recently-discovered genus which was first isolated in 2017 from the faecal sample of a 1-yo Senegalese patient with kwashiorkor (Guilhot *et al.*, 2017). Several strains of *Anaeromassilibacillus,* including *sp An250* have since been identified from the caecal microbiota of chickens (Medvecky *et al.*, 2018; Glendinning *et al.*, 2020). *Anaeromassilibacillus* is a member of the Clostridiales order and Clostridiaceae family of bacteria (Guilhot *et al.*, 2017), taxonomic groups which are increased in the gut microbiota of patients with ASD (Ho *et al.*, 2020a), depression (Barandouzi *et al.*, 2020) and schizophrenia (Zhu *et al.*, 2020). Interestingly, significant shifts in the abundance of Clostridiales taxa appears to be common to many psychiatric disorders and may represent disease-shared microbial responses (Li *et al.*, 2020). Given the very recent addition of *Anaeromassilibacillus* to human microbiome databases, there is little in the existing literature about its role in human health and disease. It was one of several genera found to be enriched in untreated patients with MDD compared to those receiving antidepressant treatment, suggesting that it could be altered by psychotropic medication or be an indicator of treatment response (Fontana *et al.*, 2020). Additionally, the relative abundance of *Anaeromassilibacillus* reduced significantly in the faeces of children with ASD after guar gum prebiotic supplementation, which was associated with reduced irritability and improved constipation (Inoue *et al.*, 2019), again suggesting that reduction of this genus may be associated with improved psychopathology. Thus, although the literature is sparse, *Anaeromassilibacillus* appears be of relevance in ASD and depression, psychiatric conditions which are highly comorbid with SAD (Maddox and White, 2015; Chartier, Walker and Stein, 2003) and which may involve shared pathophysiological processes. Of note, we did not see a difference in the relative abundance of *Anaeromassilibacillus* in medicated, compared to, unmedicated SAD patients although given the small sample size, this should be interpreted with caution.

Gordonibacter is another genus about which relatively little is known. It is a member of the Eggerthellaceae family and Coriobacteriia class (Würdemann *et al.*, 2009) and is notable in its ability to produce urolithins from the metabolism of polyphenols (Selma *et al.*, 2014), which may offer mental health benefits (Westfall and Pasinetti, 2019). *Parasutterella* has been more extensively studied. It is a member of the Sutterellaceae family and in humans, is largely represented by a single species, *Parasutterella excrementihominis* (Morotomi and M, 2014). Similar to our finding of lower *Parasutterella* levels in SAD, this genus has also been found to be reduced in ASD (Ding *et al.*, 2020). Weight and dietary factors appear to be important influences. *Parasutterella* is negatively associated with BMI and waist circumference (Zeng *et al.*, 2019) and conversely, can be induced by high sugar (Noble *et al.*, 2017) and high-fat diets (Kreutzer *et al.*, 2017). Our SAD group had elevated sugar

intake and did not differ in terms of fat intake but, although the group difference for *Parasutterella excrementihominis* remained after adjusting for BMI, increased BMI in the SAD group could contribute to its reduced abundance in SAD patients.

Using GBMs and GMMs, which are microbiome-related functional pathways that have been manually curated from existing literature (Valles-Colomer *et al.*, 2019; Vieira-Silva *et al.*, 2016), we identified one functional pathway that was enriched in the SAD group – aspartate degradation I. According to Metcyc, a comprehensive reference database of metabolic pathways and enzymes (Caspi *et al.*, 2020), this pathway involves the conversion of the amino acid, L-aspartate to the corresponding keto acid, oxaloacetate, by the enzyme, aspartate aminotransferase (AspAT). Several bacteria and archaea have demonstrated this enzymatic ability including *Haloferax mediterranei* (Muriana, Alvarez-Ossorio and Relimpio, 1991), *Pseudoalteromonas translucida TAC125* (Birolo *et al.*, 2000), *Saccharolobus solfataricus* (Marino *et al.*, 1988) and *Escherichia coli* (Fotheringham *et al.*, 1986; Powell and Morrison, 1978). Interestingly, bacterial AspAT enzyme activity may represent a link between gut microbiome function and the tryptophan-kynurenine pathway, a key physiological system in psychiatric disorders. Tryptophan metabolism involves the downstream conversion of kynurenine to kynurenic acid (KYNA) by the enzyme kynurenine aminotransferase (KAT). KYNA is an important neuroactive substance which is elevated by chronic stress in animal models (Fuertig *et al.*, 2016; Kiank *et al.*, 2010) as well as in psychiatric conditions such as schizophrenia (Plitman *et al.*, 2017; Chiappelli *et al.*, 2014), and as we have shown, SAD. Notably, KAT activity has been detected in *E. coli* cells in vitro, and authors suggested that the source of KYNA in the rat small intestine could be the gut bacteria (Kuc *et al.*, 2008). Interestingly, this bacterial KAT enzyme protein has been identified as being identical to the bacterial AspAT enzyme (Han, Fang and Li, 2001) and thus, an elevation in the 'aspartate degradation I' functional pathway may represent increased KAT, as well as AspAT potential, by the microbiome. While currently a hypothetical supposition, it is possible that the elevated peripheral KYNA we observed in SAD patients may be linked with the key functional difference seen in the microbiome of this group. In support of this hypothesis is the fact that D-cycloserine, an orally-administered, broad-spectrum antibiotic, has been found to enhance the treatment response to exposure therapy for SAD (Hofmann *et al.*, 2006; Guastella *et al.*, 2008), an effect which could plausibly be related to its ability to inhibit KAT activity and lower KYNA (Baran and Kepplinger, 2014).

This is the first study to investigate the gut microbiome in patients with SAD and has several notable strengths. Firstly, our sample consisted of carefully selected patients with a pre-existing clinical diagnosis of SAD who had sought treatment from a mental health professional. Secondly, we used a whole genome shotgun sequencing method, providing information on the functional capacity of the

microbiome, as well as offering greater resolution of bacterial species identification, than the more commonly used 16S rRNA gene sequencing (Matias Rodrigues *et al.*, 2017; Sunagawa *et al.*, 2013). Thirdly, we took into account many of the important host variables known to confound gut microbiota studies in human disease. Stool quality is a particularly strong source of gut microbiota variance (Vujkovic-Cvijin *et al.*, 2020; Falony *et al.*, 2016) which has often been neglected in psychiatric microbiome studies. Stool consistency, as measured by the BSC, was matched between our groups, as were other important variables including age, sex, birth delivery mode, smoking status and alcohol. Our groups were not matched in terms of BMI and exercise levels, variables which may be of relevance to the gut microbiota (John and Mullin, 2016; Mailing *et al.*, 2019) Although adjusting these variables did not affect group differences, it would, of course, be preferable to have samples with equivalent BMI and exercise scores. Additionally, we collected detailed dietary information which has often been lacking in studies of the microbiome in psychiatric conditions. Our groups were well-matched in terms of overall dietary intake. The only difference seen was in relation to carbohydrate consumption, driven by higher sugar intake in the SAD group, and this was adjusted for in our statistical analyses.

The main study limitations were the small sample size and the single-time point nature of the study, which limits the establishment of any causal relationships. Additionally, two thirds of our SAD patients were taking psychotropic medication, which may have had an impact on microbiota composition (Cussotto *et al.*, 2019). The majority of our medicated patients were taking an SSRI antidepressant, escitalopram being the most commonly prescribed. Escitalopram has antibacterial activity against some gut commensal strains in-vitro (Ait Chait *et al.*, 2020; Cussotto *et al.*, 2018) although this effect did not translate to an in-vivo animal model (Cussotto *et al.*, 2018). Other prescribed SSRIs in our patient group included fluoxetine, citalopram, sertraline, paroxetine and vortioxetine, all of which have shown varying levels of antibacterial activity in-vitro (Bohnert *et al.*, 2011; Cussotto *et al.*, 2018; Ayaz *et al.*, 2015; Younis *et al.*, 2017), with in-vivo evidence available for fluoxetine (Lyte, Daniels and Schmitz-Esser, 2019; Lukić *et al.*, 2019; Ramsteijn *et al.*, 2020). The SNRI venlafaxine, conversely, does not appear to impact common gut commensals in-vitro (Ait Chait *et al.*, 2020; Cussotto *et al.*, 2018), although an influence on microbial richness and on the abundance of certain genera were seen in a mouse model (Lukić *et al.*, 2019). Thus, the translatability of studies using isolated in-vitro strains to animal models is unclear, with even more uncertainty in relation to their applicability to the human gut microbiota. Limited human data in relation to the effect of antidepressants is available. A small study of 17 depressed patients commenced on escitalopram, found no significant differences in beta-diversity or in any taxa levels between pre-treatment and 6-week post-treatment time-points, although increased alpha diversity was evident (Liśkiewicz *et al.*,

2019). Furthermore, a longitudinal study of 40 patients with depression and/or anxiety revealed no difference in beta diversity between those taking, and not taking, antidepressant medications and no change in alpha diversity in antidepressant-treated patients between baseline and endpoint timepoints. Antipsychotic medications, conversely, did appear to exert an effect on the gut microbiota (Tomizawa *et al.*, 2021), consistent with previous findings (Flowers *et al.*, 2017). Two of our patients were prescribed low-dose Quetiapine, a second-generation antipsychotic which thus may have had an impact. All in all, although there is clear evidence that many antidepressants have antibacterial effects, this evidence is based primarily on in-vitro and animal studies, and the impact of these medications on the human gut microbiota structure and function remain largely unknown. Although we did not find any differences in the relative abundance of any taxa between medicated and unmedicated patients, we cannot rule out a potential influence.

Finally, some of our SAD patient group had a past history of depression and/or a comorbid anxiety disorder. However, patients with a current depressive episode were excluded and in all, the primary diagnosis was SAD with any comorbid anxiety disorder representing a secondary diagnosis. This was a clinically representative sample and we believe that, including such patients makes our findings more generalizable given the significant overlap between depression and anxiety disorders. Given the paucity of studies exploring the gut microbiome in any clinical anxiety disorders, our findings, despite the limitations, are important in generating a foundational base for larger, prospective and interventional microbiome studies in these highly prevalent and disabling psychiatric conditions. Similar early compositional microbiome studies in depression (Naseribafrouei *et al.*, 2014; Kelly *et al.*, 2016) and ASD (Song, Liu and Finegold, 2004; Parracho *et al.*, 2005) have paved the way for a variety of successful interventional studies utilising probiotics (Liu, Walsh and Sheehan, 2019), dietary change (Jacka *et al.*, 2017) and FMT (Kang *et al.*, 2017; Kang *et al.*, 2019) in these conditions. It is hopeful that microbiome-based therapeutic interventions may also be realised for patients with clinical anxiety disorders.

3.6 Conclusion

The gut microbiome of patients with SAD differs in composition and function to that of healthy controls, raising the possibility that the MGB axis may represent a biomarker reservoir and potential therapeutic target for this early-onset, chronic disorder. However, larger, longitudinal studies are needed to validate our results, understand the clinical implications (if any) and investigate the impact of psychotropic medication and treatment on the gut microbiome in SAD.

Chapter 4: Intestinal Permeability in Social Anxiety Disorder

4.1 Abstract

Background: There is growing interest in the role of gut barrier function in stress-related psychiatric conditions. Intestinal hyperpermeability has been reported in several psychiatric disorders including depression, autism spectrum disorder (ASD) and schizophrenia. Gut barrier function has not previously been investigated in social anxiety disorder (SAD) or indeed in any clinical anxiety disorder.

Methods: In a cohort of 32 patients with SAD and 36 age- and sex-matched healthy controls, we investigated gut barrier function using a panel of gut permeability blood markers including sCD14, lipopolysaccharide-binding-protein (LBP), intestinal fatty acid binding protein (I-FABP), anti-endotoxin IgA, IgG and IgM antibodies, C-reactive protein (CRP)and inducible protein (IP)-10.

Results: SAD patients had elevated sCD14, LBP and IgA levels compared to controls. Zonulin and CRP levels were also elevated in the patient group, although the group differences were lost after controlling for BMI levels, which were higher in the patient group. No differences were seen in I-FABP, anti-endotoxin IgG and IgM and IP-10.

Conclusions: Increased gut permeability is seen in patients with SAD. Larger, prospective studies are warranted to further investigate the role of compromised gut barrier function in clinical anxiety disorders.

4.2 Introduction

The role of the gut-brain axis in the pathogenesis of psychiatric disorders is of major interest in recent years. Specifically, increased gut barrier permeability, leading to translocation of bacteria and antigenic bacterial components, into the bloodstream and a resultant low-grade inflammatory response, could underlie some neuropsychiatric disorders. The intestinal barrier is a tightly-controlled, multi-layer system which regulates the absorption of nutrients while restricting the transport of toxic substances and bacteria to the bloodstream and beyond (Ghosh *et al.*, 2020; Klumbies *et al.*, 2014). Gut barrier integrity can be compromised by many factors including psychological stress (Vanuytsel *et al.*, 2014), smoking (Zuo *et al.*, 2014), alcohol (Wang *et al.*, 2014), poor diet, obesity and metabolic dysfunction (Leech *et al.*, 2019), factors which are all of relevance in psychiatric populations.

There are several methods to assess gut barrier function including oral administration of sugar probes, such as lactulose or mannitol, which can be measured in the urine after crossing the intestinal epithelium via paracellular transport, providing an indirect measure of intestinal permeability (Rao *et al.*, 2011). Blood biomarkers, which offer a more convenient method of gut permeability assessment, are increasingly being used (Keane *et al.*, 2021). Circulating markers of intestinal epithelial damage, and thus gut hyperpermeability, include intestinal-fatty-acid-binding-protein (I-FABP) (Kanda *et al.*, 1996), citrulline (Crenn *et al.*, 2000) and glutathione S-transferase (GST) (McMonagle *et al.*, 2006). Elevated blood levels of zonulin, an important regulator of epithelial tight junctions, are purported to reflect increased gut permeability (Fasano *et al.*, 2000) and are also frequently reported, although issues have been raised in relation to commercially available assays (Massier *et al.*, 2020). Compromised gut barrier function can also be inferred by the presence of bacterial endotoxin, or lipopolysaccharide (LPS), in the blood. While the direct measurement of LPS in blood is possible, absolute quantitation can be unreliable (Munford, 2016) and surrogate markers such as soluble cluster of differentiation (sCD)-14 (Yaegashi *et al.*, 2005) and lipopolysaccharide-binding-protein (LBP) (Citronberg *et al.*, 2016) as well as anti-endotoxin antibodies (Hoke *et al.*, 2018) are successfully used.

In this study, we investigate a panel of intestinal permeability (IP) blood markers, encompassing measures of tight-junction protein dysfunction (zonulin), gut epithelial cell injury (I-FABP) and endotoxemia (sCD14, LBP, anti-endotoxin IgA, IgG and IgM antibodies along with more general inflammatory markers such as C-reactive protein (CRP)and inducible protein (IP)-10). Given the emerging evidence of increased gut permeability in stress-related psychiatric conditions (Kelly *et al.*, 2015), we hypothesize that gut barrier dysfunction is present in SAD patients.

4.3 Methods

Participants and Procedures:

Patients and healthy controls were recruited and assessed as outlined in Chapter 2.

Biological Samples

Serum samples were collected into serum tubes and centrifuged at 1500g for 10 minutes. The supernatant was then aliquoted into 1ml plastic tubes, capped and frozen at -80 °C for later analysis.

Measurement of intestinal permeability markers

Levels of IFABP and sCD14 (Quantikine Immunoassays, R&D Systems, Bio-Techne), LBP (Hycult Biotech), and anti-endotoxin core antibodies (EndoCAb IgA IgG IgM, Hycult Biotech) were determined using commercially available quantitative ELISAs. For each assay, serum samples were used, analysed in duplicate according to the manufacturer's instructions with absorbances measured at 450 nm on a Synergy™ HT, BioTek® plate reader. Results were calculated on a 4-parameter logistics curve generated using Gen5 BioTek Microplate Data Collection and Analysis software. Also, serum levels of IP-10 and CRP were assessed by means of ECL immunoassay using the V-PLEX Human IP-10 and CRP Kit, respectively (Meso Scale Diagnostics). For each assay, samples were analysed in duplicate according to the manufacturers' instructions, concentration was obtained and analysed using the MSD QuickPlex SQ 120 Instrument. The dilution factor for each assay were as follows: IFABP (5-fold dilution), sCD14 (200-fold dilution), LBP (1000-fold dilution), anti-endotoxin IgA (50-fold dilution), anti-endotoxin IgG (200-fold dilution), anti-endotoxin IgM (100-fold dilution), IP-10 (4-fold dilution), CRP (1000-fold dilution). Serum zonulin was determined using a competitive ELISA kit (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions. The assay sensitivity was <0.01 ng/ml. The ELISA kit detects the active (uncleaved) form of zonulin.

Statistics

All data were analysed using SPSS 25 (IBM, Armonk, NY, USA). Visual inspection of box plots was used to identify outliers and consideration given to removal of those lying more than three times the interquartile range (IQR) below the first quartile or above the third quartile. Missing values were excluded from analysis. Normality of data was assessed by visual inspection of histograms along with examination of skewness and the Shapiro-Wilk statistic. A Log10 transformation was applied to IgA, to normalise the data for univariate analysis. Independent T-tests were used to compare means between groups. Welch corrections were used where there was violation of homogeneity of variances. Categorical data were analysed using the Chi-squared or Fisher's Exact test. Univariate general linear modelling was used to compare means while adjusting for fixed factors such as sex

and covariates including age, BMI and exercise. Bonferroni adjustment was used to correct for multiple comparisons. Correlations were carried out using Pearson correlation coefficient for normally distributed data and Spearman rank sum for non-parametric data. Data are presented as mean ± SD unless stated otherwise. For descriptive purposes, summary statistics are reported in original units (in the text and in figures) along with statistical results from analysis of transformed data.

4.4 Results

Serum concentration of sCD14 was higher in patients with SAD compared to controls (t(66)=3.68, =<0.0005), a mean difference of 247,175.82 pg/ml. Mean serum IgA levels were also significantly higher in SAD patients (mean difference of 14.47 AMU/ml; t(65)=2.14, p=0.04) as were serum zonulin levels (mean difference of 5.71 ng/ml; t(64)=3.082, p=0.003) and CRP levels (t(59)=2.168, p=0.034). Conversely, LBP levels were lower in the SAD group, a mean difference of -4171.93 ng/ml (t(66)=-3.573, p=0.001). No group differences were seen in IgM (t(66)=0.572, p=0.57), IgG (t(63)=-0.498, p=0.62), I-FABP (t(63)=0.56, p=0.58) or IP-10 (t(65)=0.28, p=0.78) (Figure 1).

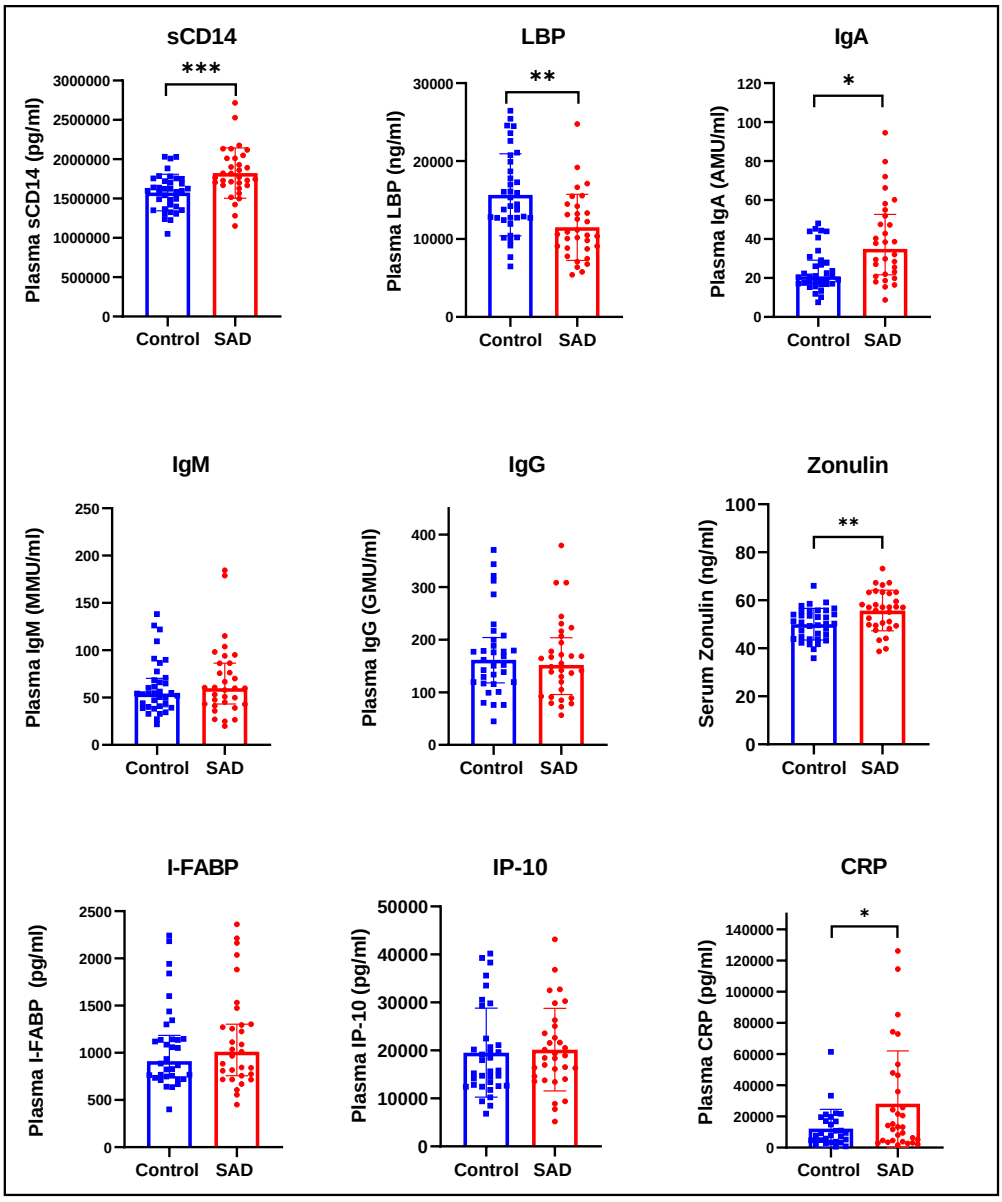
The difference in sCD14 between SAD patients and controls remained significant after adjusting for sex, age, BMI and exercise (F(1,58)=8.688, p=0.005). Similarly, whilst adjusting for these same factors, the group difference remained significant for IgA (F(1, 57)=5.315, p=0.025) and LBP (F(1, 56)=24.56, p=<0.0005). BMI was a significant covariate in the LBP model (F(1,56)=26.36, p=<0.0005) and displayed positive corelations with LBP across the total sample (Rs=0.248, p=0.043) as well as in the SAD (R=0.687, p=<0.0005) and HC (Rs=0.423, p=0.01) groups (Figure 2).

The group difference for zonulin was lost (F(1,56)=0.927, p=0.34) on adjusting for BMI which was a significant covariate (F(1,56)=25.7, p=<0.0005). Similarly, the group difference for CRP was lost (F(1,51)=8.324, p=0.43) on adding BMI, which was also a significant covariate in the general linear model (F91, 51)=8.324, p=0.006). Correlation analysis revealed strong positive associations between zonulin and BMI across the entire sample (R(65)=0.586, p=<0.0005) as well as within the control (Rs(35)=0.640, p=<0.0005) and SAD (R(31)=0.489, p=0.006) groups. A weaker association was seen between CRP and BMI in the total sample (Rs(66)=0.358, p=0.005) but not in either the control (Rs(28)=0.298, p=0.124) or SAD groups (Rs(31)=0.305, p=0.1) (Figure 2).

No correlations were noted between any IP markers and LSAS subgroup or total scores. No differences were seen for any IP markers in SAD patients with or without a history of MDD or between those taking psychotropic medication and those who were medication-free. Serum IgM

levels were higher in those SAD patients with a comorbid anxiety disorder in comparison to those with no such comorbidity (t(13.6)=-2.487, p=0.027). No other differences in IP markers were seen between those with or without a comorbid anxiety disorder.

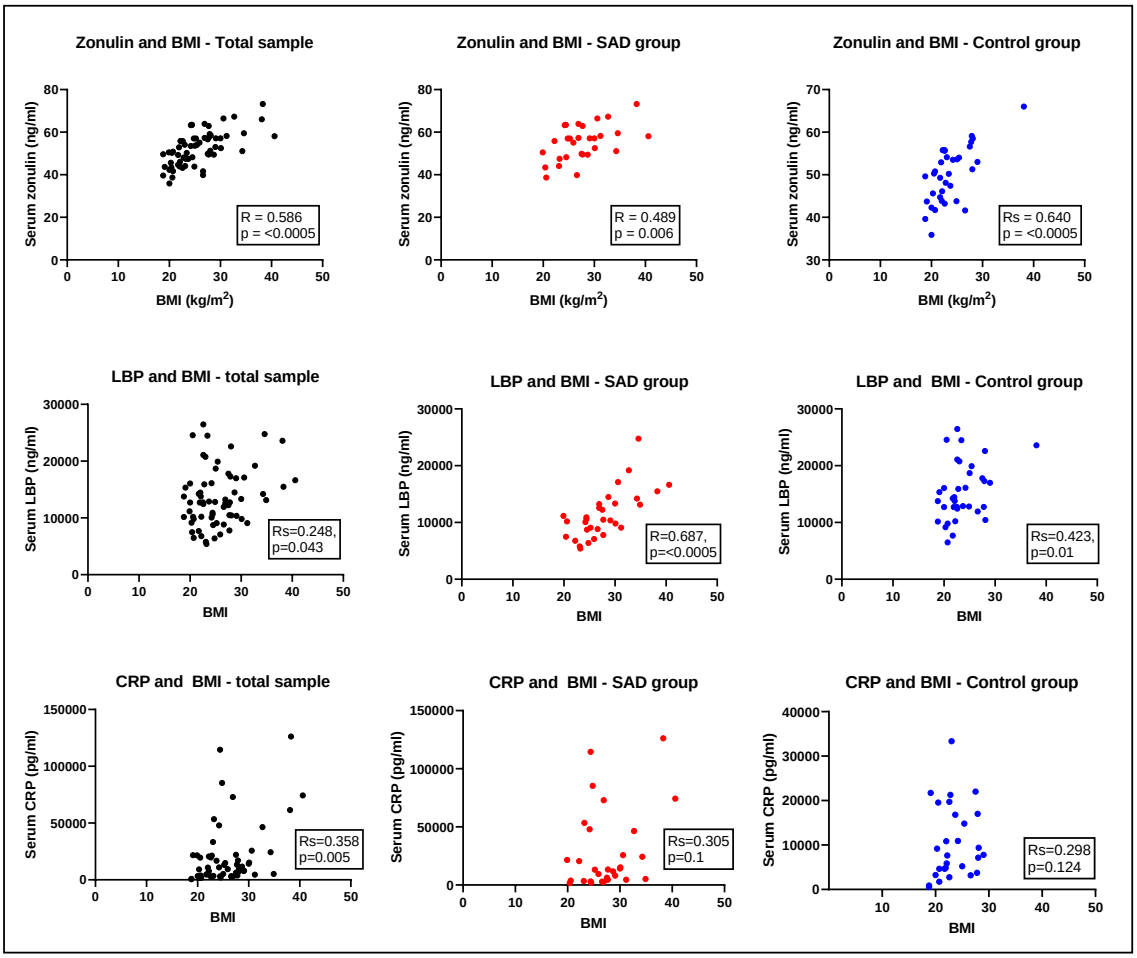
Figure 1: Differences in intestinal permeability markers between SAD patients and controls. The graphs show unadjusted values. Group differences remained for sCD14, LBP and IgA after adjusting for sex, age, BMI and exercise but were lost for zonulin and CRP after adjusting for BMI.



*(Scatter plot with bar: Top of bar represents the mean, error bars represent SD. * p=<0.05, ** p=<0.01, *** p=<0.001*

CRP: C-reactive protein, I-FABP: Intestinal fatty acid binding protein, LBP: Lipopolysacharride-binding protein)

Figure 2: Correlations between BMI and intestinal permeability markers, zonulin, LBP and CRP across total sample, SAD and control groups



(BMI: Body mass index, CRP: C-reactive protein, LBP: Lipopolysacharride-binding protein, R= Pearson correlation coefficient, Rs= Spearman's Rank Correlation Coefficient)

4.5 Discussion

Our findings of increased sCD14 and anti-endotoxin IgA in SAD patients point to increased gut permeability in this group. Elevated zonulin levels are also suggestive of gut hyperpermeability although the group difference was lost after adjusting for mean BMI, which was higher in the SAD group. It is well recognised that zonulin levels are increased in overweight and obesity and associated with abnormal gut barrier function in such individuals (Küme *et al.*, 2017; Moreno-Navarrete *et al.*, 2012b; Kim *et al.*, 2018). Additionally, the elevated CRP in the SAD group was no longer evident after adjusting for BMI, again unsurprising given that elevated CRP is a feature of obesity (Visser *et al.*, 1999). However, sCD14, IgA and LBP differences were not altered whilst

adjusting for BMI, indicating that the gut barrier disfunction seen in SAD patients could not just be attributed to the higher mean BMI levels in this group. Indeed, although LBP is elevated in obesity (Moreno-Navarrete *et al.*, 2012a; Kim *et al.*, 2016) and significant positive associations were evident between LBP and BMI in both the SAD and control groups, SAD patients had lower LBP concentrations despite elevated BMI levels.

The concept of compromised gastrointestinal permeability being involved in stress-related psychiatric conditions is now appreciated (Kelly *et al.*, 2015). Stress can lead to increased gut permeability resulting in the translocation of bacterial endotoxin from the gut lumen into the bloodstream (de Punder and Pruimboom, 2015). The presence of LPS in the blood can thus stimulate an inflammatory response, such as that seen in several psychiatric conditions including MDD, bipolar disorder (BD) and schizophrenia (Yuan *et al.*, 2019). This inflammatory response involves LPS binding by LBP and sCD14 with subsequent activation of the innate and adaptive immune responses and the ensuing production of pro-inflammatory cytokines and anti-endotoxin antibodies (Wright *et al.*, 1990; Hurley, 2013). Thus, the elevated anti-endotoxin IgA along with raised sCD14 in our SAD group suggest the presence of endotoxemia and fit with this gut hyperpermeability theory.

Of note is the discordance between sCD14 and LBP levels. While sCD14 concentrations were significantly elevated in SAD patients, LBP levels were reduced in comparison to healthy controls. However, the relationship between sCD14 and LBP has been shown to be complex and corresponding changes in both markers may not necessarily be evident due to different biological mechanisms (Barbosa *et al.*, 2012; Romero-Sánchez *et al.*, 2012). While LPS is the only ligand of LBP, sCD14 represents a broader measure of monocyte activation and has been shown to recognise other pattern-associated molecular patterns, including heat shock protein-60 (Kol *et al.*, 2000) and certain gram-positive bacterial components and mycobacteria (Pugin *et al.*, 1994). Additionally, LBP has been shown to play a dual role in the immune response to LPS. At low constitutive levels, LBP binding to LPS results in a pro-inflammatory response. However, at higher levels, as seen in an acute phase reaction, LBP binding actually neutralises LPS and moderates the inflammatory response (Gutsmann *et al.*, 2001; Wurfel *et al.*, 1994). Thus, reduced LBP levels may actually be seen in states of increased gut permeability, as has been reported in studies of patients with Parkinson's disease (PD) (Forsyth *et al.*, 2011; Pal *et al.*, 2015). In these studies, serum LBP levels were found to be reduced compared to controls, although patients had increased gut permeability based on a urinary sucralose challenge test, as well as increased extra-luminal exposure to gut bacteria, manifested by more intense staining of *E. coli* in both the epithelial and lamina propria zones of the sigmoid mucosa. No differences in sCD14 were seen between the PD and control groups (Forsyth *et al.*, 2011). Discordant patterns between LBP and sCD14 have also been demonstrated in patients in the

prodromal phase of schizophrenia (Weber *et al.*, 2018) as well as in those with established schizophrenia and BD (Severance *et al.*, 2013) with patients having elevated sCD14 levels which were not matched by increased LBP concentrations.

No differences in I-FAPB were seen in SAD patients in comparison to controls. I-FABP is present exclusively in enterocytes and is not a direct marker of intestinal permeability but rather provides evidence of injury to enterocytes (Kanda *et al.*, 1996) and resultant impaired transcellular and/or paracellular permeability. Elevated zonulin, due to its role in the regulation of epithelial tight junctions, represents gut hyperpermeability via the paracellular pathway. Therefore, our findings of elevated zonulin, in the absence of elevated I-FABP levels, suggest impaired paracellular, as opposed to transcellular, gut permeability in SAD patients. This is consistent with a recent meta-analysis of gut permeability markers in patients with severe mental illness including MDD, bipolar disorder and schizophrenia along with chronic fatigue syndrome (CFS) which reported elevated zonulin levels but equivocal I-FABP levels (Safadi *et al.*, 2021).

To our knowledge, this is the first study to investigate intestinal permeability in patients diagnosed with SAD, or any specific clinical anxiety disorder. One study has reported elevated IP markers in 22 patients, apparently recruited through a gastroenterology clinic, with a diagnosis of depression and/or anxiety. However, no details were given in relation to the subjects or the type of anxiety and it appears that a general umbrella diagnosis of depression and/or anxiety was used (Stevens *et al.*, 2018). Given the high rates of comorbidity of SAD with schizophrenia (Pallanti, Quercioli and Hollander, 2004), MDD, alcohol abuse (Schneier *et al.*, 2010) and autism (Simonoff *et al.*, 2008), conditions which have all been associated with increased gut permeability (Safadi *et al.*, 2021; de Timary *et al.*, 2015; de Magistris *et al.*, 2010), it is unsurprising that evidence of increased gastrointestinal permeability is present in SAD and may represent a trans-diagnostic pathophysiological marker for these psychiatric illnesses.

Several limitations of this study must be recognised. Although we did not find differences in any IP markers between medicated and unmedicated patients we cannot exclude the possibility that psychotropic medications might modulate these parameters. However, in a recent meta-analysis of similar gut permeability markers in patients with severe mental illness, medications showed no effect (Safadi *et al.*, 2021). Secondly, our groups were unmatched in terms of BMI and exercise, with the SAD group having higher mean BMI scores and lower exercise levels compared to controls. Exercise is associated with increased gut permeability (Chantler *et al.*, 2021; Pires *et al.*, 2017), and given that our SAD group had lower exercise levels, this is unlikely to be a factor in explaining their elevated IP markers. Nonetheless, we controlled for exercise levels in our statistical analyses. Given

the evidence of a link between obesity and increased gut permeability, it would, of course, be preferable to have groups with equivalent BMI levels. However, whilst adjusting for BMI did influence the group difference with regards to zonulin and CRP, other marker differences were unaffected. Thirdly, concerns have been raised recently in relation to the reliability of zonulin as an intestinal permeability biomarker (Barbaro *et al.*, 2020) and the inadequacy of current commercial enzyme assays (Talley *et al.*, 2020; Massier *et al.*, 2020). Thus, while the other markers may offer more robust evidence of gut barrier integrity, zonulin has utility when used as part of this biosignature panel.

4.6 Conclusion

Our findings suggest that gut barrier dysfunction is present in patients with SAD. Given the emerging evidence of the role of the MGB axis in psychiatric disorders, further studies are warranted to explore the potential role of increased gastrointestinal permeability in the aetiology of SAD and other clinical anxiety disorders.

Chapter 5: The Immune-Kynurenine Pathway in Social Anxiety Disorder

5.1 Abstract

Background: The tryptophan-kynurenine pathway is of major interest in psychiatry and is altered in patients with depression and schizophrenia. Stress and immune alterations can impact this system, through cortisol- and cytokine-induced activation. There is emerging evidence that the kynurenine pathway (KP) is associated with suicidality. There have been no studies to date exploring the immune-kynurenine system in SAD, and indeed very limited human studies on the KP in any clinical anxiety disorder.

Methods: We examined plasma levels of several KP markers, including kynurenine (KYN), tryptophan (TRYP) and kynurenic acid (KYNA), along with the KYN/TRYP and KYNA/KYN ratios, in a cohort of 32 patients with SAD and 36 healthy controls. We also investigated a broad array of both basal and lipopolysaccharide (LPS)-stimulated blood cytokine levels including IFN-γ, interleukin (IL)-10, IL-1β, IL-2, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α.

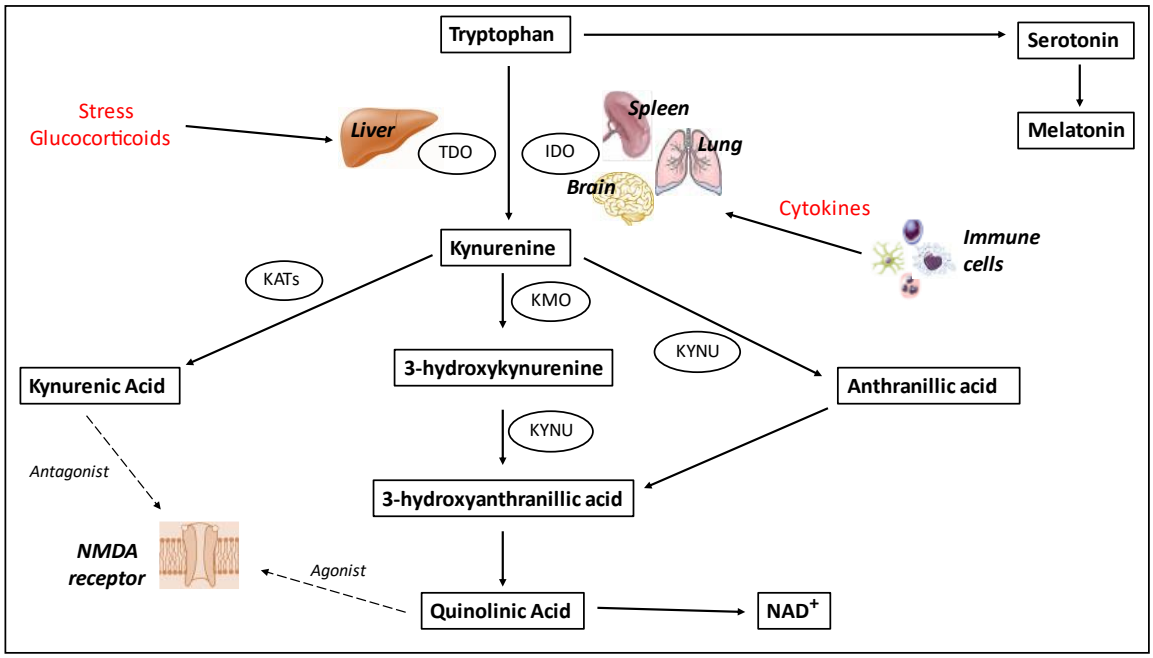
Results: SAD patients had elevated plasma KYNA levels and an increased KYNA/KYN ratio compared to healthy controls. No differences in KYN, TRYP or the KYN/TRYP ratio were seen between the two groups. SAD patients with a history of past suicide attempt showed elevated plasma KYN levels and a higher KYN/TRYP ratio compared to patients without a history of suicide attempt. No differences were seen in basal or LPS-stimulated pro-inflammatory cytokine levels between the patients and controls. However, unstimulated IL-10, an anti-inflammatory cytokine, was significantly lower in the SAD group. A significant sex influence was evident with SAD males having lower levels of IL-10 compared to healthy males but no difference seen between SAD and healthy females.

Conclusions: The peripheral KP is altered in SAD and preferentially directed towards KYNA synthesis. Additionally, KP activation, as evidenced by elevated KYN and KYN/TRYP ratio, is evident in SAD patients with a history of past suicide attempt. While no differences in pro-inflammatory cytokines is apparent in SAD patients, lower anti-inflammatory IL-10 levels are seen in SAD males. Further investigation of the role of the immune-kynurenine pathway in SAD and other clinical anxiety disorders is warranted.

5.2 Introduction

There has long been an interest in the role of serotonin across all psychiatric disorders (Marazziti, 2017). Serotonin is produced from the amino acid, tryptophan (TRYP) and has been extensively researched and utilised as a therapeutic target for mood and anxiety disorders (Otte *et al.*, 2016; Craske *et al.*, 2017). However, in recent years there has been more emphasis placed on the potential role of the kynurenine pathway (KP), an alternative metabolic route for TRYP (Savitz, 2020). While best known in neuroscience and psychiatry for its role as a precursor of serotonin, the vast majority of TRYP is actually converted into kynurenine (KYN) and its multiple downstream metabolites (for review and current status see (Schwarcz and Stone, 2017) (Figure 1)). This conversion of TRYP to KYN is the rate-limiting step in the KP and controlled by two enzymes, tryptophan 2,3-dioxygenase (TDO) and indolamine 2,3-dioxygenase (IDO), of which there are two subtypes, IDO-1 and IDO-2 (Ball *et al.*, 2009). TDO, induced by stress and glucocorticoids, is predominantly expressed in the liver which is the main source of TRYP degradation under physiological conditions. IDO, in contrast, is largely extrahepatic and induced by pro-inflammatory cytokines. KYN can be metabolised through three branches (Figure 1); firstly, it can be converted into anthranilic acid (AA) by kynureninase; secondly, it can be converted by kyurenine 3-monooxygenase (KMO) to 3-hydroxykynurenine (3-HK) and subsequently to 3-hydroxyanthranilic acid (3HAA), quinolinic acid (QA) and ultimately nicotinamide adenine dinucleotide (NAD^+); lastly, it can be irreversibly transaminated to kynurenic acid (KYNA) by the kynurenine aminotransferase (KAT) enzymes, of which there are 4 recognised subtypes in the mammalian brain (Han *et al.*, 2010), the KAT II enzyme thought to be responsible for the majority of the brain KYNA synthesis (Fujigaki, Yamamoto and Saito, 2017). In the brain, KYNA is produced predominantly in astrocytes (Guillemin *et al.*, 2001) and acts as a glutamate receptor antagonist capable of inhibiting N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite receptors (Stone, 1993; Mok *et al.*, 2009) as well as the alpha7 nicotinic acetylcholine receptor (Hilmas *et al.*, 2001). It is thus recognised as having neuroprotective and anticonvulsant properties (Foster *et al.*, 1984). In contrast, QA is an agonist of the NMDA receptor (Stone and Perkins, 1981) and has excitatory, convulsant and neurotoxic effects (Lapin, 1978).

Figure 1: Tryptophan Metabolism and the Kynurenine Pathway



(IDO: indoleamine 2, 3-dioxygenase, KATs: kynurenine aminotransferases, KMO: kynurenine monooxygenase, KYNU: kynureninase, NAD: nicotinamide adenine dinucleotide, NMDA: N-methyl-D-aspartate, TDO: tryptophan 2, 3-dioxygenase)

There have, to our knowledge, been no studies to date exploring the KP in SAD, and indeed very limited human studies on the KP in any clinical anxiety disorder. However, KP activity in MDD and schizophrenia has received much attention in recent years (Marx *et al.*, 2020a; Barry *et al.*, 2009). The neurodegeneration hypothesis of depression suggests that the low-grade inflammation seen in depression could, via cytokine-induced IDO upregulation and subsequent KP activation, result in an imbalance between neuroprotective and neurodegenerative KP metabolites which may underlie the pathogenesis of MDD (Myint and Kim, 2003; Myint *et al.*, 2007). This theory is supported by the findings of raised plasma and cerebrospinal fluid (CSF) levels of KYN in patients with hepatitis C undergoing treatment with interferon (IFN)-alpha/ribavirin, a treatment associated with high levels of depression. There is a high correlation between plasma levels and CSF concentration of KYN in these patients, which in turn correlates with depressive symptoms (Raison *et al.*, 2010). Additional support for this hypothesis is the growing body of evidence showing cross-sectional reductions in peripheral concentrations of KYNA and the KYNA/QA balance in depression (Savitz, 2020). In contrast, KYNA has consistently been shown to be elevated in the central nervous system (CNS), both in CSF analysis and post-mortem brain samples, in patients with schizophrenia (Plitman *et al.*, 2017). Central KYNA levels are also elevated selectively in that subset of bipolar disorder patients who have experienced psychotic symptoms (Olsson *et al.*, 2012; Sellgren *et al.*, 2019), suggesting

that it is related in some way to psychotic psychopathology, likely through its impact on glutamatergic and thus, dopaminergic, neurotransmission.

We examined plasma levels of several KP markers, including KYN, TRYP, KYNA, along with the KYN/TRYP and KYNA/KYN ratios in a cohort of patients with SAD and healthy controls. We also investigated a broad array of both basal, and lipopolysaccharide (LPS)-stimulated, blood cytokine levels including IFN-γ, interleukin (IL)-10, IL-1β, IL-2, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α. We hypothesized that there is increased KP activation in SAD patients and that differences in KP metabolite concentrations may be associated with pro-inflammatory cytokine abnormalities. Additionally, given the emerging literature on KP activation and suicidality (Bryleva and Brundin, 2017), we hypothesized that there are differences in KP metabolites between SAD patients with and without a history of a suicide attempt.

5.3 Methods

Participants and Procedures:

Patients and healthy controls were recruited and assessed as outlined in Chapter 2.

KP metabolites

Tryptophan and kynurenine pathway metabolites were determined as previously described (Kennedy *et al.*, 2015). Briefly, plasma samples were spiked with internal standard (3-Nitro-L-tyrosine) prior to being deproteinised by the addition of 20 µl of 4 M perchloric acid to 200 µl of sample. Samples were centrifuged at 21,000g on a Hettich Mikro 22R centrifuge (AGB, Dublin, Ireland) for 20 min at 4 °C and 100 µl of supernatant transferred to a HPLC vial for analysis on the HPLC system (UV and FLD detection). All samples were injected onto a reversed phase Luna 3 µm C18 (2) 150 × 2 mm column (Phenomenex), which was protected by KrudKatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex). The mobile phase consisted of 50 mM acetic acid and 100 mM zinc acetate with 3 % (v/v) acetonitrile and was filtered through Millipore 0.45-µm HV Durapore membrane filters (AGB) and vacuum degassed prior to use. Compounds were eluted isocratically over a 30-min runtime at a flow rate of 0.3 ml/min after a 20-µl injection. The column was maintained at a temperature of 30 °C, and samples/standards were kept at 8 °C in the cooled autoinjector prior to injection. The fluorescent detector was set at an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The UV detector was set to 330 nm. L-Tryptophan and kynurenine were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Analyte:

Internal standard peak height ratios were measured and compared with standard injections, and results were expressed as nanogram per millilitre of plasma.

Cytokines

Whole blood was diluted 1:10 with Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich) supplemented with 10% foetal calf serum and 5% penicillin streptomycin. Each blood sample was cultured with and without the toll-like receptor (TLR)-4 receptor agonist, E. coli K12 LPS, from the Human TLR agonist kit (InvivoGen, San Diego, CA, USA) for 24 h. After the 24 h culture period, the supernatant from both untreated and stimulated cells was aspirated and stored at -80°C. Levels of IFNγ, IL-10, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNFα were assayed in duplicate using high-sensitivity commercially available electrochemiluminescence MULTI-SPOT_ Meso Scale Discovery kits (MSD, Rockville, MD, 75USA) as per manufacturer's instructions.

Statistics

All data was analysed using SPSS 25 (IBM, Armonk, NY, USA). Visual inspection of box plots was used to identify outliers and consideration given to removal of those lying more than three times the interquartile range (IQR) below the first quartile or above the third quartile. Missing values were excluded from analysis. Normality of data was assessed by visual inspection of histograms along with examination of skewness and the Shapiro-Wilk statistic. Independent t-tests were used to compare means between groups where assumptions were met. Welch corrections were used where there was violation of homogeneity of variances. Where parametric assumptions were violated, Mann-Whitney U tests were applied. Univariate general linear modelling was used to compare means while adjusting for fixed factors such as sex and covariates including age, BMI and exercise. Bonferroni adjustment was used to correct for multiple comparisons. Correlations were carried out using Pearson correlation coefficient for normally distributed data and Spearman rank sum for non-parametric data. Data are presented as mean ± SD unless stated otherwise. For descriptive purposes, summary statistics are reported in original units (in the text and in figures) along with statistical results from analysis of transformed data.

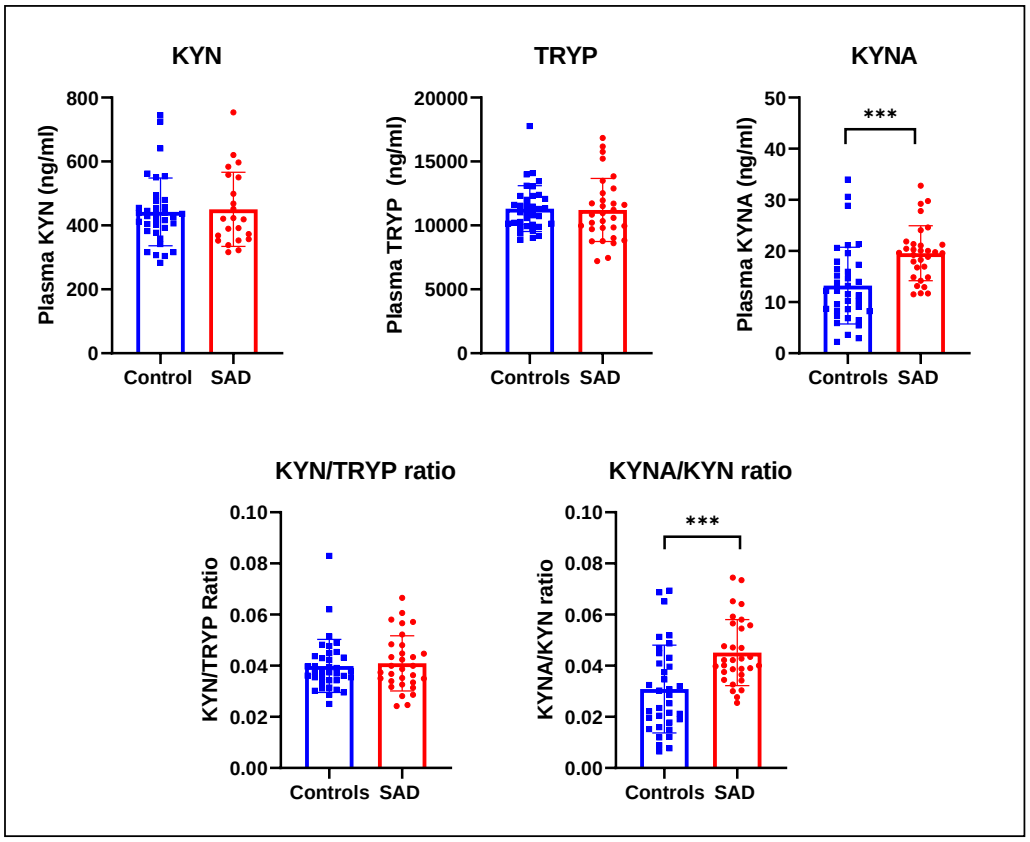
5.4 Results

Increased plasma KYNA in SAD patients

There were no significant differences in KYN ($t(65)=-.281$, $p=0.78$), TRYP ($t(65)=-.553$, $p=0.58$) or the KYN/TRYP ratio ($t(65)=0.398$, $p=0.70$) between SAD patients and healthy controls. However, KYNA

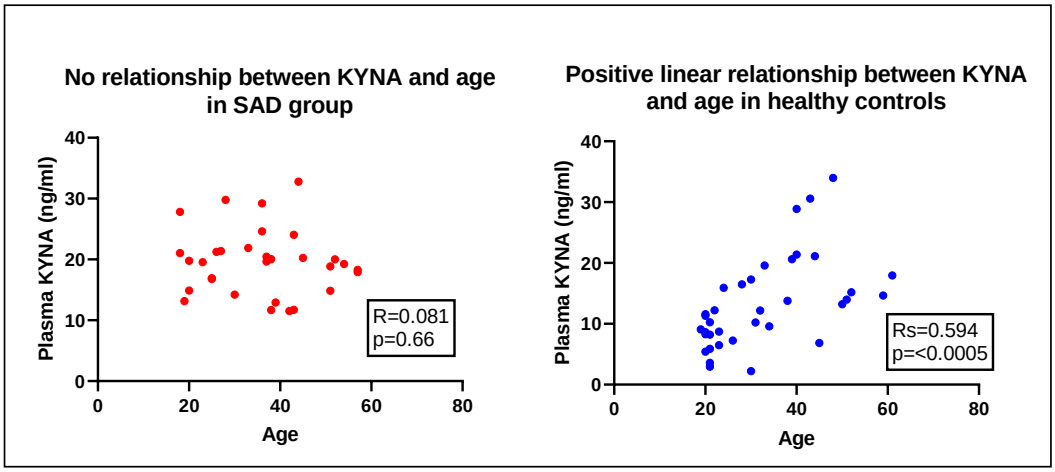
levels (mean ± SD) were markedly higher in SAD patients (19.56±5.38 ng/ml) than controls (13.21±7.5 ng/ml), a statistically significant mean difference of 6.354 ng/ml (t(65)=3.925, p=<0.0005) (Figure 2). The group difference in KYNA remained after controlling for gender, age, BMI and exercise (F(1, 56)=15.01, p=<0.0005). A significant group*age effect was seen (F(1,56)=7.09, p=0.01) with evidence of KYNA increasing with age in the control group but no such relationship seen in the SAD group (Figure 3). The KYNA/KYN ratio was also significantly elevated in the patient group (t(65)=3.927, p=<0.0005) (Figure 2), a difference which remained after controlling for age, gender, BMI and exercise (F(1,56)=18.235, p=<0.0005).

Figure 2: Differences in KP markers between SAD and healthy control groups



(*Scatter plot with bar: Top of bar represents the mean, error bars represent SD. * p=<0.05, ** p=<0.01, *** p=<0.001)*

Figure 3: Correlation between age and KYNA in SAD patients and healthy controls.

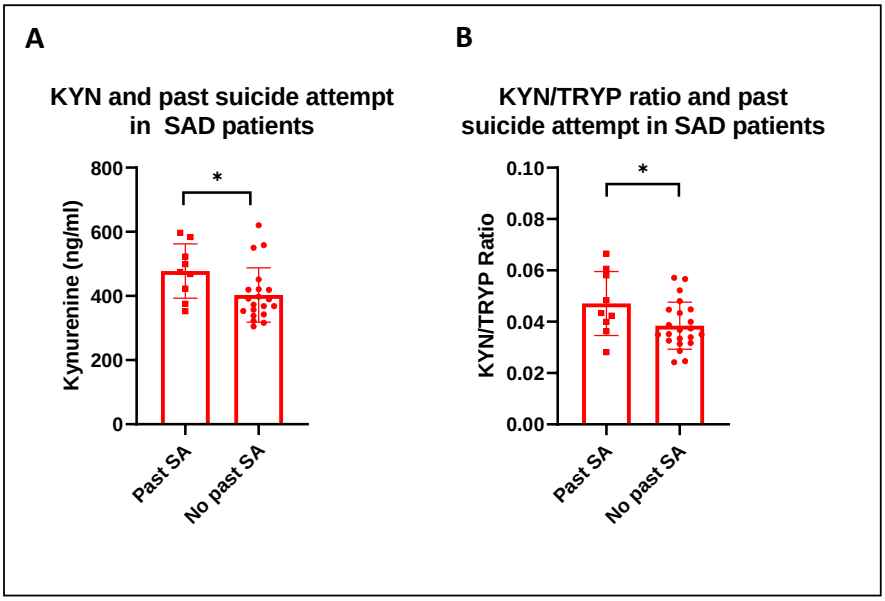


There was no difference in any KP markers between those SAD patients with a history of MDD and those with no history of depression. Likewise, no differences were seen in any KP markers between those SAD patients with or without a comorbid anxiety disorder. The SAD patients taking psychotropic medication had higher mean KYN levels (486.09±129.29) than those who were medication-free (367.86±33.21) (t(24.9)=-3.927, p=0.001) as well as a higher KYN/TRYP ratio (t(29)=-2.451, p=0.021). Other KP parameters did not differ between medicated and unmedicated patients. Of note, those patients taking psychotropic medications (n=21) had higher total LSAS scores (89±29.69) in comparison to unmedicated patients (68±16.1) (t(30)=-2.393, p=0.023). There were no correlations seen between any KP parameter and social anxiety symptom scores based on the LSAS total or subgroup scores.

Increased plasma KYN and KYN/TRYP ratio in patients with a history of a suicide attempt:

We analysed KP markers in those SAD patients with (n=9) and without a history of a suicide attempt. Plasma KYN levels were elevated in those with a history of a suicide attempt (477.459±84.56 ng/ml) in comparison to those without such a history (403.236±84.67 ng/ml), a mean difference of 74.22 mg/ml (t(27)=-2.185, p=0.038). In addition, the KYN/TRYP ratio was elevated in those with a history of suicide attempt (t(29)=-2.146, p=0.04) (Figure 4). No difference was seen in TRYP, KYNA or the KYNA/KYN ratio in those with a history of suicide attempt. There was no difference in KP markers seen between those SAD patients who did or did not report suicidal ideation in the previous month.

Figure 4: 4A: KYN levels in SAD patients with a history of suicide attempt compared with those without a history of suicide attempt. **4B:** KYN/TRYP ratio in SAD patients with a history of suicide attempt compared with those without a history of suicide attempt.



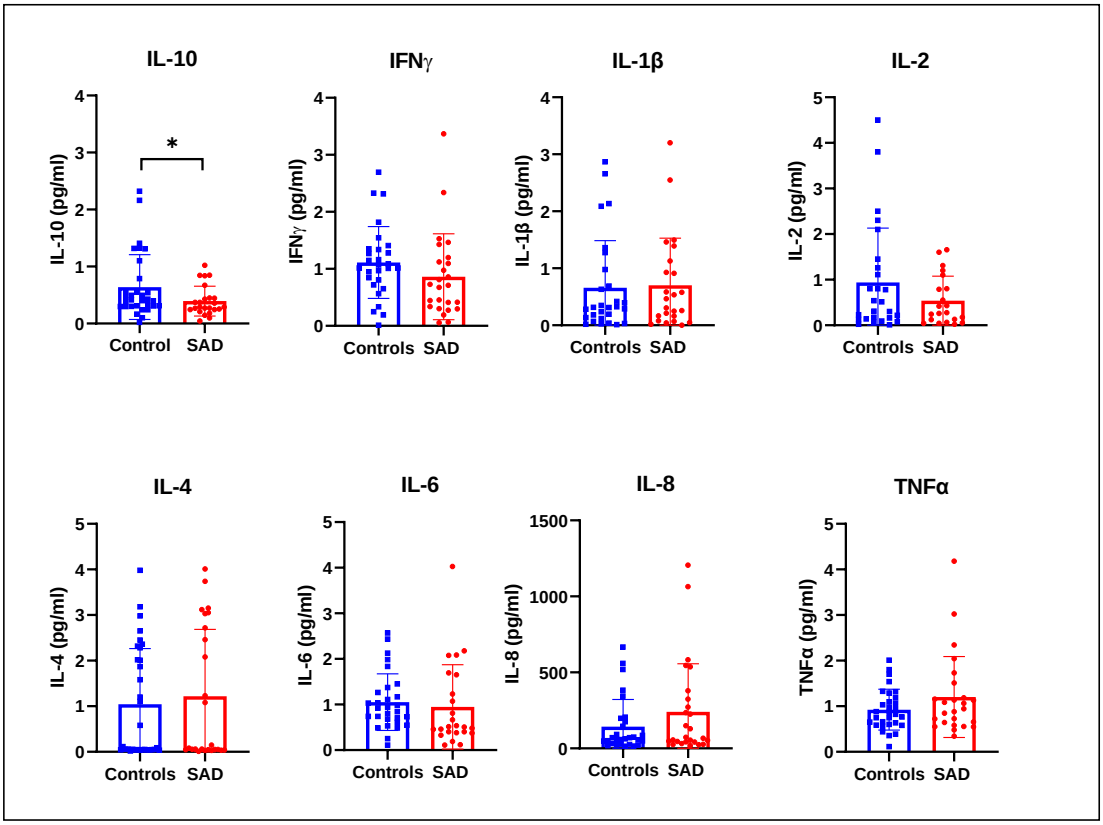
*(Scatter plot with bar: Top of bar represents the mean, error bars represent SD. * p=<0.05, ** p=<0.01, *** p=<0.001. KYN: kynurenine, SA: suicide attempt, TRYP: tryptophan)*

Reduced peripheral anti-inflammatory cytokine, IL-10, levels in SAD patients:

Unstimulated IL-10 (mean ± SD) concentrations were lower in the SAD group (0.394 ± 0.26 pg/ml) compared to the control group (0.639 ± 0.57 pg/ml), a statistically significant difference (mean difference = -0.25 pg/ml, t(44.28)=-2.13, p=0.04) (Figure 5). No differences in IL-10 were seen between those SAD patients taking psychotropic medication and those who were medication-free (t(23)=0.993, p=0.33). Similarly, no differences were seen between SAD patients with or without a history or MDD (t(23)=0.430, p=0.67) or those with or without a comorbid anxiety disorder (t(23)=-0.151, p=0.88). No group differences were seen in the remaining unstimulated cytokines (Figure 4).

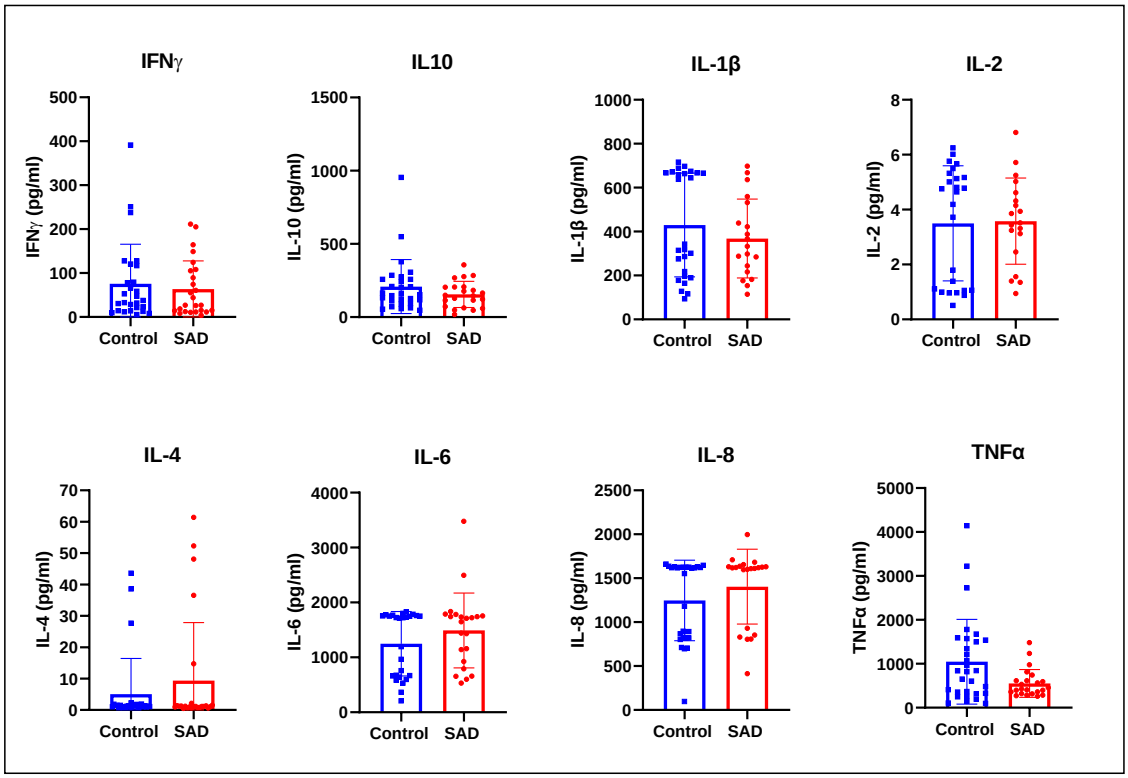
No differences were seen in any of the LPS-stimulated cytokines between SAD and control groups (Figure 6).

Figure 5: Differences in unstimulated cytokines between SAD and healthy control groups



(*Scatter plot with bar: Top of bar represents the mean, error bars represent SD. * p=<0.05, ** p=<0.01, *** p=<0.001)*

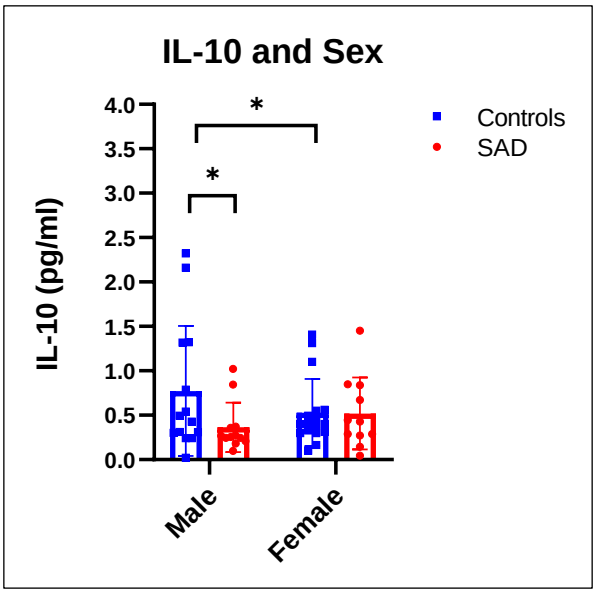
Figure 6: Differences in LPS-stimulated cytokines between SAD and healthy control groups



*(Scatter plot with bar: Top of bar represents the mean, error bars represent SD. * p=<0.05, ** p=<0.01, *** p=<0.001)*

Whilst controlling for age, exercise and BMI, a significant group*sex interaction was seen for IL-10 ($F(1,52)=7.130$, $p=0.011$) Males in the SAD group had lower IL-10 levels compared to healthy males ($F(1,45)=6.266$, $p=0.016$), while females in the SAD group did not differ from those in the healthy control group ($F(1,45)=1.135$, $p=0.30$). Healthy males had higher IL-10 levels compared to healthy females ($F(1,45)=6.259$, $p=0.016$) while no difference was seen between SAD males and females ($F(1,45)=1.538$, $p=0.22$) (Figure 7).

Figure 7: Serum IL-10 levels in males and females in control and SAD groups.



*(Scatter plot with bar: Top of bar represents the mean, error bars represent SD. * p=<0.05, ** p=<0.01, *** p=<0.001)*

Associations between KP markers and cytokine levels:

Correlations between KP markers and cytokines can be seen in Table 2. Several pro-inflammatory cytokines, including TNFα, IL-8 and IFNα, were associated negatively with TRYP levels and/or positively associated with KYN levels and the KYN/TRYP ratio (Table 2). Conversely, anti-inflammatory cytokines such as IL-10 and IL-4 correlated negatively with the KYN/TRYP ratio.

Table 2: Correlations between KP markers and cytokines in SAD and healthy control groups

SAD patients										
	KYN		**TRYP**		**KYNA**		**KYN/TRYP ratio**		**KYNA/KYN ratio**	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Unstimulated										
IFNγ	-0.053	0.806	-0.100	0.642	-0.239	0.260	0.118	0.582	-0.111	0.605
IL-10	0.215	0.324	-0.079	0.720	-0.195	0.373	0.271	0.211	**-0.422**	**0.045***
IL-1β	0.008	0.971	-0.039	0.861	0.025	0.911	0.038	0.865	-0.112	0.612
IL-2	-0.361	0.118	0.256	0.277	0.095	0.691	**-0.579**	**0.007****	0.186	0.431
IL-4	-0.212	0.308	0.041	0.847	-0.276	0.181	-0.262	0.205	-0.226	0.277
IL-6	0.089	0.687	0.229	0.293	0.208	0.342	-0.015	0.946	0.032	0.886

IL-8	0.153	0.465	0.087	0.679	0.138	0.122	0.118	0.575	0.186	0.373
TNFα	**0.415**	**0.044***	0.136	0.527	0.200	0.349	0.263	0.215	-0.257	0.266
LPS-Stimulated										
IFNγ	-0.214	0.316	0.173	0.419	0.008	0.971	-0.390	0.059	0.095	0.660
IL-10	-0.008	0.973	0.198#	0.388	0.122#	0.599	-0.170#	0.461	0.134#	0.562
IL-1β	0.132	0.578	-0.149	0.531	0.114	0.631	0.230	0.329	0.015	0.950
IL-2	0.135	0.581	0.185#	0.448	0.348#	0.144	0.004#	0.986	0.330#	0.168
IL-4	0.085	0.692	0.352	0.091	0.222	0.298	-0.208	0.330	-0.068	0.753
IL-6	0.191	0.407	0.303	0.182	0.361	0.108	-0.068	0.771	0.221	0.336
IL-8	-0.840	0.724	**-0.541**	**0.014***	-0.287	0.220	0.364	0.115	-0.274	0.243
TNFα	0.329	0.135	0.241	0.280	0.198	0.377	0.066	0.770	-0.030	0.895
Healthy Controls										
	KYN		**TRYP**		**KYNA**		**KYN/TRYP ratio**		**KYNA/KYN ratio**	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Unstimulated										
IFNγ	-0.177	-0.161	-0.014	0.945	-0.019#	0.923	-0.010#	0.959	0.012	0.952
IL-10	0.027	0.886	0.261	0.164	0.281	0.125	**-0.371**	**0.040***	0.259	0.167
IL-1β	-0.026	0.897	0.017	0.933	0.323	0.094	-0.121	0.540	0.327	0.096
IL-2	-0.069	0.744	-0.311	0.130	-0.120	0.560	-0.028	0.892	-0.113	0.592
IL-4	-0.142	0.448	0.063	0.737	**0.404**	**0.022***	**-0.360**	**0.043***	**0.473**	**0.007****
IL-6	-0.017	0.932	-0.017	0.932	-0.109	0.572	-0.009	0.962	-0.074	0.707
IL-8	0.359	0.061	0.164	0.405	-0.240	0.209	**0.493**	**0.007****	**-0.409**	**0.031***
TNFα	-0.033	0.867	-0.025	0.897	-0.179#	0.344	-0.028#	0.884	-0.157	0.416
LPS-Stimulated										
IFNγ	-0.358	0.073	**-0.481**	**0.034***	-0.233	0.242	-0.131	0.514	-0.091	0.660
IL-10	-0.152	0.449	0.034	0.868	-0.020	0.919	-0.148	0.451	0.042	0.837
IL-1β	0.127	0.545	0.102	0.627	-0.177	0.388	0.222	0.276	-0.241	0.246
IL-2	0.304	0.148	0.361	0.083	-0.106	0.614	0.183	0.381	-0.230	0.281
IL-4	0.005	0.978	0.080	0.692	0.032	0.873	-0.138	0.482	0.058	0.774
IL-6	**0.435**	**0.030***	**0.417**	**0.038***	0.024	0.909	0.267	0.187	-0.114	0.588
IL-8	0.199	0.329	**0.391**	**0.048***	0.098	0.628	0.109	0.587	0.012	0.954
TNFα	-0.173	0.370	0.109	0.574	-0.051	0.791	-0.188	0.319	0.045	0.817

*(# = Pearson correlation coefficient. All others Spearman rank coefficient. * P=<0.05, ** p=<0.01, ***p=<0.001)*

5.5 Discussion

In the present study we found increased plasma levels of KYNA in patients with SAD. In addition, we observed an elevated KYNA/KYN ratio in this group, suggesting increased KAT enzyme activity. SAD males also had lower basal IL-10 levels in comparison to healthy control males, although no other significant differences in baseline or LPS-stimulated cytokines were evident.

There have been few studies looking at the KP in clinical anxiety disorders. A small older study demonstrated that induction of anxiety in healthy volunteers using a caffeine challenge test resulted in increased KYN levels (Orlikov and Ryzov, 1991). A later study reported that patients with 'endogenous anxiety' demonstrated higher levels of plasma KYN in comparison to patients with 'endogenous depression' who had lower levels of KYN. (Orlikov, Prakhye and Ryzov, 1994). More recently it was shown that patients with panic disorder (PD) had an elevated KYN/TRYP ratio (Quagliato, Freire and Nardi, 2019). These clinical findings are in keeping with preclinical studies which have reported stress-induced increases in KYN and KYNA in various animal models of stress (Fuertig *et al.*, 2016; Kiank *et al.*, 2010; Klausing *et al.*, 2020). In addition to the patient-control group differences in the KYNA/KYN ratio and KYNA levels, we also found that SAD patients taking psychotropic medication had higher KYN levels than those who were medication-free. Notably this subgroup of medicated SAD patients had significantly higher LSAS scores than their unmedicated counterparts, suggesting a possible link between elevated KYN levels and anxiety symptom severity. Overall, our results are consistent with the existing literature that, although sparse, is suggestive of KP activation in anxiety states, which in SAD appears to be preferentially directed toward KYNA synthesis. Interestingly, a discordant age effect on KYNA levels was evident between the SAD patients and controls. KYNA concentrations increase with age in humans, both peripherally (Theofylaktopoulou *et al.*, 2013) and centrally (Kepplinger *et al.*, 2005). Our findings of a strong positive association between KYNA and age in the control group was in keeping with this. However, this normal age-related increase in KYNA was not seen in the SAD group.

While the route by which stress activates the KP through TDO induction is well recognised (O'Farrell and Harkin, 2017), there is less empirical evidence in relation to what activates KAT activity and specifically shifts KP metabolism towards KYNA production. It appears from animal studies that stress can preferentially point the KP in the direction of KYNA synthesis as opposed to down the QA route. Chronic social defeat stress in mice resulted in increased plasma KYN and KYNA levels although no corresponding significant increases in 3-HK or QA were seen (Fuertig *et al.*, 2016). Similarly, restraint stress in pregnant mice resulted in TDO activation and significant elevations of KYN and KYNA, both peripherally and centrally, in maternal and fetal tissues. In contrast, 3-HK and

QA levels remained unchanged (Notarangelo and Schwarcz, 2016). Furthermore, mice subjected to repeated psychological (acoustic and restraint) stress had reduced plasma TRYP and elevated KYNA while no changes in KYN or QA levels were evident (Kiank *et al.*, 2010). Thus, it is possible that in SAD, the chronic stress to which patients are exposed due to repeated social interactions, could not only stimulate conversion of TRYP to KYN through TDO activation, but shift downstream metabolism towards production of KYNA.

Although no studies have previously investigated KYNA levels in clinical anxiety disorders, a study in schizophrenia patients (Chiappelli *et al.*, 2014) supports the idea that KYNA may be abnormal in psychiatric cohorts exposed to stress. Researchers measured salivary KYNA before and after a psychological stressor and found that a subgroup of schizophrenia patients that exhibited distress intolerance (defined by quitting the stressful task early) had significantly higher KYNA levels both at baseline and in response to the stressor. On repeating the salivary sample with patients at rest, there was no difference in KYNA levels in this subset. Thus, is appears that this subgroup of individuals was particularly sensitive to stress such that even the anticipation of a stressful event caused a rapid elevation in salivary KYNA. It is possible that the distress-intolerant phenotype seen in these schizophrenia patients crosses diagnostic categories and that the same biological mechanism involving excessive KYNA responses to stressful events, and even to the anticipation of such events, may be at play for patients with schizophrenia or SAD, disorders which are commonly comorbid (Pallanti, Quercioli and Hollander, 2004; Michail and Birchwood, 2009). It is notable that KYNA has repeatedly been shown to be elevated in the CSF and post-mortem brain tissue of patients with schizophrenia (Plitman *et al.*, 2017) and has also been found to be raised in peripheral blood samples of such patients (Fazio *et al.*, 2015; Ravikumar *et al.*, 2000), though results are not so consistent (Myint *et al.*, 2011; Barry *et al.*, 2009; Fukushima *et al.*, 2014). Although we did not subject patients to a specific stressor, it is possible that the social interaction involved in meeting a clinician prior to blood-sampling could have precipitated an elevation in KYNA, which was not seen in controls.

Other explanations for the raised plasma KYNA in SAD patients could exist. Physical exercise induces KAT expression in skeletal muscle and raises peripheral KYNA levels (Agudelo *et al.*, 2014; Agudelo *et al.*, 2019). However, given that our SAD patients had significantly lower exercise levels than controls this is unlikely to be a factor here. Administration of β-agonists can increase central KYNA levels in rats (Luchowska *et al.*, 2009). Thus, although hypothetical, it is plausible that excessive β-adrenergic receptor stimulation in the context of an overactive sympathetic nervous system activity, as seen in SAD (García-Rubio *et al.*, 2017; van Veen *et al.*, 2008), could result in an elevation of KYNA. Another source of peripheral KYNA concentration could be the gastrointestinal tract (GIT). KYNA is found in

certain foods and herbs and can be ingested directly in the diet (Turski *et al.*, 2009). Potatoes are a particularly rich source of KYNA (Turski *et al.*, 2012), as are broccoli and honey (Turski *et al.*, 2009). Furthermore, certain gut bacteria possess KAT activity (Han, Fang and Li, 2001) and can produce and release KYNA into the gut lumen (Kuc *et al.*, 2008). In addition, an important site of KYNA degradation appears to be the GIT (Kaihara and Price, 1962), most likely mediated by the gut microbiota (Hayaishi *et al.*, 1961; Dagley and Johnson, 1963). Thus, the gut dysbiosis seen in many neuropsychiatric disorders (Butler, Cryan and Dinan, 2019) could also contribute to abnormal KYNA levels both through direct synthesis and impaired metabolism of the compound. Interestingly, a recent meta-analysis involving studies of healthy individuals and patients with a variety of clinical conditions, reported that pro- and prebiotic supplementation can affect tryptophan-kynurenine pathway metabolites (Purton *et al.*, 2021), thus supporting a role for the MGB axis in the regulation of tryptophan metabolism.

An immune-mediated mechanism may also be at play. We did not find any elevation in pro-inflammatory cytokines which could possibly explain an IDO-mediated increase in KP activation in SAD patients. However, the anti-inflammatory cytokine, IL-10, reduces LPS-induced IDO expression in mice (Jung *et al.*, 2009). Thus, it is possible that the low levels of IL-10 seen in the SAD group could, through reduced inhibition of IDO, stimulate KP activity with subsequent downstream KYNA production. A negative feedback mechanism may also propagate this system. KYNA has significant immunomodulatory properties (Wirthgen *et al.*, 2018) and can inhibit the production of IL-10 in mice (Metghalchi *et al.*, 2015), although in humans its main immunosuppressant effects appear to be directed toward the pro-inflammatory cytokines (Maes *et al.*, 2007). Interestingly, increased rates of SAD have been found in patients with immune-mediated inflammatory disorders such as inflammatory bowel disease (IBD), multiple sclerosis (MS) and rheumatoid arthritis (RA) (Reinhorn *et al.*, 2020), disorders which are also associated with abnormal KYNA levels (Hartai *et al.*, 2005; Forrest *et al.*, 2003; Parada-Turska, Zgrajka and Majdan, 2013) .

Given the emerging evidence of an association between KP metabolites and suicidality (Bryleva and Brundin, 2017), we explored for such associations in our SAD group. Although a history of depression in SAD patients was not associated with any difference in KP markers, those patients who had attempted suicide did have elevated KYN and KYN/TRYP ratio levels in comparison to those without a history of suicidality. A similar finding was first reported 10 years ago in depression (Sublette *et al.*, 2011) when a research team found elevated plasma KYN levels in patients with MDD and a history of suicide attempt compared to MDD patients with no history of suicidal behaviour. This association was confirmed in a later study of depressed adolescents (Bradley *et al.*, 2015) and subsequent work

has suggested that KP metabolites could serve as biomarkers of suicide risk (Erhardt *et al.*, 2013; Brundin *et al.*, 2016). Our results are in keeping with the emerging evidence that KP dysfunction may be associated with suicidal behaviour in some psychiatric patients.

Our findings of equivalent plasma pro-inflammatory cytokines, at baseline and after in vitro LPS-stimulation, in SAD patients and controls are consistent with the few case-control studies which have investigated immune marker levels in this disorder. A small study found no differences in serum IL-2 levels or soluble IL-2 receptors between subjects with generalized social phobia and healthy volunteers (Rapaport and Stein, 1994). Several years later the same author reported increased CD16 (natural killer) cell numbers in social phobia patients, although no changes in other lymphocyte markers (Rapaport, 1998). A more recent study investigated LPS-induced cytokine production in SAD and found no difference in IL-6, IL-8 or TNFα (Fluitman *et al.*, 2010) while a large population-based study reported that those with social phobia, in particular women, had lower levels of CRP and IL-6 in comparison to GAD, PD and agoraphobia (Vogelzangs *et al.*, 2013).

To our knowledge, no study has previously investigated levels of anti-inflammatory cytokines in SAD. IL-10 is a potent anti-inflammatory cytokine, which plays an important regulatory role in suppressing pro-inflammatory responses (Couper, Blount and Riley, 2008). It appears to be of relevance in depression with IL-10 knockout mice displaying increased depressive-like behaviour, which can be reversed by administration of IL-10 (Mesquita *et al.*, 2008). Additionally, peripheral IL-10 levels are reduced in GAD (Hou *et al.*, 2017; Vieira *et al.*, 2010) and PD (Hoge *et al.*, 2009), although elevated levels have been reported in post-traumatic stress disorder (PTSD) (Guo *et al.*, 2012; Hoge *et al.*, 2009). Importantly, a significant sex effect was present in our study, with lower IL-10 levels present in SAD males compared to healthy males but no such difference evident in females. It is well recognised that there are sex differences in immune responses (Klein and Flanagan, 2016) with higher IL-10 production previously reported in males (Torcia *et al.*, 2012). IL-4 is also an anti-inflammatory cytokine and thought to be important in the regulation of brain immunity (Gadani *et al.*, 2012). We did not find any differences in IL-4 between the SAD and control groups. However, interestingly there was a moderate positive association between baseline levels of IL-4 and both KYNA and the KYNA/KYN ratio in healthy controls which was not seen in SAD patients.

Our study is the first to investigate the KP in patients with SAD. Additionally, this is the first study to investigate such a broad range of cytokines, including anti-inflammatory cytokine profile, in such patients. However, several limitations must be recognised. Obviously, conclusions are limited by the single time-point, cross-sectional nature of this study and further evaluation of the immune-KP response to stressful events in SAD is warranted. Secondly, elevated peripheral KP metabolite levels

may not reflect corresponding central changes in SAD. While KYNA has a poor ability to penetrate the blood brain barrier, KYN can cross easily and the majority of central KYN is sourced from the periphery (Fukui *et al.*, 1991). A recent study found no correlation between plasma or CSF KYNA levels in patients with bipolar disorder or healthy controls. (Sellgren *et al.*, 2019). In contrast, central KYN levels correlated well with peripheral levels in patients on hepatitis C treatment with depressive symptoms (Raison *et al.*, 2010). Hence, increased peripheral KYNA levels in our SAD cohort may not reflect a corresponding increase in central KYNA levels although KYN differences are likely to indicate similar central changes. Unfortunately, investigation of CSF KP profile is limited by the necessity for an invasive lumbar puncture procedure. Another potential confounding factor in our study is that some patients were taking psychotropic medication. There is evidence that various serotonergic antidepressant medications may upregulate IL-10 levels (Kenis and Maes, 2002; Kubera *et al.*, 2001) and increase production of KYNA (Kocki *et al.*, 2012). We did not find any difference in these two markers between medicated and unmedicated SAD patients. However, those SAD patients taking psychotropic medication did have elevated plasma KYN levels, although this may have been reflective of higher social anxiety symptom scores in that subgroup.

5.6 Conclusion

The peripheral KP in patients with SAD is preferentially directed towards KYNA synthesis as evidenced by an elevated KYNA/KYN ratio and raised plasma levels of KYNA in these patients. KYNA levels have not previously been investigated in clinical anxiety disorders, although similar KP changes have been reported in preclinical stress studies and in clinical cohorts involving schizophrenia patients. Additionally, consistent with emerging literature on the association between the KP and suicidality, SAD patients with a history of suicide attempts have raised KYN levels and an increased KYN/TRYP ratio compared to those without a history of suicidal attempts. While patients with SAD do not exhibit any increase in circulating pro-inflammatory cytokines, we have shown for the first time that males with SAD have lower peripheral levels of the potent anti-inflammatory cytokine, IL-10 compared to healthy males. Further evaluation of the role of inflammation and the immune-kynurenine pathway in SAD through large prospective studies is warranted.

Chapter 6: Neuroendocrine Alterations in Social Anxiety Disorder

6.1 Abstract

Background: Despite being a common, chronic and severely disabling condition, research into the neurobiological basis of social anxiety disorder (SAD) is limited. In particular, the literature on neuroendocrine dysfunction in this disorder is scarce and inconsistent.

Methods: Several neuroendocrine markers were investigated in 32 patients with a clinical diagnosis of SAD and 36 healthy controls, including plasma oxytocin, the salivary cortisol awakening response (CAR), chronic hair cortisol concentrations and morning salivary alpha amylase (sAA) levels under basal non-stressed conditions. A variety of psychometric tests were administered to investigate associations between these markers and particular psychological parameters including social anxiety symptoms, general anxiety and stress, empathy, autistic traits and childhood trauma.

Results: Patients with SAD had lower plasma oxytocin levels in comparison to controls. A significant group*sex interaction was evident with SAD males having significantly lower oxytocin levels compared to healthy males while no difference between SAD and healthy females was evident. SAD patients exhibited a blunted CAR but higher hair cortisol concentrations than controls. In addition, SAD patients had a higher sAA level one-hour post waking. Levels of general anxiety and stress were associated with plasma oxytocin in the SAD group. SAD patients displayed higher levels of empathy and autistic traits, although no significant associations with oxytocin levels were found. SAD patients reported significantly higher levels of childhood trauma, which were associated with a reduced CAR.

Conclusions: Patients with SAD exhibit significant neuroendocrine alterations under basal non-stressed conditions. Further investigation using larger prospective studies is warranted to understand the impact and changing nature of neuroendocrine system in this early-onset, chronic anxiety disorder.

6.2 Introduction

Unfortunately, our knowledge in relation to the neuroendocrine basis for SAD is limited. Given that the age of onset for many sufferers is in the early teen years (Lijster *et al.*, 2017) and thus around the age of puberty, an exceptionally sensitive period for neurodevelopment, the influence of hormonal and neuroendocrine processes may be of particular significance. Oxytocin, a neuropeptide synthesized in the hypothalamus and involved in reproduction and social behaviour, has been the subject of much interest in psychiatry in recent years, particularly in disorders which involve impairments in social functioning including schizophrenia, autism spectrum disorder (ASD) and SAD. In addition, oxytocin has been shown to play a significant role in regulating anxiety and the stress response, leading to interest in its therapeutic potential in anxiety disorders (Naja and Aoun, 2017). Although there is a large body of preclinical work investigating the role of oxytocin in anxiety and social behaviour, few studies have analysed plasma levels of oxytocin in patients with relevant psychiatric disorders and results are somewhat inconsistent. There have been several small studies investigating plasma oxytocin levels in patients with SAD with results varying from no difference (Hoge *et al.*, 2008; Çetinkaya, Üneri and Göker, 2018) to both lower (Hoge *et al.*, 2012) and higher (Oh *et al.*, 2018) levels in comparison to controls. However, the majority of these studies used enzyme immuno-assay techniques with unextracted samples which have been shown to produce highly unreliable results (Leng and Sabatier, 2016).

Abnormalities in hypothalamic-pituitary-adrenal (HPA) axis function have been widely studied in depression and are considered important aetiological factors (Herbert, 2013). However, there has been far less research of HPA-axis dysfunction in anxiety disorders, despite the significant overlap between depressive and anxiety disorders in terms of comorbidity (Lamers *et al.*, 2011), symptomatology (Eysenck and Fajkowska, 2018) and risk factor profiles (Blanco *et al.*, 2014). In particular, dysfunction of this system should be of major interest in SAD given that stressors with a socio-evaluative basis elicit stronger HPA-axis responses than other types of stressors (Dickerson and Kemeny, 2004). One method of investigating HPA-axis activity is by measuring the cortisol awakening response (CAR), a distinct pattern of increase in cortisol levels seen in the first 30-45 minutes after awakening (Wilhelm *et al.*, 2007; Pruessner *et al.*, 1997). Few studies have investigated the CAR in patients with SAD with results to date suggesting a lack of difference in comparison to controls (van Veen *et al.*, 2008; Vreeburg *et al.*, 2010; Hek *et al.*, 2013). An alternative measure of more long-term HPA-activity is the analysis of hair cortisol concentrations. Human hair grows on average 1cm/month (Pecoraro, 1990) so analysis of the 1cm of hair closest to the scalp provides information on cortisol levels over the preceding month. Only one study has measured hair

cortisol concentrations in patients with SAD and reported no differences in comparison to healthy controls (Klumbies *et al.*, 2014).

Another facet of neuroendocrine dysfunction of relevance to anxiety disorders is the autonomic nervous system (ANS). It consists of two branches, the sympathetic nervous system (SNS) which mediates the 'fight or flight' response to stress, and the parasympathetic nervous system (PNS) which facilitates recovery from stress and a more relaxed 'rest and digest' state (Wehrwein, Orer and Barman, 2016). Two studies which did not find any differences in HPA-axis parameters in SAD patients, did report altered ANS markers (van Veen *et al.*, 2008; Tamura *et al.*, 2013) raising the possibility that ANS dysfunction may be of more relevance in SAD than HPA-axis abnormalities. Salivary alpha amylase (sAA) has gained recognition in recent years as a simple, non-invasive, valid measure of ANS activity and has become popular as a biomarker in stress research. The sAA awakening response is in the opposite direction to the CAR with an observed decrease in the first 60 minutes after awakening and a rise in levels thereafter (Nater *et al.*, 2007). Basal levels of sAA appear to be elevated in SAD (van Veen *et al.*, 2008; Tamura *et al.*, 2013) although studies investigating sAA responses to the Trier Social Stress Test (TSST) have reported no differences (Klumbies *et al.*, 2014; Krämer *et al.*, 2012; García-Rubio *et al.*, 2017).

In this study, we investigate a broad range of neuroendocrine markers in a clinical population of patients with SAD. To our knowledge, this is the first study to simultaneously investigate such an array of basal, non-stressed neuroendocrine markers, including plasma oxytocin (extracted samples), salivary CAR, chronic hair cortisol levels and the sAA awakening response in this population. In addition, we administered a broad spectrum of psychometric scales which allowed us to explore associations between these biological markers and various psychological parameters including stress, anxiety and depressive symptoms along with childhood trauma, autistic traits and empathy measures.

6.3 Methods

Participants and Procedures

Recruitment and assessment of patients and controls were as outlined in Chapter 2. While we included subjects taking hormonal contraception in the analysis of cortisol and alpha amylase, we excluded these subjects (3 from the SAD group, 8 from the control group) for oxytocin analysis.

Biological Samples

Salivary CAR and alpha amylase:

Morning saliva samples were collected using the Salivette system (Sarstedt, Germany). Participants were instructed to collect the saliva samples in the morning of the day of their study visit. A total of four samples was collected, with the first one collected upon awakening, the second one 30 minutes later, the third at 45-minutes post-waking and the fourth at 60-minutes post-waking. Participants were instructed to not brush their teeth until after all saliva samples were collected, to not eat or drink anything prior to the first sample, and to avoid eating and drinking 15 min prior to the remaining samples. Saliva samples were centrifuged at 1500g for 5 min, aliquoted, and stored at−80 °C for later analysis.

Salivary cortisol was analysed in duplicate using the ENZO Life Sciences enzyme-linked immunosorbent assay (ELISA) kits (Exeter, UK) as per manufacturer's instructions. The lower limit of detection was 0.156nmol/L. Inter- and intra-assay coefficients of variability were 13.4% and 10.5%.

Salivary alpha amylase was measured using a kinetic enzyme assay that utilizes a chromagenic substrate, 2-chloro-p-nitrophenol, linked to maltotriose. The procedure was carried out according to the manufacturer's instructions [Salimetrics, State College, PA, USA]. sAA data are expressed in U/mL.

Plasma oxytocin:

Blood was collected in EDTA-tubes and centrifuged at 1500g for 10 minutes. The plasma was then aliquoted into 1ml plastic tubes, capped and frozen at -80 °C. Oxytocin concentration was measured, following extraction, using a radioimmunoassay performed by RIAgnosis (Sinzing; Germany).

Hair cortisol:

We analysed the centimetre of hair closest to the scalp. Hair samples were weighed, cut into small pieces and placed into tubes. 1 mL of methanol was added to each tube in a fume hood and incubated at 50°C overnight (ON). Each tube was sonicated for 30 minutes, then placed on heated shaker for 90 minutes at 50°C with a final incubation ON at 50°C. Methanol was transferred into a clean 2 mL tube and evaporated to dryness under nitrogen for 90 minutes at 37˚C and the residue reconstituted in 250 µL PBS with vortexing. Extracted samples were stored at -80˚C prior to analysis. Cortisol concentration was determined by the ENZO Life Sciences ELISA kits (Exeter, UK) as per manufacturer's instructions.

<u>Statistics</u>

All data was analysed using SPSS 25 (IBM, Armonk, NY, USA). Visual inspection of box plots was used to identify outliers and consideration given to removal of those lying more than three times the interquartile range (IQR) below the first quartile or above the third quartile. Missing values were excluded from analysis. Areas under the curve (AUC) for cortisol and alpha amylase measurements were calculated with respect to ground (AUCg) and increase (AUCi) (Pruessner *et al.*, 2003). Normality of data was assessed by visual inspection of histograms along with examination of skewness and the Shapiro-Wilk statistic. Data was transformed by various methods based on the direction and extent of the skew, including a square-root transformation for AUCg cortisol data and a log10 transformation for alpha amylase and hair cortisol data. Independent T-tests were used to compare means between groups where assumptions were met. Welch corrections were used where there was violation of homogeneity of variances. Where parametric assumptions were violated, Mann-Whitney U tests were applied. A Related-Samples Friedman's Two-way Analysis of Variance by Ranks test with pairwise comparisons was used to determine if there were differences in cortisol and sAA concentration at the four different time-points. Univariate general linear modelling was used to compare means while adjusting for fixed factors such as gender and covariates including age, BMI and exercise. Bonferroni adjustment was used to correct for multiple comparisons. Correlations were carried out using the Pearson correlation coefficient for normally distributed data and the Spearman rank sum for non-parametric data. Partial correlations were undertaken to account for confounding factors. Data are presented as mean ± SD unless stated otherwise. For descriptive purposes, summary statistics are reported in original units (in the text and in figures) along with statistical results from analysis of transformed data.

6.4 Results

Thirty-two patients with SAD and 36 healthy controls participated in the study and demographic characteristics were as outlined in Chapter 2. However, given that we excluded subjects taking hormonal contraception for oxytocin analysis, these subgroups involved 29 SAD patients and 28 healthy controls. The differences in demographic characteristics did not change with these smaller groups and can be viewed in Table 1.

Table 1: Demographic Characteristics and Psychological Scales for SAD and control groups, excluding participants taking hormonal contraception

	SAD (n=29)	**Controls (n=28)**	**p-value**
Age (years); mean (SD)	37.14 (11.5)	34.29 (13.4)	0.40
Gender; % female (n)	41.4 (12)	42.9 (12)	0.91
Race; % Caucasion (n)	100 (30)	88 (22)	0.44
Years of Education; mean (SD)	17.55 (4.2)	17.75 (4.3)	0.86
BMI (units); mean (SD)	27.2 (4.5)	24.0 (4.1)	0.008 *
Alcohol (units per week); mean (SD)	5.52 (7.3)	5.16 (6.2)	0.84
Smoking status; % smokers (n)	13.8 (4)	7.1 (2)	0.41
Exercise (IPAQ score)	3465.5 (3957.9)	5336.1 (3890.1)	0.008 *
Sleep Quality (PSQI score); mean (SD)	7.7 (4.4)	4.6 (3.8)	0.013 *
Comorbidity: % (n)			
Past history of MDD	76 (22)	N/A	
Other anxiety disorder	31 (9)	N/A	
• Agoraphobia	24 (7)	N/A	
• GAD	3.4 (1)	N/A	
• Panic Disorder	3.4 (1)	N/A	
• Multiple	0 (0)	N/A	

Psychotropic medication % (n)
• No medication
• Taking medication
  ○ SSRI
  ○ SNRI
  ○ Other regular anxiolytic
  ○ PRN Beta-blocker
  ○ PRN Benzodiazepine
	
	38 (11)
	62 (21)
	44.8 (13)
	9.4 (3)
	20.7 (6)
	10.3 (3)
	6.3 (2)
		
		100 (36)
		0 (0)
		N/A
		N/A
		N/A
		N/A
		N/A	

Social anxiety scale scores			

LSAS, mean (SD)
  ○ Fear Subscale
  ○ Avoidance subscale
  ○ Social interaction subscale
  ○ Performance subscale
  ○ Total
	
	43.03 (12.5)
	36.31 (13.5)
	36.86 (13.5)
	42.17 (14.4)
	79.38 (25.3)
		N/A	

FQ, mean (SD)	40.5 (14.7)	N/A	
FNE	49 (8.7)	N/A	
Psychological scale scores			
• PSS, mean (SD)	20.3 (7.2)	8.2 (4.5)	<0.0005 *
• STAI-S, mean (SD)	45.5 (12.5)	26.2 (6.3)	<0.0005 *
• STAI-T, mean (SD)	55.3 (11.5)	28.7 (5.8)	<0.0005 *

• HADS-D, mean (SD)	6.5 (4)	1.4 (1.6)	<0.0005 *
• HADS-A, mean (SD)	9.1 (3.8)	2.8 (3.2)	<0.0005 *
• HADS-T, mean (SD)	15.6 (7.2)	4.1 (4.4)	<0.0005 *
Personality Scales			
• AQ, mean (SD)	22.4 (6.9)	12.5 (6.4)	<0.0005 *
• Davis IRI; mean (SD)			
○ Perspective taking	24.9 (5.2)	24 (6.1)	0.247
○ Fantasy	21.1 (6)	19.4 (5.8)	0.431
○ Empathic Concern	27.4 (4.9)	23.5 (5.5)	0.003 *
○ Personal Distress	23.9 (5.4)	11.3 (5)	<0.0005*
• Childhood trauma questionnaire			
○ Emotional Abuse	12.2 (5.8)	7 (2.3)	<0.0005*
○ Physical Abuse	7.1 (3.2)	5.8 (1.4)	0.131
○ Sexual Abuse	6.5 (4.2)	5 (0)	0.015*
○ Emotional Neglect	13.4 (5.)	7.1 (2.5)	<0.0005*
○ Physical Neglect	7.4 (2.8)	5.6 (1.2)	0.004*
○ Total	46.7 (14)	30.5 (5.4)	<0.0005*

(AQ: Autism Quotient, BMI: Body Mass Index, CTQ: Childhood Trauma Questionnaire, GAD: Generalised Anxiety Disorder, HADS: Hospital Anxiety and Depression Scale; HADS-A: HADS-Anxiety, HADS-D: HADS-Depression, HADS-T: HADS-Total, IRI: Interpersonal Reactivity Index, LSAS: Liebowitz Social Anxiety Scale, PSS: Perceived Stress Scale, MDD: Major Depressive Disorder, SAD: Social Anxiety Disorder, SD: Standard Deviation, SNRI: Serotonin and Norepinephrine Reuptake Inhibitor, SSRI: Selective Serotonin Reuptake Inhibitor, STAI: State-Trait Anxiety Inventory, STAI-S: STAI-State, STAI-T: STAI-Trait)

Plasma oxytocin concentration

Mean oxytocin levels were lower in the SAD group (1.93 ± 0.17 pg/ml) compared to healthy controls (2.17 ± 0.44 pg/ml), a statistically significant difference mean difference of -0.24 pg/ml (t(34.704)= -2.968, p=0.011) (Figure 1A). Whilst controlling for age, BMI and exercise, a significant group*sex interaction was seen (F(1,43)=4.391, p=0.042). SAD males showed significantly lower oxytocin levels compared to healthy males (mean difference -0.410 pg/ml, p=0.003, 95% CI [-0.671, -0.149]) while no such group difference was evident in females (mean difference -0.01 pg/ml, p=0.95, 95% CI [-0.308, 0.289]). No differences were seen between males and females in the SAD group (mean difference 0.013 pg/ml, p=0.93, 95% CI [-0/262, 0.288]) while healthy males had significantly higher oxytocin levels compared to healthy females (mean difference 0.413 pg/ml, p=0.003, 95% CI [0.144, 0.682]) (Figure 1B).

Figure 1: Plasma oxytocin levels in SAD patients compared to healthy controls

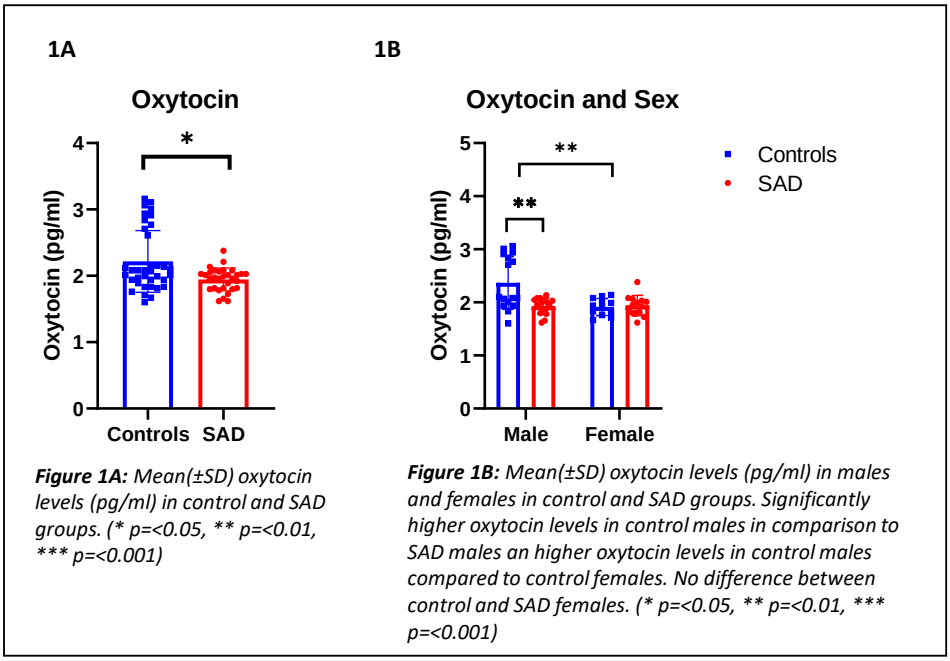


Figure 1A: *Mean(±SD) oxytocin levels (pg/ml) in control and SAD groups. (* p=<0.05, ** p=<0.01, *** p=<0.001)*

Figure 1B: *Mean(±SD) oxytocin levels (pg/ml) in males and females in control and SAD groups. Significantly higher oxytocin levels in control males in comparison to SAD males an higher oxytocin levels in control males compared to control females. No difference between control and SAD females. (* p=<0.05, ** p=<0.01, *** p=<0.001)*

Patients with SAD who had a past history of depression (n=22, 76%) had lower levels of oxytocin (1.89 ± 0.15 pg/ml) in comparison to those without a past history of depression (2.05 ± 0.18 pg/ml), a statistically significant difference of 0.156 pg/ml (t(26) = 2.245, p = .034). There was no difference in mean oxytocin levels between those with or without a comorbid anxiety disorder (t(26)=0.058, p=0.95). Patients taking medications had slightly lower oxytocin levels (1.88 ± 0.14) than unmedicated patients (2.01 ± 0.2), a mean difference of 0.13 pg/ml (t(26)=2.087, p=0.047)

No association between oxytocin levels and social anxiety symptom severity, based on the LSAS total score, was seen in the SAD group (Rs(28) -0.1, p=0.63). However, within the SAD group, oxytocin levels were positively correlated with several measures of stress and general anxiety based on PSS (R(28) 0.622, p=<0.001), STAI-S (R(26)= 0.437, p=0.025), HADS-Anxiety subscale (R_s(26)=0.464, p=0.017) scores. The strength of these relationships remained unchanged after controlling for sex. Associations between oxytocin levels and these psychological scales were not seen in the control group (data not shown). No associations were seen between oxytocin and the AQ or Davis IRI empathy scales, in either group (data not shown).

Salivary cortisol awakening response

A Friedman test was run to determine if there were differences in cortisol concentration at the four different time-points in the hour post-waking, and thus if a cortisol awakening response was present in the control and SAD groups. Pairwise comparisons were performed with a Bonferroni correction for multiple comparisons. In the healthy control group, cortisol concentrations were significantly different at the four morning time points ($\chi^2(3) = 8.88$, $p = 0.031$). Post hoc analysis revealed a statistically significant difference between CAR2 (median=8.87) and CAR4 concentration (median=8.12) while the differences between other CAR time-points were not statistically significant. A similar pattern was seen in the SAD group with an overall significant difference between time points ($\chi^2(3) = 10.59$, $p = 0.014$) and, following Bonferroni correction, a statistically significant difference between CAR2 (median=5.54) and CAR4 (median=3.85).

Subjects in the SAD group showed a significantly lower salivary CAR. Although there was no significant difference between groups in cortisol levels immediately upon waking (t(50.4) = -1.64, p=0.107), cortisol levels were significantly lower in the SAD group 30 minutes post-waking (t(51.2)= -2.76, p=0.008), 45 minutes post-waking (t(53.9) = -2.60, p=0.012) and 60 minutes post-waking (t60.3= -2.03, p=0.047) (Figure 3). AUCg analysis showed that patients with SAD exhibited reduced total cortisol output (t(59)= -2.515, p=0.015) (Figure 2B). These significant differences remained for the 30-minute (F(1,57)=3.878, p=0.05) and 60-minute (F(1,54)=4.430, p=<0.0005) post-waking timepoints as well as for AUCg (F(1,54)=4.337, p=0.042) after adjusting for sex, age, BMI and exercise. There was no significant difference between groups with respect to AUCi analysis (t(37) = -1.37, p=0.178) (Figure 2C).

Figure 2: The Cortisol Awakening Response (CAR) in SAD patients compared to healthy controls

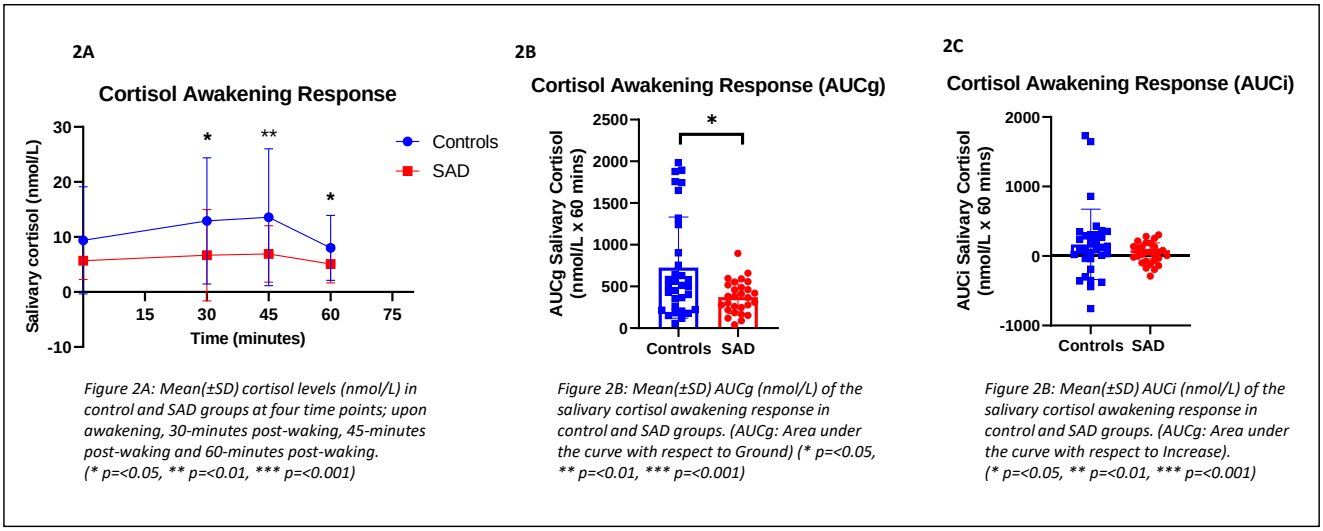


Figure 2A: Mean(±SD) cortisol levels (nmol/L) in control and SAD groups at four time points; upon awakening, 30-minutes post-waking, 45-minutes post-waking and 60-minutes post-waking. (p=<0.05, ** p=<0.01, *** p=<0.001)*

Figure 2B: Mean(±SD) AUCg (nmol/L) of the salivary cortisol awakening response in control and SAD groups. (AUCg: Area under the curve with respect to Ground) (p=<0.05, ** p=<0.01, *** p=<0.001)*

Figure 2B: Mean(±SD) AUCi (nmol/L) of the salivary cortisol awakening response in control and SAD groups. (AUCg: Area under the curve with respect to Increase). (p=<0.05, ** p=<0.01, *** p=<0.001)*

No differences were seen in the CAR AUCg between those with or without a history of MDD (t(26) = -0.816, p=0.422), those taking psychotropic meds and those not (t(26) = -0.392, p=0.698) and those patients with or without a comorbid secondary anxiety disorder (t(26) = 1.334, p=0.194).

There were no correlations seen between the CAR AUCg and the LSAS, PSS, STAI or HADS scores in either group (data not shown). Positive correlations were between CAR AUCg and the sexual abuse (Rs(27)=0.419, p=0.03) and physical neglect (Rs(24)=0.419, p=0.042) subscales of the Childhood Trauma Questionnaire (CTQ) in the SAD group.

Hair Cortisol Concentration

Hair cortisol levels were higher in the SAD group (89.6 ± 66.9 pg/mg) than in controls (38.9 ± 29.7 pg/mg), a mean difference of 50.65 pg/mg (t(52)=2.293, p=0.026) (Figure 3). After controlling for age, sex, BMI and exercise, the significance of the difference reduced slightly to the level of a trend (F(1,45)=3.811, p=0.057).

Figure 3: Hair cortisol concentrations in SAD patients compared to healthy controls

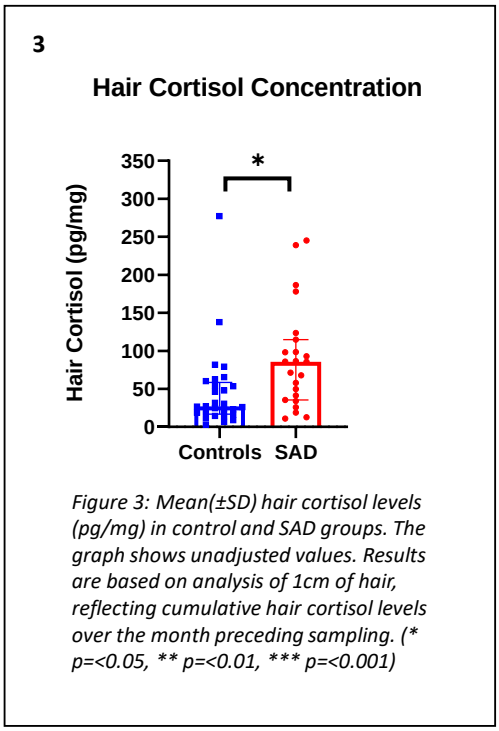


Figure 3: Mean(±SD) hair cortisol levels (pg/mg) in control and SAD groups. The graph shows unadjusted values. Results are based on analysis of 1cm of hair, reflecting cumulative hair cortisol levels over the month preceding sampling. (p=<0.05, ** p=<0.01, *** p=<0.001)*

No differences were seen in hair cortisol levels between those with or without a history of MDD ($t21=1.32$, $p=0.20$), those taking psychotropic meds and those medication-free ($t21=0.365$, $p=0.72$), and those patients with or without a comorbid secondary anxiety disorder ($t21=0.146$, $p=0.885$).

There were no associations seen between hair cortisol and the LSAS, PSS, STAI, HADS or CTQ scales (data not shown).

Salivary alpha amylase awakening response

sAA concentration was significantly different at the different time-points in the control group ($\chi^2(3) = 11.81$, $p= 0.008$). Post-hoc analysis revealed that waking sAA (median=66.34) was significantly higher ($p=0.004$) than 60-minute sAA concentration (median=31.65) with no difference evident between other time-points. No difference in sAA at the various time-points was seen in the SAD group ($\chi^2(3) = 5.28$, $p= 0.15$).

Although sAA concentrations were higher in the SAD group at all time points, these differences were not statistically significant immediately upon waking ($t(62) = 0.40$, $p=0.690$), 30 minutes post-waking ($t(57.5)= 0.173$, $p=0.863$) or 45 minutes post-waking ($t(62) = 1.77$, $p=0.24$). However, sAA levels in the SAD group (1.7±0.316) were significantly higher than the control group (1.48±0.531) at 60 minutes post-waking ($t(58.38)= 2.31$, $p=0.024$) (Figure 4A), a difference which remained after adjusting for age, gender, BMI and exercise ($F(1,56)=7.857$, $p=0.007$). No differences between the groups were seen in relation to sAA AUCg ($t59= 1.32$, $p=0.19$) (Figure 4B) or AUCi ($t60= -0.69$, $p=0.49$).

Figure 4: Salivary Alpha Amylase (sAA) concentrations in SAD patients compared to healthy controls

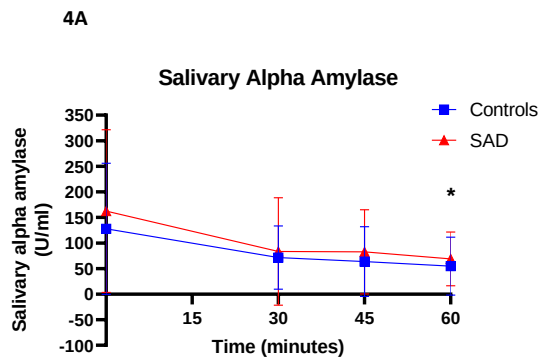


Figure 4A: Mean(±SD) alpha amylase levels (nmol/L) in control and SAD groups at four time points; upon awakening, 30-minutes post-waking, 45-minutes post-waking and 60-minutes post-waking. A significant difference is seen between the two groups at the 60-minutes post-waking time point (p=<0.05, ** p=<0.01, *** p=<0.001)*

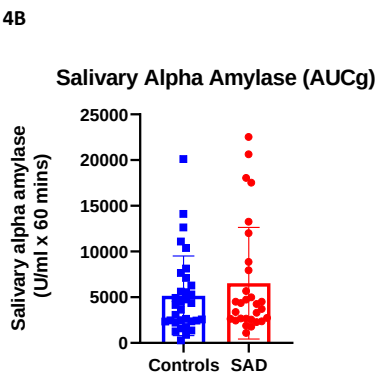


Figure 4: Mean(±SD) AUCg (U/ml) of the salivary alpha amylase awakening response in control and SAD groups. (AUCg: Area under the curve with respect to ground)
(p=<0.05, ** p=<0.01, *** p=<0.001)*

No differences were seen in the sAA awakening response (at any time point or in the cumulative measures) between those taking psychotropic medications and unmedicated patients. Similarly, no differences were seen between those SAD patients with or without a history of depression or between those with or without a comorbid anxiety disorder (p>0.05 at all time-points and for AUCg and AUCi).

6.5 Discussion

The present study demonstrates that patients with SAD exhibit significant neuroendocrine differences in comparison to healthy controls under basal, non-stressed conditions. Such differences include lower plasma oxytocin levels, a blunted salivary CAR, higher one-month hair cortisol levels and higher sAA concentrations one-hour post-waking. Given the paucity of studies investigating the neuroendocrine basis for SAD, these results provide important aetiological clues for a common, complex and chronic condition.

<u>Oxytocin in SAD</u>

Firstly, SAD patients had lower plasma oxytocin levels in comparison to healthy controls. Although oxytocin is of major interest in anxiety and in conditions associated with social difficulties, few studies have analysed plasma levels of oxytocin in patients with SAD and results are somewhat inconsistent. Two studies have reported no difference in plasma oxytocin between SAD patients and

controls (Hoge *et al.*, 2008; Çetinkaya, Üneri and Göker, 2018) while others have reported both lower (Hoge *et al.*, 2012) and higher (Oh *et al.*, 2018) basal concentrations. One probable reason for the inconsistency is variation in assay methods. There has been much debate with regards to oxytocin assays, the primary issue being whether or not an extraction step (to eliminate interfering substances present in the samples) prior to enzyme immunoassay (EIA) or radioimmunoassay (RIA) (McCullough, Churchland and Mendez, 2013). Unextracted plasma oxytocin values are often 100-fold higher than unextracted samples and considered to be highly erroneous and biologically implausible, going from 1.8 pg/mL after extraction up to 358 pg/mL without extraction (Szeto *et al.*, 2011). Three of the four prior studies investigating plasma oxytocin in SAD used unextracted samples for EIA and reported mean baseline levels ranging from 51.6 to 354 pg/ml (Çetinkaya, Üneri and Göker, 2018; Hoge *et al.*, 2008; Hoge *et al.*, 2012). In comparison, using RIA with extraction, considered the foundational gold standard method for peripheral oxytocin measurement (Lefevre *et al.*, 2017), plasma concentrations are almost always <10 pg/ml and in the range of ∼1–3 pg/ml for most people (Szeto *et al.*, 2011). Our results are consistent with these values. The fourth aforementioned study (Oh *et al.*, 2018) did use an extraction step prior to EIA but still reported high values of >100pg/mL, levels far in excess of previously reported concentrations using a similar EIA with extraction technique (Grewen, Davenport and Light, 2010; Holt-Lunstad, Birmingham and Light, 2008). Thus, our study is the first to measure plasma oxytocin in SAD using the gold standard method of RIA with prior extraction and the first to produce biologically plausible values which are consistent with the human literature.

Interestingly, those patients who had a past history of depression had significantly lower oxytocin levels in comparison to those without a past history of depression. A similar finding was reported by Hoge et al (2012) although their results were not statistically significant. Although the human literature on oxytocin and depression is inconsistent and insufficient, the majority of studies of depressed women, both pregnant and non-pregnant, suggest an inverse relationship between oxytocin levels and depressive symptoms (Massey, Backes and Schuette, 2016). However, sex differences appear to exert an important influence with the direction of the relationship between oxytocin and depression in men apparently going in the opposite direction (Yuen *et al.*, 2014). We found that sex significantly impacted oxytocin levels with our group difference being driven by higher values in healthy males. This is consistent with a previous large cohort study involving 473 participants which demonstrated significantly higher oxytocin levels in men and found that sex was an important moderator in the relationship between anxiety and oxytocin (Weisman *et al.*, 2013). Sex influences may also explain some of the discrepancies in oxytocin differences reported in several other SAD studies. A small study in children with SAD, which reported no difference in oxytocin

levels in comparison to controls (Çetinkaya, Üneri and Göker, 2018), involved groups which were unmatched with respect to gender while a Korean study reporting higher oxytocin levels in SAD patients used an exclusively male cohort (Oh *et al.*, 2018).

Although we found no association between plasma oxytocin levels and social anxiety symptom severity, we did note positive correlations between several general anxiety and stress measures including PSS, STAI-S and HADS-A scores. The relationship between plasma oxytocin, stress and anxiety is complex and incompletely understood. On one hand, elevated peripheral oxytocin levels have been shown to be an indicator of the acute stress response in humans, for example, in response to psychosocial stress (Jong *et al.*, 2015; Pierrehumbert *et al.*, 2010), physical exercise (Jong *et al.*, 2015) and uncontrollable noise stress (Sanders, Freilicher and Lightman, 1990). On the other hand, there is evidence that intranasal oxytocin has significant anxiolytic properties, inhibiting the stress-induced HPA response in response to social rejection (Linnen *et al.*, 2012), psychosocial stress (Heinrichs *et al.*, 2003; Quirin, Kuhl and Düsing, 2011), physical stress (Cardoso *et al.*, 2013) and interpersonal conflict (Ditzen *et al.*, 2009), with evidence that this effect of dampening the cortisol response is most pronounced in events that elicit a robust HPA-axis response and in clinical populations as opposed to healthy cohorts (Cardoso, Kingdon and Ellenbogen, 2014). A possible unifying theory was recently suggested by a German team (Engert *et al.*, 2016) who reported that stress-induced peripheral oxytocin elevation resulted in faster vagal rebound and parasympathetic recovery, rather than reduced cortisol reactivity following the stress. Thus, the stress-buffering effect of oxytocin may occur predominantly in later stages of the stress response and the initial stress-induced elevation in plasma oxytocin may boost stress recovery rather than reduce the early stress reactivity response. In the context of SAD, lower basal peripheral oxytocin levels may reflect an impaired ability to respond to stress with oxytocin elevation and subsequent impaired stress recovery. This hypothesis is supported by a recent study (Ho *et al.*, 2020b) which reported that dominant SNS activity during stress recovery is a key physiological mechanism which specifically mediates associations between stress sensitivity and social anxiety symptoms in females. Similar aberrant parasympathetic recovery responses to psychosocial stress are seen in schizophrenia (Andersen, Lewis and Belger, 2018), a disorder also characterised by low peripheral oxytocin levels (Liu *et al.*, 2019).

Oxytocin has been shown to play an important role in the development and regulation of social bonding and empathy. Our SAD patients had significantly higher scores on the 'Personal Distress' and 'Empathic Concern' subscales of the IRI, a tool used for multidimensional assessment of empathy (Davis, 1983). While the finding of low plasma oxytocin levels and elevated empathy scores

in our SAD group may seem contradictory given the widely-accepted idea that oxytocin is a prosocial, pro-empathic neuropeptide, the relationship is not so simple. Although intranasal oxytocin certainly has prosocial influences, it has also been shown to produce more contradictory effects such as an increase in envy and gloating (Shamay-Tsoory *et al.*, 2009), derogation of those outside one's group (De Dreu *et al.*, 2011) and reduced trust and cooperation in people with borderline personality disorder (Bartz *et al.*, 2011a). It is suggested that this variability in social and emotional effects may be explained by an ability of oxytocin to increase sensitivity to social salience cues, which will produce differential effects based on contextual and inter-individual factors (Bartz *et al.*, 2011b). When social cues in a particular environment are perceived as 'safe' by an individual, oxytocin may promote pro-social reactions but when social cues are interpreted as negative and threatening the opposite may occur (Olff *et al.*, 2013). For those with SAD, who are generally hypersensitive to social cues and have a bias towards negative interpretation (Chen, Short and Kemps, 2020), oxytocin may actually stimulate a more defensive and distrustful response. Thus, lower basal oxytocin levels may actually represent an adaptive response to chronic social stress in these patients. Given that the source of peripheral oxytocin is the CNS (synthesized in the hypothalamus with release into the blood via the posterior pituitary) a key consideration is to what extent peripheral oxytocin levels reflect central levels. A recent meta-analysis reported positive correlations between plasma and central oxytocin levels. However, this correlation was only seen across studies involving intra-nasal oxytocin administration and following a stressor, and not under basal conditions (Valstad *et al.*, 2017).

Cortisol in SAD

The CAR, which can be defined as the period of cortisol secretory activity in the first 60 min immediately post-awakening (Clow *et al.*, 2004), typically involves a significant peak in cortisol concentration in the first 30 minutes after waking (Pruessner *et al.*, 1997). While we did observe this cortisol peak, visible as an increase from CAR1 (waking sample) to CAR2 (30 minutes post-waking), this was not statistically significant on post-hoc analysis. However, and consistent with the expected CAR profile, we did see a statistically significant difference between CAR2 and CAR4 (60 minutes post-waking) in both groups, whereby CAR2, was significantly higher than CAR4. A possible explanation for the lack of significant increase between CAR1 and CAR2 may be the be that the CAR1 was not collected immediately on waking and thus not representative of a true awakening baseline value. Although participants were instructed to collect CAR1 immediately upon waking, collection was done at home and not under monitoring. Detection of the CAR requires adequate participant adherence to the sampling schedule including strict referencing to the time of waking and duration

of sampling (Smyth *et al.*, 2015; Stalder *et al.*, 2016). Approximately, one quarter of people do not show a CAR, and while these people may be true non-responders, it has been suggested that it may also be due to missing the true awakening time, for example, if participants wake but then doze for a short period before they start to collect the first saliva sample or if they take longer than recommended to provide the samples (Wüst *et al.*, 2000). Most CAR sampling occurs in the domestic setting and it has recently been proposed that electronic monitoring would be helpful to verify the time of waking (Smyth *et al.*, 2016). Other potential confounding factors about which we did not have information included sleep duration, time of awakening and the use of an alarm clock. However, these features do not appear to influence the CAR (Pruessner *et al.*, 1997; Wüst *et al.*, 2000) although sleep quality is a potentially important factor we cannot rule out (Fekedulegn *et al.*, 2018).

It is interesting that we see opposite findings in the CAR and hair cortisol levels in this study. While the CAR was blunted in our SAD group, they exhibited higher hair cortisol levels. It is necessary here to consider the different components of HPA-axis output. The CAR is a distinct feature of the diurnal cortisol pattern and involves a sharp increase after awakening (Pruessner *et al.*, 1997). Levels of cortisol then tend to decrease gradually throughout the day with lowest levels seen just before bedtime, the degree of change between morning and evening being referred to as the diurnal cortisol slope (DCS) (Adam *et al.*, 2017). However, superimposed on this rhythmic pattern of basal cortisol release are the acute reactive cortisol bursts precipitated by stressful events during the day (Dickerson and Kemeny, 2004). A recent study found that 1-month hair cortisol concentration correlated significantly with total integrated salivary cortisol concentrations measured repeatedly at several time-points throughout the day over the same 1-month period (Short *et al.*, 2016). However, hair cortisol concentration did not correspond to the CAR or the DCS, suggesting that it was driven by something other than just these chronobiological metrics. The authors did not measure cortisol reactivity in this study and thus could not account for the impact of stress-induced fluctuations in cortisol on chronic hair cortisol concentrations. Thus, it seems likely that the higher hair cortisol levels seen in our SAD group are more reflective of excessive cortisol reactivity to stressful conditions than representative of any increase in basal diurnal cortisol measures. On the contrary, we found a blunted CAR in this patient group. However, we did not measure basal cortisol levels at other times of day so cannot exclude that the higher hair cortisol concentrations were not due to an altered DCS. Of relevance to SAD with regard to stress-induced cortisol reactivity is the finding that, while different stressors, both physical and psychological, can elicit an HPA-axis response, stressors with a socio-evaluative component have been shown to generate the largest cortisol and adrenocorticotropin hormone changes, as well as being associated with prolonged recovery from

stress (Dickerson and Kemeny, 2004). Given that the hallmark of SAD is a persistent and excessive fear of social situations and negative evaluation by others, patients with this chronic condition can be exposed to frequent, extreme and prolonged episodes of HPA hyperactivation throughout the day. Subsequent downregulation of the adrenocortical system may be necessary to allow such individuals adapt and cope with their environment and may account for the observation of a blunted HPA-axis with respect to basal measures such as the CAR (Fries *et al.*, 2005). Indeed, it has been shown that the CAR is blunted in states of chronic stress (Pruessner, Hellhammer and Kirschbaum, 1999; Duan *et al.*, 2013) as well as in conditions associated with fatigue, burnout and exhaustion (Chida and Steptoe, 2009). The CAR has also been shown to be reduced in post-traumatic stress disorder (PTSD) (Wessa *et al.*, 2006) and older adults with generalised anxiety disorder (GAD) (Hek *et al.*, 2013), although normal (Petrowski *et al.*, 2010; Jakuszkowiak-Wojten *et al.*, 2016) or higher (Vreeburg *et al.*, 2010) levels have been reported in panic disorder.

Few studies have investigated the CAR in patients with SAD. A small case-control study found no difference in the salivary CAR between adults with SAD and controls (van Veen *et al.*, 2008) and a similar lack of difference was reported in a subset of patients with SAD as part of a large population-based cohort study of anxiety disorders (Vreeburg *et al.*, 2010). Another large cohort study in older adults (>55 years), found that those with an anxiety disorder had a significantly lower CAR than those without anxiety disorder but again no difference was found specifically in the subset suffering from SAD (Hek *et al.*, 2013). Several other studies have measured different markers of basal cortisol levels in SAD. Two small studies found no differences in 24 hour-urinary free cortisol levels (Potts *et al.*, 1991; Uhde *et al.*, 1994) while a study in adolescent girls with SP found no difference in salivary cortisol levels measured at four time-points throughout the day (Martel *et al.*, 1999). In contrast to the lack of difference in basal cortisol measures, many studies investigating cortisol reactivity to psychosocial stress in clinical SAD samples have reported increased HPA-axis responses to the TSST or similar speech tasks (Condren *et al.*, 2002; Furlan *et al.*, 2001; Roelofs *et al.*, 2009; van West *et al.*, 2008). However, a few studies have reported equivalent (Klumbies *et al.*, 2014; Krämer *et al.*, 2012; Martel *et al.*, 1999) or blunted (Beaton *et al.*, 2006) stress reactivity measures. Such discrepancies may in part be explained by the theory of HPA-adaptation to chronic stress with a change from elevated cortisol responses in the early course of the disorder to subsequent lack of difference and ultimately attenuated responses. Longitudinal studies are valuable in this regard in allowing us to untangle the relationship between cortisol levels and psychiatric disorders and indeed findings to date support the notion of changes in cortisol reactivity over the course of an anxiety or depressive disorder. One such study in adolescents with SAD found that an increase in social anxiety over time was associated with a lower cortisol response, with the level of pubertal development representing

an important moderator (van den Bos, Tops and Westenberg, 2017). Another large prospective study found that a higher CAR in late adolescence was a strong and significant predictor of the onset of SAD over the following 6 years (Adam *et al.*, 2014). A study involving a large cohort of adolescents with depression revealed that, while depressive problems initially increased the cortisol responses to stress, this pattern reversed when symptoms persist over prolonged periods of time (Booij *et al.*, 2013). Similar findings are evident in adults. Using data from the Netherlands Study of Depression and Anxiety, a research group followed 837 adult patients with a DSM-IV based depressive and/or anxiety disorder at baseline and found that a lower CAR was associated with an increased risk of an unfavourable, chronic trajectory of the disorder(s) over 2 years (Vreeburg *et al.*, 2013).

Comorbidity may also account for the heterogeneity of results. One study investigated the cortisol response to a social stressor in patients with SAD both with and without a comorbid current depressive episode (Yoon and Joormann, 2012). They found higher cortisol reactivity in the SAD group but this effect was dampened in those with comorbid MDD. Of note, in our study, no participants had a current depressive episode and a past history of MDD was not associated with a difference in the CAR or hair cortisol levels.

Salivary Alpha Amylase in SAD

In the past decade sAA has emerged as a useful non-invasive surrogate marker of SNS activity which has not only been shown to be highly sensitive to stress-related changes in healthy subjects, but also a useful indicator of ANS dysregulation in psychiatric disorders (Schumacher *et al.*, 2013). The diurnal profile of sAA involves a pronounced decrease within 60 min after awakening and a steady increase thereafter over the course of the day (Nater *et al.*, 2007). Our results in the control group reflected this with a significant decrease between waking and 60-minute post-waking sAA levels. However, this decrease was not statistically significant in the SAD group.

Although we did not observe any group differences in sAA at earlier time points, we found sAA levels to be significantly higher in SAD patients at 60-minutes post-waking. Our findings are in line with those from a previous study (van Veen *et al.*, 2008) which found no differences in awakening sAA between SAD and controls but reported higher sAA levels throughout the remainder of the day and evening in SAD patients. Another study also reported higher baseline sAA levels in SAD patients and increased sAA responses to an electrical stimulation stress (Tamura *et al.*, 2013). In contrast, however, several studies have reported no elevated sAA responses to the TSST in SAD patients (Klumbies *et al.*, 2014; García-Rubio *et al.*, 2017) despite elevated subjective feelings of stress and

anxiety (Klumbies *et al.*, 2014). It is interesting that basal levels of sAA are elevated in SAD patients although sAA reactivity to a social stress intervention appear to be similar to healthy controls. Perhaps the continuous and chronic stress of everyday social interaction in SAD with tonically elevated SNS activity is of more relevance from a neurobiological perspective than an excessive reactivity to performance situations such as that posed by the TSST.

Strengths and Limitations

An important strength of this study is that our cohort consisted of carefully selected patients with a pre-existing clinical diagnosis of SAD who had sought treatment. Some similar studies have used healthy subjects whereby participants were included on the basis of self-reported social anxiety symptoms (Miers *et al.*, 2011; Losiak *et al.*, 2016; Shirotsuki *et al.*, 2009). Other strengths were the matching of patients and controls in terms of age and sex, something which was not seen in some other studies (Çetinkaya, Üneri and Göker, 2018; Oh *et al.*, 2018) and may have contributed to inconsistency in results in those studies, in particular in relation to oxytocin which has strong sex influences. Although the two groups differed in relation to mean BMI and exercise scores, we took this into account in statistical analysis. Another strength is that cortisol and alpha amylase were measured in saliva which has the advantage of non-stressful sampling as opposed to blood measurements where the stress of venepuncture could potentially impact levels (Gozansky *et al.*, 2005; Rohleder, 2014). In addition, the saliva samples were taken at home, providing a context of basal unstressed conditions. It is unclear whether basal versus challenge conditions are more important in SAD from a neuroendocrine perspective. One would expect, given the nature of the disorder, that a public-speaking intervention would produce reliable and consistent neuroendocrine changes in this group of patients. However, this is not the case and such interventional studies vary widely in their findings of salivary cortisol and alpha amylase reactivity in SAD as outlined in the discussion above. SAD is a complex and heterogenous disorder and there are many influences to consider when investigating stress reactivity. Symptoms and stress triggers can vary and the chronicity of the disorder with the resultant potential adaptation and downregulation of stress response systems over time are likely to influence how patients with SAD respond to stress from a neurobiological perspective.

With regards to limitations, our sample size is small and limited conclusions can be drawn from a case-control study design. Given the chronic nature of SAD, larger longitudinal studies would, of course, offer greater insights into the enduring and dynamic nature of neuroendocrine systems and

the potential adaptation and dynamic quality of these systems throughout the course of the disorder. Secondly, our SAD group involved both unmedicated and medicated patients. However, we investigated the effect of psychotropic medications on each biological parameter by a subgroup analysis. Additionally, there is evidence that serotonergic psychotropics do not impact oxytocin levels (Humble *et al.*, 2013) , the CAR (Ronaldson *et al.*, 2018) or sAA (de Almeida *et al.*, 2008).

Another limitation of this study was that we did not collect information on menstrual cycle which may have had an impact on oxytocin levels (Salonia *et al.*, 2005). However, we did exclude participants taking hormonal contraception in oxytocin analysis, given the significant effect of such medications on oxytocin concentrations (Silber *et al.*, 1987). The major strength of our study in relation to oxytocin is that we are the first to measure plasma oxytocin in SAD using a reliable RIA technique with prior extraction and produce results that are biologically plausible and consistent with the human literature. This is particularly important given the recent problems associated with peripheral oxytocin assays and the profound discrepancies in results (Leng and Sabatier, 2016).

6.6 Conclusion

Patients with SAD exhibit significant neuroendocrine alterations measured under basal non-stressed conditions. Plasma oxytocin is present at significantly lower levels in such patients, with group differences largely being driven by male subjects. HPA-axis dysfunction also plays a role although the relationship is not straightforward. The salivary CAR, a distinct feature of diurnal HPA-axis function, is blunted in patients with SAD while longer-term cortisol concentrations, measured over 1 month in hair samples, are significantly higher in comparison to controls. We propose that elevated chronic cortisol concentrations may be reflective of increased stress-induced cortisol reactivity in patients with SAD while blunted basal measures such as the CAR may reflect HPA-axis adaptation and down-regulation secondary to chronic stress. Abnormalities of the ANS are also seen with increased levels of salivary alpha amylase one-hour after waking. Given the chronic and disabling nature of SAD along with the frequent psychiatric comorbidity, it is vital that we develop a greater understanding of the neurobiological mechanisms. Further evaluation of neuroendocrine abnormalities in large prospective longitudinal studies is recommended.

Chapter 7: Recipe for a Healthy Gut: Intake of Unpasteurised Milk Is Associated with Increased *Lactobacillus* Abundance in the Human Gut Microbiome

7.1 Abstract

Introduction: The gut microbiota plays a role in gut-brain communication and can influence psychological functioning. Diet is one of the major determinants of gut microbiota composition. The impact of unpasteurized foods on the microbiota is unknown. In this observational study, we investigated the effect of a dietary change involving intake of unpasteurised dairy on gut microbiome composition and psychological status in participants undertaking a residential 12-week cookery course on an organic farm.

Methods: Twenty-four participants were recruited. The majority of food consumed during their stay originated from the farm itself and included unpasteurized milk and dairy products. At the beginning and end of the course, participants provided faecal samples and completed self-report questionnaires on a variety of parameters including mood, anxiety and sleep. Nutrient intake was monitored with a food frequency questionnaire. Gut microbiota analysis with performed with 16S sequencing. Additionally, faecal short chain fatty acids (SCFAs) were measured**.**

Results: Relative abundance of the genus *Lactobacillus* increased significantly between pre- and post-course time points. This increase correlated positively with participants intake of unpasteurized milk and dairy products. An increase in faecal SCFAs, valerate, and to a lesser extent propionate, was observed. While there was no overall change in psychological measures, stress and anxiety scores decreased significantly in participants with higher baseline scores.

Conclusions: While concerns in relation to safety need to be considered, intake of unpasteurized milk and dairy products promote the growth of the probiotic bacterial genus, *Lactobacillus* in the human gut. Such changes in microbiome composition may be important in promoting reduced stress and anxiety levels. More research is needed on the effect of dietary changes on gut microbiome composition and subsequent impact on mental health.

7.2 Introduction

A growing body of evidence over the past decade has demonstrated the importance of the gut microbiome in all aspects of physical and mental health. While it is still unclear what exactly constitutes a 'healthy' gut microbiome, certain bacterial groups have been strongly associated with better health outcomes. *Lactobacillus* is one of the foremost genera considered to have probiotic properties (Di Cerbo *et al.*, 2016). There have been a wide variety of studies undertaken in recent years which have demonstrated the benefit of a *Lactobacillus* probiotic, both mono- and multi-strain, for improving a range of health outcomes including obesity (John *et al.*, 2018), diabetes (Hsieh *et al.*, 2018), liver disease (Wong *et al.*, 2013), cardiovascular disease (DiRienzo, 2014), gastrointestinal conditions (Wilkins and Sequoia, 2017) and neuropsychiatric disorders such as depression, anxiety and autism (Butler, Cryan and Dinan, 2019).

A key present-day challenge involves identifying the most effective ways of maintaining a healthy gut microbiome and promoting the growth of probiotic bacteria. While commercial probiotic products are widely available, there are concerns in relation to regulation, quality control, efficacy and cost (Kolacek *et al.*, 2017). Dietary intake is one of the main factors regulating gut microbiome composition and food-based interventions can be tailored to each individual to modify their bacterial profile (Johnson *et al.*, 2019). While unravelling the diet-microbiome relationship is a formidable task given the many confounding factors, attempts to do so have been made over the past decade. Gut microbiome profile has been shown to be distinctly different in those living in rural areas with a traditional diet in comparison to urban-based westernized populations (Yatsunenko *et al.*, 2012; Clemente *et al.*, 2015; De Filippo *et al.*, 2010). Even when one accounts for contributions of human genetic and geographical factors between populations, subsistence methods and diet significantly impact gut microbiota composition (Jha *et al.*, 2018). It is hypothesized that a 'microbiota insufficiency syndrome' has resulted from modern lifestyle with its highly processed diets, overuse of antibiotics and increased sanitation and that the 'industrialized' microbiota may be a major contributing factor in the rise of many non-communicable chronic diseases in westernized societies (Sonnenburg and Sonnenburg, 2019). Even as one moves from looking at the early ancestral microbiota to more recent times, significant changes in lifestyle have continued until relatively recently. Ireland, as with many countries in the developed world, was a predominantly agrarian society up until the mid-late 20th century. In 1966, over 30% of the workforce were employed in agriculture with this figure estimated at less than 5% in 2016 (Office, 2016). Consumption of unpasteurized milk was a common part of the diet of those living on farms and

epidemiological studies suggest that it may have played a protective role against the development of allergies and atopic diseases (Braun-Fahrlander and von Mutius, 2011).

Despite food safety concerns, the consumption of unpasteurized milk appears to be growing in popularity (Buzby *et al.*, 2013; Fagnani, Ito Eleodoro and Osti Zanon, 2019). To our knowledge, there are no studies exploring the impact of unpasteurized milk intake on the gut microbiome. In this observational study, we investigated the effect of a dietary change involving the intake of unpasteurized milk on gut microbiota composition, metabolites and psychological status in 24 participants undertaking a residential, farm-based, 12-week cookery course. Our centre had previously published a study (Quigley *et al.*, 2013a) on the microbiota composition of unpasteurised milk taken from Irish cows which would thus be representative of the expected microbiota composition of the raw milk that would be consumed by participants in our study. Given the reported high proportion of viable probiotic bacteria such as *Lactobacillus* (and other lactic acid bacteria including *Lactococcus* and *Leuconostoc*), along with the fact that *Lactobacillus* are considered intrinsically resistant to gastric acid (Tannock, 2004), we hypothesized that a dietary change involving raw milk consumption would alter the gut microbiome of participants with a potential differential increase in the relative abundance of these probiotic bacterial groups.

7.3 Methods

Study Site and Subjects

Ballymaloe Cookery School, Organic Farm and Gardens is located in East Cork, Ireland and runs bi-annual 12-week residential courses where students live on-site, learn about organic farming methods and undertake intensive cookery classes. The majority of food consumed by participants during their stay originates from the organic farm itself or consists of high-quality, locally-sourced produce. The farm has a small herd of Jersey cows whose milk is used in its raw unpasteurized state for direct consumption, cooking and the production of other dairy products including cream, butter, cheese and yoghurt. There is an emphasis on eating, and cooking with, local seasonal fruit and vegetables, the vast majority of which is organic. Meat and fish are also locally sourced and, for the most part, organic.

Approval of the study protocol was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Protocol number DOP001) and conducted following the ICH Guidelines on Good Clinical Practice, and the Declaration of Helsinki. Written informed consent was obtained from all subjects before study procedures were conducted. Course participants were emailed in advance

informing them of the study and a short talk on the gut microbiome was given at an introductory session prior to commencement of the course. In order to be eligible for the study, participants had to be between the age of 18-65 years and be generally healthy, with no chronic or current, physical or mental illness. Exclusion criteria included the use of medications which were likely to interfere with the objectives of the study (including any psychotropic medications) as well as intake of antibiotics, probiotics or prebiotics within the month prior to commencement of the study.

Subject Metadata

Demographic data was collected for each individual including information on age, sex and race. Weight and height were measured and used to calculate body mass index (BMI). Information in relation to medical and psychiatric history, along with medication use, was also obtained at interview. At the beginning and end of the 12-week course participants completed self-report questionnaires on a variety of parameters including mood, anxiety, sleep, exercise (Perceived Stress Scale (PSS), Hospital Anxiety and Depression Scale (HADS), Pittsburgh Sleep Quality Index (PSQI), International Physical Activity Questionnaire (IPAQ)).

Diet Quantification

To monitor nutrient intake, participants completed the self-administered 152-item SLAN-06 (Survey of Lifestyle, Attitudes and Nutrition in Ireland) food frequency questionnaire (FFQ) (Harrington J, 2008) which is validated to be used in an Irish population. An additional eight food items as well as questions about type and frequency of milk, salt and fried food consumption were added. These items are included in the EPIC Norfolk questionnaire (Riboli and Kaaks, 1997) from which the SLAN-06 FFQ was adapted. An extra section was added to the FFQ by the authors to quantify intake of unpasteurized milk and dairy products before and during the course, as this information would not otherwise have been captured. These extra questions followed the same response format as the other food items.

Participants were asked to estimate the frequency with which they consumed a specified portion size of each of the foods listed over the preceding month. The FFQ has nine possible responses ranging from “never or less than once per month” to “6+ per day”. Participants completed the FFQ before and after the stay at Ballymaloe. The FFQs were analysed for nutrient intake using the FETA software (Mulligan *et al.*, 2014).

The 160 foods items were grouped into 29 food groups (e.g., fruits, vegetables, grains, sweets) using methods similar to those described in previous studies of dietary patterns (Arthur *et al.*, 2013). To estimate the number of servings of any food group, each response was converted to the

corresponding frequency factor and summed over all the food items to get the average servings of a specific food group per day. Intake of unpasteurised milk and dairy products was analysed in a similar way.

Faecal sample collection and 16S rRNA gene sequencing and processing

Faecal samples were collected at the beginning and end of the 12-week period in disposable plastic containers with a Thermo Scientific™ Oxoid AnaeroGen 2.5L Sachet in situ to generate anaerobic conditions within the container. Participants were instructed to keep the sample containers in a refrigerator at 4^{o}C. Samples were collected and transferred to a -80^{o}C freezer within 12 hours. DNA was extracted using the DNA Fast Stool DNA extraction kit (Qiagen) using the protocol for Gram positive bacteria and including an additional bead beating step at the beginning of the procedure. DNA was quantified using the Qubit High Sensitivity Kit (Life Technologies), standardized and then used as a template for PCR. 16S metagenomic libraries were prepared using primers to amplify the V3-V4 region of the bacterial 16S rRNA gene, with Illumina adaptors incorporated as described in the Illumina 16 s Metagenomic Library Preparation guide. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. The pooled libraries were assessed using an Agilent high sensitivity DNA kit and examined by quantitative PCR (qPCR) using the Kapa Quantification kit for Illumina (Kapa Biosystems, USA) according to the manufacturer's guidelines. Libraries were then diluted and denatured following Illumina guidelines and sequenced (2 × 300 bp) on the Illumina MiSeq platform.

Sequence table generation

Three hundred base pair paired-end reads were prefiltered based on a quality score threshold of > 28 and trimmed, filtered for quality and chimeras using the DADA2 library in R (Callahan *et al.*, 2016). Taxonomy was assigned with DADA2 against the SILVA SSURef database release v132. Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. ASVs were cut off at genus level, those that were unknown on the genus level were not considered in downstream analysis, as were genera that were only detected as non-zero in five percent or fewer of total samples.

Short chain fatty acid (SCFA) measurements

The concentration of SCFAs, acetate, propionate, Iso-butyrate, butyrate, Iso-valerate, and valerate were analyzed by gas chromatography flame ionization detection (GC-FID) using a Varian 3800 GC

system, fitted with a 5m guard column (Restek) connected to an Agilent DB-FFAP column (30 m L x 0.32mm ID x 0.25 µm df) and a flame ionization detector with a CP-8400 auto-sampler.

Statistical analysis

Statistical analysis for changes in dietary measures was performed using SPSS Statistical Packages version 25 (SPSS, Inc., Chicago, IL, USA). Normality of outcome measures was assed using Shapiro Wilk's test of normality. Differences in nutrient and food group intake pre- and post-course participation were analysed using the paired t-test or the non-parametric Wilcoxon Rank sum test.

Microbiome data-handling was done in R (version 3.6) with the Rstudio GUI (version 1.2.1555). In all cases, the iNEXT library was used to calculate alpha diversity (Hsieh, Ma and Chao, 2016). Principal component analysis (PCA) was performed on centred-log ratio transformed (clr) values using the ALDEx2 library (Fernandes *et al.*, 2014). Number of permutations was always set to 1000. Aitchison distance was used as a distance metric for beta-diversity. Piphillin (Iwai *et al.*, 2016) was used for functional inference from 16S rRNA gene sequences of stool samples in the form of Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthologues. Gut-brain modules were calculated using the R version of the Gomixer tool (Darzi *et al.*, 2016). Differential abundance of microbes between groups was assessed using the ALDEx2 library. As part of testing for correlations between microbial abundance and metadata, skadi, an implementation of jack-knifing and Grubb's test, was used to assess the reliability of the data and detect outliers (R scripts available online, https://github.com/thomazbastiaanssen/Tjazi; (Bastiaanssen T, 2018)). Correlation was assessed using Spearmans's rank correlation coefficient. The relationship between categorical variables was assessed using Pearson's Chi-squared test. For datasets in which the condition of normality was violated the non-parametric Kruskal-Wallis test was used and post-hoc analysis was done using the Wilcoxon test. A p-value of < 0.05 was deemed significant. To correct for multiple testing in tests correlating volatility and specific microbiota, KEGG orthologues or pathways, the Q-value post-hoc procedure was performed with a q-value of 0.1 as a cut-off (Storey J, 2019).

7.4 Results

Participant characteristics:

A total of 62 participants who were completing the 12-week course between May-July 2018 were informed about the study. Twenty-eight participants volunteered and underwent screening. Two

were excluded; one had a chronic gastrointestinal disorder and another had taken antibiotics in the previous month. Twenty-six participants were enrolled with 24 (13 females, 11 males) completing the study; 2 failed to provide faecal samples. Of note, subject metadata and faecal samples were collected within the first three days of the course and again at week 11. The final week of the course (week 12) involved several examinations for students and thus, the associated increased stress during this week may have had the potential to influence findings. Our study sample comprised 24 participants; 13 females and 11 males. Baseline characteristics of participants, including age, body mass index (BMI), smoking status, sleep quality and exercise levels are shown in Table 1.

Table 1: Baseline Characteristics of participants

	Pre-course	**Post-course**	**P-value**
Number of participants	24		
Female; n (%)	13 (54%)		
Mean age; n (range)	30.25 (18-59)		
Smoking status; n (%)	7 (29)		
BMI (kg/m)	24.87 (3.42)	25.33 (3.61)	0.1
Physical activity (as measured by IPAQ score)	4757.52 (4614.74)	3271.52 (7280.05)	0.32
Sleep quality (as measured by PSQI)	5.36 (2.87)	4.95 (2.91)	0.25
Bristol stool scale score	3.78 (1.085)	4.04 (0.706)	0.39
GI-Visual Analogue Scale; Satisfaction with bowel habit	38.37 (33.757)	27.29 (27.98)	0.25

(BMI: Body Mass Index, IPAQ: International Physical Activity Questionnaire; PSQI: Pittsburgh Sleep Quality Index)

Changes in diet:

Based on food frequency questionnaire (FFQ) analysis (Tables 2 and 3), there was no change in total calorie intake during the course. In terms of macronutrient intake, protein and carbohydrate intake remained unchanged and though total fat consumption increased, this change did not reach statistical significance (mean increase (g) from 94±35 to 128±66, p=0.08). With regards to micronutrients, Vitamin A (µg) intake increased significantly (715±577 to 1505±975, p=0.005) as did Vitamin B12 (µg) (7.8±3.6 to 11±5.8, p=0.04). Although intake of fruit reduced slightly (2.02±1.2 to

1.38±0.84, p=0.04) consumption of vegetables and wholegrains did not change, nor did intake of alcohol or unhealthy foods such as sweets or snacks.

Table 2: Changes in dietary components between pre-course and post-course time points, obtained from analysis of food frequency questionnaires. (P-values reaching statistical significance are in bold and accompanied by an asterix)

Nutrient	Recommended daily intake*	Pre-course	Post-course	p-value
Kilocalories	2,000 – 2,400 (males; depending on activity level)	2264±1006	2723±1494	0.47
Protein (g)	10-35% of total energy	97±40 (17%)	109±57 (16%)	0.54
Fat (g)	20-35% of total calories	94±35 (37%)	128±66 (42%)	0.08
Carbohydrate (g)	45-65% of total calories	246±158 (43%)	275±178 (40%)	0.77
Alcohol (ml)	21 standard drinks (1/2 pint of beer, small glass of wine, one measure of spirits)	15±13	14±13	0.98
Monounsaturated fatty acids (g)	>12% of total energy	38±17 (15%)	51±27 (17%)	0.13
Polyunsaturated fatty acids (g)	>6% of total energy	16±7 (6%)	22±13 (7%)	0.20
Saturated fatty acids (g)	<10% of total energy	34±13 (14%)	49±25 (16%)	**0.04***
Cholesterol (mg)	300 mg	381±173	469±236	0.13
Total sugar (g)	<10% of total energy	115±67 (20%)	125±76 (18%)	0.81
Starch (g)		128±96	147±104	0.62
Fibre (g)	>25g	19±15	20±15	0.88
Vitamin A (µg)	800 µg	715±577	1505±975	**0.005***
Thiamine (mg)	1.1 mg	1.8±1.2	1.9±1.2	0.87
Riboflavin (mg)	1.4 mg	2.3±1.5	2.6±1.7	0.45
Niacin (mg)	16 mg	27±15	29±19	1.00
Vitamin B6 (mg)	1.4 mg	3.1±3.0	2.9±1.9	0.81
Vitamin B12 (µg)	2.5 µg	7.8±3.6	11±5.8	**0.04***

Folate (µg)	200 µg	339±284	328±236	0.58
Vitamin C (mg)	80 mg	104±60	79±41	0.16
Vitamin D (µg)	5 µg	3.6±2.3	5.1±3.5	0.09
Vitamin E (mg)	12 mg	14±7	16.6±9.7	0.41
Phosphorous (mg)	700 mg	1612±763	1787±942	0.64
Calcium (mg)	1000 mg	914±387	1062±548	0.34
Iron (mg)	7 mg	15±11	16±11	0.81
Selenium (µg)	55 µg	67±30	79±43	0.49
Zinc (mg)	10 mg	12±6	13±7	0.58
Sodium (mg)	1600 mg	2983±1559	3385±1964	0.59
Potassium (mg)	2000 mg	3798±1603	4015±1958	0.85
Magnesium (mg)	375 mg	359±200	343±189	0.67
Copper (mg)	1 mg	1.2±0.6	1.5±0.8	0.31
Chloride (mg)	800 mg	4407±2407	4885±2886	0.64
Manganese (mg)	2 mg	3.1±1.5	3.2±1.8	0.85
Iodine (µg)	15 µg	169±76	201±100	0.28

Table 3: Change in food group intake between pre-course and post-course time points, obtained from analysis of food frequency questionnaires. (P-values reaching statistical significance are in bold and accompanied by an asterix)

Food Group	**Pre-course**	**Post-course**	**P-value**
Red meats	0.62±0.35	0.85±0.56	0.07
Processed meats	0.58±0.77	0.33±0.27	0.08
Poultry	0.31±0.26	0.25±0.22	0.73
Organ meats	0.04±0.07	0.11±0.09	**0.01***
Fish	0.55±0.43	0.68±0.49	**0.04***
Fried foods	0.21±0.14	0.30±0.16	**0.03***
Refined carbohydrates	0.84±0.56	1.28±1.17	0.26
Whole grains	0.66±0.41	0.95±0.75	0.29
Cereal	0.69±1.45	0.47±0.55	0.76
Potatoes	0.34±0.26	0.49±0.34	0.06
Pasta meals	0.42±0.31	0.34±0.25	0.66
High-fat dairy products	2.03±1.15	3.59±3.07	0.09

Low-fat dairy products	0.21±0.27	0.36±0.28	**0.02***
Egg dishes	0.61±0.49	0.43±0.29	0.34
Fruit	2.02±1.2	1.38±0.84	**0.04***
Green leafy vegetables	0.77±0.42	1.25±1.03	0.11
Cruciferous vegetables	0.68±0.61	0.44±0.32	0.34
Starchy vegetables	0.42±0.56	0.37±0.24	0.16
Other vegetables	3.98±2.17	3.72±2.03	0.66
Legumes	0.30±0.24	0.22±0.25	0.10
Sweets	1.77±1.29	2.45±2.00	0.31
Snacks	0.48±1.00	0.37±0.66	0.40
Soups	1.15±1.24	1.11±1.17	0.48
Sauces	0.19±0.17	0.27±0.28	0.31
Condiments	2.68±1.65	3.17±2.12	0.58
Non-alcoholic beverages	2.39±1.71	1.99±1.42	0.45
Alcoholic beverages	1.43±1.21	1.42±1.29	0.91
Fruit Juice	0.42±0.71	0.39±0.53	0.50
Sweetened beverages	0.81±1.03	0.78±0.74	0.56

Participant's intake of milk and dairy products are summarised in Table 4. In relation to participants overall intake of milk, this did not change during the course (mean increase from 177mls±120 to 192mls±134, p=0.60). However, a switch to unpasteurised milk was evident for most participants. Only one of 24 (4%) participants reported consuming unpasteurised milk prior to commencing the residential course while 23 participants (96%) reported its consumption during the course (mean increase from 23±116mls to 239±51mls, p<0.0001). Pre-course, 3 participants consumed skimmed milk, 7 semi-skimmed, 11 whole and 3 non-specific and post-course only one participant consumed semi-skimmed while the remaining participants consumed whole milk, consistent with unpasteurized milk intake. Total intake of dairy products (cream, yoghurt, dairy desserts, cheese; salad cream or mayonnaise, butter, cottage cheese) did increase slightly though not to a statistically significant level (mean increase from 2.24±1.23 daily servings to 3.35±3.16, p=0.07). Two participants (8%) reported intake of unpasteurised dairy products prior to the course whereas 21 (87.5%) consumed these products during the course (mean increase from 0.01±0.04 servings per day to 1.2±1.4, p<0.0001).

Table 4: Change in participants consumption of milk and dairy products between pre-course and post-course time points, obtained from analysis of food frequency questionnaires. (P-values reaching statistical significance are in bold and accompanied by an asterix)

Dairy intake	Pre-course	Post-course	P-value
Total Milk (mL)	177mls±120	192mls±134	0.6
High-fat dairy products (servings/day)	2.03±1.15	3.59±3.07	0.09
Low-fat dairy products (servings/day)	0.21±0.27	0.36±0.28	**0.02***
Total dairy products	2.24±1.23	3.35±3.16	0.07
Unpasteurised milk (mL)	23±116	239±51	**<0.0001***
Unpasteurised dairy products (servings/day)	0.01±0.04	1.2±1.4	**<0.0001***

Change in Microbiome composition:

We quantified the microbial diversity within each subject (α-diversity) before and after the course, and the difference between each subject's pre-course and post-course gut microbiota (β-diversity). No significant differences were found in either α-diversity (simpson; p = 0.41, shannon; p = 0.26) or β-diversity (p= 0.998) (Figures1B and 1C). No differences were found between males and females. Analysis of the differential relative abundance of bacterial taxonomic groups revealed a total of 578 amplicon sequence variants (ASVs) within our samples (Figure 1D). Undirected pairwise analysis of all ASVs, (Wilcoxon signed rank test, allowing for Storey's q-value post-hoc correction) revealed only one ASV which changed significantly between pre-course and post-course time points. This ASV corresponded to the genus, *Lactobacillus* which increased significantly (p=0.0003728; q = 0.0498) (Figure 1A). Identification of ASVs at a species level was not possible. We subsequently performed a directed search in relation to other lactic acid bacteria (LAB), a dominant population in bovine milk prior to pasteurization, based on what was previously reported in the literature on the subject (Quigley *et al.*, 2013a). On the genus level, the relative abundance of *Leuconostoc* (p=0.09) and *Enterococcus* (p=0.14) did not change but that of *Lactococcus* increased significantly (p=0.01; q = 0.0108). Other major components of unpasteurized milk include *Pseudomonas* and *Acinetobacter* (Quigley *et al.*, 2013a). We did not detect these genera in any of our samples.

We directly tested for associations between changes in dietary components and changes in microbiome composition within each subject over time. We observed a positive correlation between *Lactobacillus* abundance and combined intake of unpasteurized milk and dairy products (Spearman correlation, r=0.618, p=0.0000027) Upon closer inspection, *Lactobacillus* abundance appeared to fall into two groups based on the centred log-ratio (clr) transformation of relative abundance scores of

>2.5 or <2.5, prompting us to dichotomize the data for Pearson's Chi-squared test. We defined these groups as low versus high *Lactobacillus* abundance and found a positive association between these two groups and the change in intake of unpasteurized milk and dairy products (combined score), binned into four groups based on the amount of portions consumed; 0-2, 3-4, 5-6 and 7-8. (Pearson's Chi-squared, X-squared = 13.265, df = 3, p-value = 0.004096) (Figure 2). This association also held when looking at the relationship between *Lactobacillus* abundance and unpasteurized milk or unpasteurized dairy products individually. We analysed this *Lactobacillus* grouping against our other metadata (including age, sex, BMI, sleep, exercise and gastrointestinal parameters) but found no other factors associated with the high versus low split.

Figure 1: 1A; Relative abundance of *Lactobacillus* at pre-course and post-course time points. **1B**; Alpha diversity of gut microbiome at pre-course and post-course time points. **1C** Beta diversity of gut microbiome at pre-course and post-course time points. **1D**; Relative abundance of genus-level taxa for each participant. Each column represents one participant with pre-course taxa on the left and post-course taxa on the right. (Box plots: Body represents median and interquartile range, whiskers represent the extreme values)

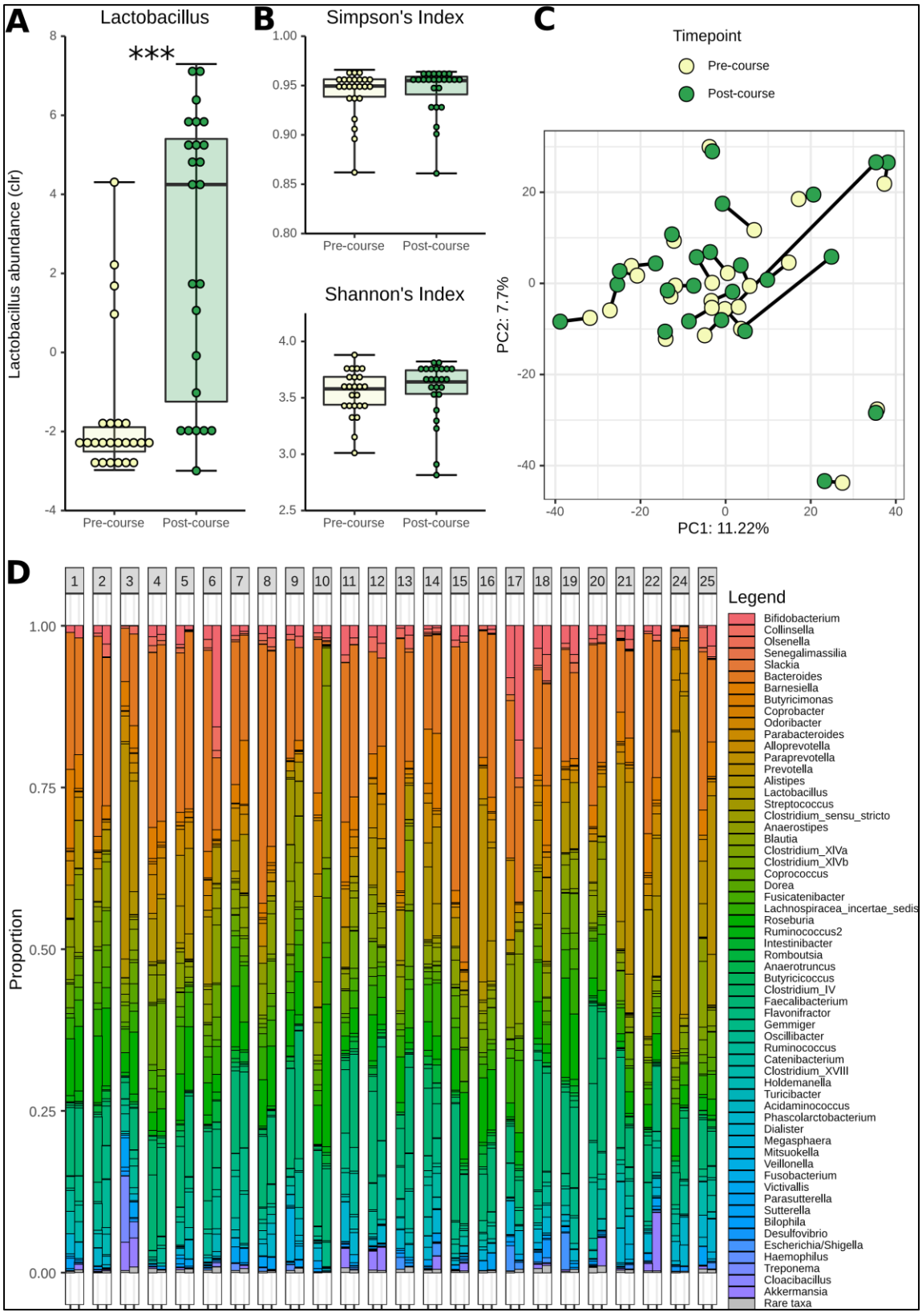
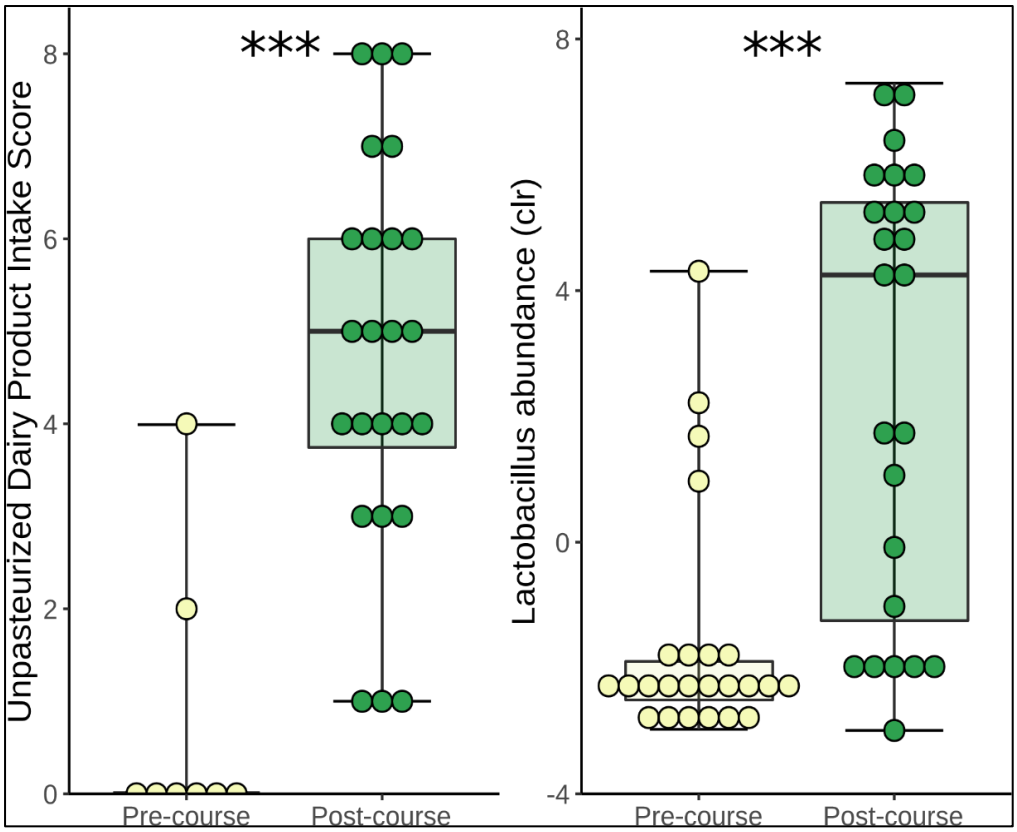


Figure 2: Box plots showing the change in combined unpasteurised dairy score and *Lactobacillus* abundance between pre- and post-course time points. (Body represents median and interquartile range, whiskers represent the extreme values; As some scores overlap, each participant is not visible as an individual point on the graph)

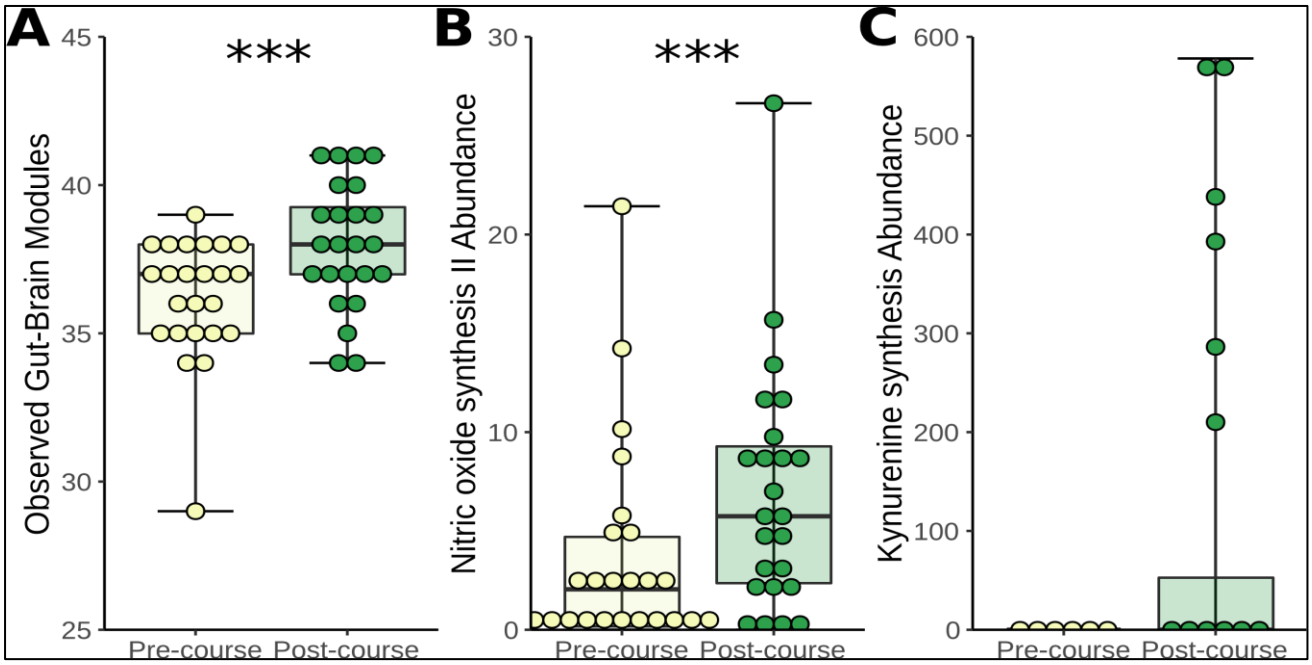


Functional Prediction and Application of Gut-Brain Modules:

Functional analysis of our microbiome data was performed using Piphillin (Iwai *et al.*, 2016) and further extended by subjecting our metagenomic data to a module-based analytical framework which targets microbial pathways involved in microbiota-gut-brain communication, thus generating a predication of the neuroactive potential of a microbiome sample (Valles-Colomer *et al.*, 2019). Within our sample, we observed 43 of the 56 gut-brain modules (GBMs) described previously by the authors. In addition, we observed an increase in the functional richness of the microbiome profile, as determined by the number of GBMs present (Wilcoxon signed rank test; mean increase of 1.79, p=0.00087), following the 12-week course (Figure 3A). On analysis of the individual GBMs, one consistently increased significantly; GBM026: Nitric oxide synthesis II (nitrite reductase) (p = 0.001; q = 0.061) (Figure 3B). Notably, GBM004: Kynurenine synthesis was never found in participants pre-course, but was detected in 6 out of 24 participants post-course at very high levels. This observation

did not pass the post-hoc correction (p = 0.036, q = 0.361) (Figure 3C). Functional alpha diversity, measured here by calculating the alpha diversity of the floored KEGG Orthologue tables generated by Piphillin, did not differ between pre- and post-course time points (chao1; p = 0.14, simpson; p = 0.19, shannon; p = 0.85).

Figure 3: 3A: Functional richness of microbiome, as measured by observed number of gut-brain modules (GBM) at pre-course and post-course time points. **3B**: Increase in abundance of GBM 026: Nitric oxide synthesis II (nitrite reductase) between pre- and post-course time points. **3C:** Increase in GBM 004: Kynurenine synthesis between pre- and post-course time points. (Box plots: Body represents median and interquartile range, whiskers represent the extreme values; As some scores overlap, each participant is not visible as an individual point on the graph)



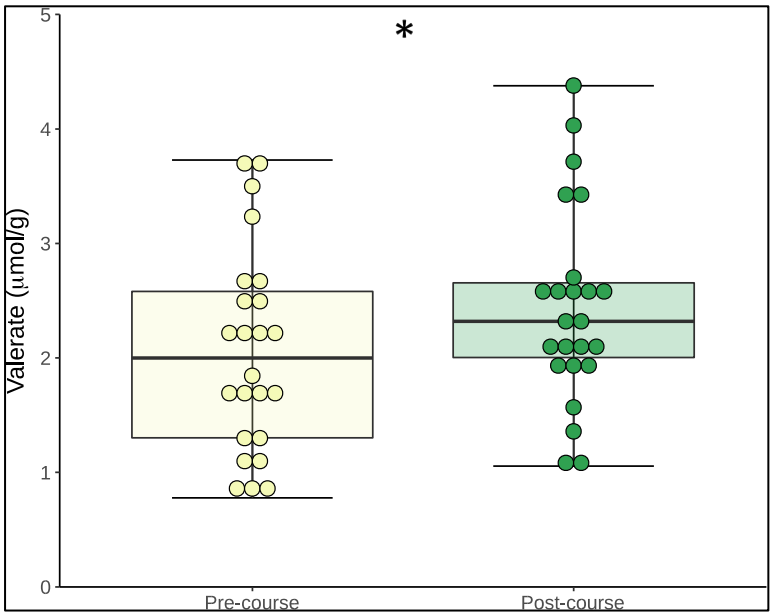
Change in microbiome metabolites:

Analysis of faecal short-chain fatty acids (SCFAs) revealed a significant increase in Valerate (p=0.04) over the 12 weeks. Propionate also increased, although not to a statistically significant level (p=0.09) while no change was observed in butyrate, iso-butyrate, iso-valerate or acetate. (Table 5)

Table 5: Short-chain-fatty-acid (SCFA) concentrations; pre- and post-course results

SCFA (µmol/g)	**Pre-course** Mean (SD)	**Post-course** Mean (SD)	**P-value**
Acetate	27.0 (8.6)	29.3 (10.3)	0.268
Propionate	14.0 (7.0)	16.3 (7.6)	0.09
Iso-butyrate	2.4 (1.1)	2.6 (0.9)	0.485
Butyrate	17.6 (9.6)	19.0 (10.8)	0.156
Iso-valerate	3.2 (1.9)	3.4 (1.7)	0.498
Valerate	2.3 (0.8)	2.5 (0.8)	**0.049 ***
Total BCFA	5.6 (2.9)	6.0 (2.5)	0.44
Total SCFA	66.6 (24.7)	73.1 (27.7)	0.113

Figure 4: Concentration of Valerate at pre- and post-course time points. (Body represents median and interquartile range; whiskers represent the extreme values)



Change in psychological measures:

There was no change in total scores on the Cohen's Perceived Stress Scale (PSS), Hospital Anxiety and Depression Scale (HADS)-total score, HADS-anxiety subscale or HADS-depression subscale

between pre- and post-course time points. However, because our study involved a healthy population, baseline anxiety and stress scores were low and mood scores were within the normal range (Table 6). Further analysis was considered taking into account baseline scores. The sample was dichotomized based on the median score of the above scales. Participants with higher baseline scores on the PSS showed a mean reduction of 4.42 points, whereas the rest of the participants reported a mean increase of 1 point (Wilcox test, p=0.026) between pre-course and post course time points. Participants with higher anxiety scores than the median on the HADS-A also showed a statistically significant reduction compared to the rest of the participants (mean reduction of 2 vs a mean increase of 2, Wilcox test p=0.0043). We did not find any relationship between microbes and psychological scales. No differences were found between males and females.

Table 6: Results of psychological scales at pre- and post-course time points.

Scale	**Pre-course Mean (SD)**	**Post-Course Mean (SD)**		**p-value**
PSS	14.96 (6.23)	13.13 (5.12)		0.149
HADS-A	5.61 (3.72)	4.83 (3.21)		0.274
HADS-D	3.04 (2.82)	3.83 (3.42)		0.198
HADS-T	8.65 (5.37)	8.65 (5.93)		1
Subgroup Analysis	**Pre-course Mean (SD)**	**Post-course Mean (SD)**	**Change (delta) between pre- and post-course scores Mean (SD)**	**p-value†**
PSS -Highest	19.58 (3.12)	15.17(5.15)	-4.42 (5.9)	**0.026***
PSS-Lowest	10.08 (4.42)	10.91 (4.25)	1.0 (4.58)	
HADS-A-Highest	7.67 (2.85)	5.56(3.44)	-2.11 (2.30)	**0.004***
HADS-A-Lowest	1.75 (1.17)	4 (2.35)	2.25 (2.30)	

(HADS: Hamilton Anxiety and Depression Scale, HADS-D: HADS-Depression subscale, HADS-A: HADS-Anxiety subscale, PSS: Perceived Stress Scale. PSS-highest refers to those participants with scores above the median and PSS-lowest, to those with scores below the median. HADS-A-highest refers to those participants with scores above the median and HADS-A-lowest, to those with scores below the median. († the p-value in this case refers to the comparison between the mean scores of delta PSS-highest and delta PSS-lowest as well as the mean scores between delta HADS-A-highest and delta HADS-A-lowest)

7.5 Discussion

In this observational study, we investigated the effect of a dietary change on the gut microbiome of participants who undertook a 12-week residential cookery course on an organic farm, where the majority of food consumed and used for cooking, was locally-sourced, seasonal and produced using organic methods. Of particular interest was the use of unpasteurized milk and dairy products obtained from a small herd of Jersey cows on the farm. Most participants had not been using any unpasteurized dairy prior to the course and all used these products to some degree throughout their stay. We found that the main change in terms of microbiome composition was a dramatic increase in participants *Lactobacillus* abundance between pre-course and post-course faecal samples. This increase was strongly associated with participants intake of unpasteurized milk and dairy products. In addition, a positive change was noted in relation to microbiome metabolites with an increase in valerate and, to a lesser extent not quite reaching statistical significance, propionate.

While administration of probiotics in the form of conventional pharmaceutical agents such as tablets or capsules is a common method, the majority of probiotics commercially available are in the form of food-based delivery systems which use probiotic bacteria in their production or add these bacteria during the manufacturing process, e.g., cheese, yoghurt or fermented drinks (Govender *et al.*, 2014). There are several problems associated with pharmaceutical and commercially-produced probiotic formulations. Firstly, the probiotic potential of bacteria is species and strain-specific but efficacy is often generalized across products in the current unregulated commercial probiotic market (de Simone, 2019). Secondly, there are many aspects of the manufacturing process of such products which can alter the delivery of viable functional probiotic bacteria (Sanders *et al.*, 2014). Because probiotic products are generally categorized as food supplements, they are subject to less stringent regulatory criteria and quality control processes with regard to microorganism specification, their numbers and functional properties (Kolacek *et al.*, 2017). Thirdly, there is a cost consideration when it comes to commercial probiotic products, which may place daily probiotic supplements out of the reach of many.

An alternative to consuming commercially-produced probiotic supplements for the maintenance of a healthy gut microbiome is to alter one's diet. It is increasingly accepted that the 'Western-diet', characterized by highly-processed, low-fibre, high-sugar, high-fat foodstuffs has negative implications for health (Cordain *et al.*, 2005) which may be mediated by an unfavourable impact on the gut microbiome (Zinocker and Lindseth, 2018). In contrast, adherence to a Mediterranean-style diet (characterized by high-level consumption of olive oil, fruit, nuts, vegetables, and cereals with

moderate intake of fish and poultry) has been strongly associated with better physical (Estruch *et al.*, 2018) and mental (Lassale *et al.*, 2018) health outcomes, which again may be related to a beneficial impact on the gut microbiome and metabolome (De Filippis *et al.*, 2016). Gut microbiome composition can be rapidly and significantly altered by introducing dietary change (David *et al.*, 2014) with the impact of food choices on the microbiome being highly individualized (Johnson *et al.*, 2019). In this study, the key change in relation to dietary intake during the 12-week residential course was an increase in dairy products, which in this context were unpasteurized. This was a major change for our subjects, the vast majority of whom did not consume unpasteurized milk or dairy products prior to the course.

Cow's milk is produced on a massive scale worldwide and has long played an important role in human nutrition (Haug, Høstmark and Harstad, 2007). Cow's milk harbours a rich microbiota and typically contains a significant population of lactic acid bacteria (LAB) that includes *Lactococcus* (8.2 x 10^1–1.4 x 10^4 CFU/ml), *Streptococcus* (1.41 x 10^1–1.5 x 10^4 CFU/mL), *Lactobacillus* (1.0 x 10^2–3.2 x 10^4 CFU/ml*), Leuconostoc* (9.8 x 10^1–2.5 x 10^3 CFU /mL) and *Enterococcus spp.* (2.57 x 10^1–1.58 x10^3 CFU/mL) (Quigley *et al.*, 2013b). Other organisms present in substantial proportions are *Pseudomonas* and *Acinetobacter,* so-called psychrotrophs which can flourish during cold storage conditions and typically cause milk spoilage (Raats *et al.*, 2011). Pasteurization of milk gained widespread popularity in the early 1900's when cow's milk was linked to the spread of disease epidemics such as tuberculosis, diphtheria, typhoid fever, scarlet fever, anthrax and cholera (Rankin *et al.*, 2017). A recent Irish study, using molecular, culture-independent techniques, compared the microbial content of raw and pasteurized cow's milk (Quigley *et al.*, 2013a). Authors reported that, although the bacterial diversity of the raw and pasteurized milk were similar, raw milk contained mostly viable cells whereas the cell population in pasteurized milk were predominantly non-viable. Thus, while pasteurized milk appeared to have a somewhat similar microbiome composition to that of the raw milk, any potential probiotic LAB would have been in a nonviable state. In this study, *Pseudomonas* and *Acinetobacter,* two major genera found in unpasteurized milk, were not detected by 16S rRNA analysis of the microbiomes of the participants, either pre or post treatment. This may be due to a selective filtering effect of the human immune system or physiological barriers such as gastric acid, which is known to act as such a filter (Freedberg *et al.*, 2015; Imhann *et al.*, 2017). The consumption of raw milk is growing in popularity, although there is some debate in relation to its purported benefits and concern about the potential dangers of contracting milk-borne illnesses if the raw milk is contaminated with human pathogens (Lucey, 2015). There is a strong suggestion from epidemiological literature that the consumption of unpasteurized cow's milk or yoghurt by

children living on farms or rural areas has a protective effect against the development of asthma, allergies and atopy, a finding which seems to be independent of other farm-related exposures (Braun-Fahrlander and von Mutius, 2011). In addition, raw milk is anecdotally reported to be beneficial for people with lactose intolerance (Beals, 2008). This is thought to be due to the fact that raw milk contains high counts of LAB that produce lactase enzymes, which would otherwise be destroyed during pasteurization. However, there is little research evidence to support these anecdotal claims and, in fact, one recent pilot randomized controlled trial (RCT) involving 16 adults with lactose malabsorption, failed to find any benefit of raw milk over pasteurized milk for gastrointestinal symptoms (Mummah *et al.*, 2014). Despite this, in a survey of raw-milk consumers (Mullin and Belkoff, 2014), over one-third of responders claimed to experience gastrointestinal discomfort from drinking pasteurized milk but no discomfort after drinking raw milk, although the vast majority of these people did not have a diagnosis of lactose intolerance. Another proposed benefit of raw milk is that it contains higher quantities of vitamins. A meta-analysis (Macdonald *et al.*, 2011) reported that pasteurization reduced the concentrations of Vitamin E, Vitamin B12, Vitamin B2, Vitamin C and folate. Of these vitamins, B2 is of most importance as bovine milk contributes significantly to the recommended daily intake whereas in the case of all the others, milk is not typically an important source. In relation to the human gut microbiome, we are unaware of any studies specifically examining the effect of raw milk consumption. However, a few studies have investigated the impact of pasteurized milk on the human microbiome. One cross-sectional study reported a differential oral microbiome based on high versus low (pasteurized) milk intake (Johansson *et al.*, 2018). Another investigated the impact of whole milk supplementation on the gut microbiota and cardiometabolic biomarkers between lactose malabsorbers (LM) and absorbers (LA) (Li *et al.*, 2018). Authors found that whole milk supplementation significantly altered the intestinal microbiota composition in LM resulting in an increase in the phylum Actinobacteria along with increases in several genera; *Bifidobacterium*, *Anaerostipes* and *Blautia.* These changes occurred only in LM and not LA, suggesting that it was the increased lactose substrate reaching the colon which preferentially enhanced the growth of some micro-organisms. In addition to pasteurization, milk can be altered by skimming which is currently a widespread procedure. Prior to the course 10/24 of our participants reported consuming skimmed or semi-skimmed milk while post-course 23/24 participants consumed whole milk, reflecting the unpasteurized milk intake. Skimmed milk contains less fat than whole milk and thus also less fat-soluble vitamins such as A and E. However, regular unfortified milk is not a major contributor to a person's recommended daily allowance of these vitamins (Herrero *et al.*, 2002) and despite the variable amounts in different milk types there does not appear to be significant difference in their bioavailability (Herrero-Barbudo *et al.*, 2006). Other

micronutrients such as calcium, sodium and choline do not differ between skimmed and whole milk (Manzi, Di Costanzo and Mattera, 2013). Therefore, we considered the skimmed versus whole milk type to be of limited consequence.

An obvious limitation of this study is the inherent potential for confounding given that, in addition to a change in diet, study participants experienced a change in environment. Disentangling the impact of diet and geographical environment on the gut microbiome, however, is a very difficult task. Several large scale studies have attempted to explore the differences in microbiome composition between industrialised Western urban dwellers and those living in traditional rural communities in South America and Africa, such as the Hadza hunter-gatherers of Tanzania (Schnorr *et al.*, 2014), rural Papua New Guineans (Martínez *et al.*, 2015) children from the rural African village of Burkina Faso (De Filippo *et al.*, 2010) and communities from Malawi and Amazonian Amerindians (Yatsunenko *et al.*, 2012). Although a rural setting will likely contribute to gut microbiome differences, these farming environments are intrinsically linked to variation in diet and it is difficult to separate the impact of the farm environment itself and the farm-related dietary patterns. If a move to a rural farming environment were to account for the changes in microbiome seen in our study one could postulate that the changes would be consistent with the microbiome composition in rural dwellers from the above studies. This was not the case. While rural dwellers from PNG did have higher abundance of *Lactobacillus* than their urban counterparts (Martínez *et al.*, 2015), those from the other rural farming communities did not (Schnorr *et al.*, 2014; De Filippo *et al.*, 2010). Obviously, the rural locations in the above studies were at the extreme end in relation to geographical location and traditional lifestyle, and poorly comparable to the developed farm environment in which our participants were based. In a study more closely resembling our location, authors compared the microbiome of infants from farming and non-farming families in Wisconsin, United States, and again no differences in *Lactobacillus* or other LAB abundance were seen (Thorsen *et al.*, 2019). Furthermore, the changes in bacterial taxa in the microbiome of our subjects were consistent with those species found in unpasteurized milk, supporting our conclusion that this specific dietary change was driving the microbiome differences between pre- and post-course time points.

In this study we found that, during the 12-week course, the levels of the faecal SCFA valerate increased with a trend towards increase in proprionate. Straight-chain SCFAs (acetate, butyrate, propionate and valerate) are produced by the gut microbiota during the fermentation of partially and nondigestible polysaccharides whereas branched-chain SCFAs (isobutyrate and isovalerate) result from the metabolism of proteins (He *et al.*, 2018). SCFAs are thought to play a major role in

the maintenance of gut and immune homeostasis (Tan *et al.*, 2014) as well as in the gut-brain axis response to stress (van de Wouw *et al.*, 2018). SCFA production can be stimulated by increasing dietary fibre intake (Francois *et al.*, 2012) or protein consumption (Russell *et al.*, 2011). However, in our study, participants intake of fibre or protein did not change and thus, it is proposed that increased valerate and propionate levels may have been secondary to increased abundance of *Lactobacilli*, which, along with other LAB, are known producers of SCFA (LeBlanc *et al.*, 2017). Propionate has anti-inflammatory properties and has been shown to be of potential benefit across a range of disorders, including hypertension and cardiovascular disease (Bartolomaeus *et al.*, 2019), obesity (Chambers *et al.*, 2015) and hypercholesterolemia (Demigne *et al.*, 1995). Valerate is a less well-known SCFA with limited research to date into its therapeutic potential. However, a recent study revealed that it also appears to have an immunomodulatory effect (Luu *et al.*, 2019). Interestingly, supplementation with *Lactobacillus acidophilus* increased the concentration of valerate in the caecum of chickens infected with *Clostridium perfringens* while reducing the infection-associated gut dysbiosis (Li *et al.*, 2017). Valerate may also hold some translatable therapeutic value in the context of *Clostridium difficile* infection (CDI). Valerate was shown to be significantly reduced in the faecal samples of patients with recurrent CDI and recovered following successful treatment with FMT (McDonald *et al.*, 2018).

Changes in the functionality of the microbiome were assessed in the context of a recent study which facilitates analysis of the neuroactive potential of a microbiome sample (Valles-Colomer *et al.*, 2019). Authors achieved this using a GBM framework which targets microbial pathways known to be involved in microbiota-gut-brain communication and have made this GBM catalogue available for use by other researchers (https://raeslab.org/software/gbms.html). When applying our predictive metagenomic data to this GBM catalogue we found an increase in the functional richness of the microbiome profile, as determined by the number of GBMs present, following the 12-week course (Figure 4). Such a consistent general increase in GBMs without a significant increase in microbial alpha diversity goes somewhat against the intuition that a more diverse microbial ecosystem will necessarily display a higher functional diversity. More strikingly, the functional alpha diversity did not change during the course. GBMs represent a specific subset of microbiome function and are calculated using the values of specific KEGG Orthologues. A shift in microbial functions that specifically potentially impact the host brain without a corresponding general shift in microbial function detectible on the alpha diversity level shines light on the possibility that many more such specific shifts can occur undetected using current bioinformatics tools. Because of this, we call for a

move away from general diversity and towards informed interrogation of specific functional changes in the microbiome as a readout.

One GBM changed significantly after post-hoc correction; 'GBM026; Nitric oxide synthesis II (nitrite reductase)'. Several studies have demonstrated the ability of various *Lactobacillus* species to synthesize nitric oxide by nitrate reductase activity (Liu *et al.*, 2014; Xu and Verstraete, 2001). Nitric oxide is a complex and widespread signalling molecule which participates in virtually every organ system of the body. It is thought to play a role in the stress response and mood regulation (McLeod, Lopez-Figueroa and Lopez-Figueroa, 2001) and may represent one mechanism by which *Lactobacilli* exert psychobiotic effects. The authors believe another GBM warrants discussing here, although its increase did not satisfy significance after post-hoc correction; 'GBM004, Kynurenine synthesis'. This module was never detected in participants pre-course but was present in very high levels in 6 out of 24 participants post-course. This can be explained by the fact that the Kynurenine synthesis module requires two enzymatic steps. One of these was found in *Lactobacillus*, but the other one was not specific to a single microbe in this data set, but rather spread over several microbes and was only found in the 6 participants positive for MBG004. This finding conforms well with literature regarding emergent biosynthetic capacity of the microbiome (Chiu, Levy and Borenstein, 2014; Perisin and Sund, 2018).

Although we found no direct correlation between *Lactobacillus* abundance and psychological measures, it is notable that stress and anxiety levels reduced significantly in those with higher baseline scores on the PSS and HADS-A. This is consistent with probiotic interventional trials in healthy populations, whereby an impact is often only seen in those with higher anxiety or depression scores at baseline (Liu, Walsh and Sheehan, 2019; Ng *et al.*, 2018). Of course, there are many possible confounding factors when it comes to interpreting this reduction. Participants in this course had varying reasons for completing the course; for some, the purpose was to enhance or change their career options and thus, possibly associated with stress; for others it was simply for leisure and viewed more as a holiday incorporating cookery classes. The change in environment and daily activity, the purpose of participation in the course and interaction with new people may all have contributed to psychological status. However, given the increasing evidence that the gut microbiome is an important node in gut-brain communication and that certain psychobiotics have anxiolytic effects, it is plausible to consider the possibility that the improvement in stress and anxiety may have been partially related to the increase in *Lactobacillus*. *Lactobacillus rhamnosus* (JB-1) has been shown to reduce anxiety behaviours in mice as well as altering central levels of gamma-

aminobutyric acid (Bravo *et al.*, 2011), a key neurotransmitter in anxiety regulation. Several species of *Lactobacillus* have demonstrated the ability to reduce anxiety and stress levels in healthy subjects (Nishihira J. *et al.*, 2014; Messaoudi *et al.*, 2011; Takada *et al.*, 2016) as well as in patients with chronic fatigue syndrome (Rao *et al.*, 2009) or laryngeal cancer (Yang *et al.*, 2016).

There are several limitations to our study. Firstly, this was an observational study. While of course an RCT would be preferable, there are many challenges inherent in designing RCTs involving dietary interventions. It can be difficult to define appropriate control groups and effective blinding of participants and investigators is often extremely difficult (Weaver and Miller, 2017). In particular, it can be challenging to accomplish a high level of adherence with whole food, or dietary pattern, interventions. A major strength of our study in this regard was that our participants were based on-site for the entire duration of the study making it possible to ensure a consistency across individual diets which would be difficult to achieve outside a residential setting. The potential confounding effect of the farm environment as an independent modulator of microbiome composition is addressed earlier in the discussion. Secondly, our sample size was quite small. However, previously published studies investigating the diet-microbiome relationship have involved participant numbers of ten or less (David *et al.*, 2014; Ruggles *et al.*, 2018) and have generally been of much shorter duration (Johnson *et al.*, 2019). Another factor which may limit the generalizability of our study was participants undertaking this course were interested in food and cooking. Thus, they were likely to have good nutritional knowledge and possibly healthier than average diets at baseline. A specific limitation in this regard was an absence of any information on the use of non-nutritive sweeteners (NNS). These are being increasingly used due to the concern about the negative health impact of high-sugar diets and have been shown to significantly, and generally negatively, impact the gut microbiome (Suez *et al.*, 2015). Finally, given the limitations of 16S rRNA gene sequencing we were unable to characterize organisms beyond the genus level. More accurate taxonomic classification would have been useful had shotgun metagenomic sequencing been performed. Despite these limitations, this is, to our knowledge, the first study to report on the potential impact of unpasteurized milk and dairy products on the human gut microbiome. Given the growing popularity of consumption of raw milk and other probiotic-rich fermented foods, it is important that the effect of such products on the gut microbiome are investigated.

7.6 Conclusion

While there are understandable concerns in relation to potential contamination and safety when it comes to unpasteurized milk, it is a rich source of probiotic bacteria. Abundances of *Lactobacillus* increased significantly following a 12-week dietary change which involved consumption of unpasteurized milk and dairy products. *Lactococcus* abundance, also increased, although to a lesser extent. These changes in microbiome composition were reflected by an increase in levels of the SCFA, valerate with an observed trend towards increase of propionate, along with an increase in the predicted functional richness of the microbiome. Given the growing appreciation of the importance of a healthy gut microbiome and the limitations of commercial probiotic products, there is a need for further research into the effect of different dietary changes on the microbiome. In particular, further studies investigating the probiotic potential of natural probiotic-containing foodstuffs such as unpasteurized milk are warranted.

Chapter 8: General Discussion

8.1 Overview and summary

The neurobiological basis for SAD is poorly understood. In particular, the role of the MGB axis has not been explored in this disorder. Indeed, the MGB axis remains largely unexplored across clinical anxiety disorders in general, despite an ever-growing body of preclinical evidence that the microbiome influences anxiety-like behaviours, as well as promising human data for the therapeutic potential of microbiome-based interventions in improving anxiety and depressive symptoms. In this thesis, I investigated the hypothesis that the gut microbiome is altered in patients with SAD, and that pathways of relevance to MGB communication deviate from control subjects. I have demonstrated that SAD is associated with gut hyperpermeability and gut microbiome variations, both in terms of composition and function. Furthermore, I have demonstrated that various systems involved in gut-brain communication, including the HPA-axis, immune markers and the kynurenine-tryptophan pathway show differences from healthy controls, several of these pathways of which have never been previously explored/implicated in SAD.

In chapter 3, I demonstrate that the gut microbiome in SAD patients shows distinct changes from that of healthy controls, with evidence of beta-diversity differences between the two groups and alterations in the relative abundance of several genera and species. I report a notable difference in the relative abundance of a hitherto relatively unfamiliar genus, *Anaeromassillibacillus*, which was present in almost half of our SAD group but in only one control individual. I show that the gut microbiome is functionally different in SAD with enrichment of a metabolic pathway involving the enzyme aspartate aminotransferase (AspAT). Given the identical nature of AspAT and kynurenine aminotransferase (KAT), the enzyme responsible for kynurenic acid (KYNA) synthesis, I suggest a possible association between gut microbiome function and the tryptophan-kynurenine pathway in SAD. This is supported by the finding of peripheral elevated KYNA concentrations and an increased KYNA/KYN ratio in SAD patients, as discussed in chapter 5. In chapter 4, I demonstrate gut hyperpermeability in SAD patients based on a panel of gut permeability blood markers providing indirect evidence of epithelial tight junction dysfunction and endotoxemia. In chapter 6, I report alterations in neuroendocrine markers in SAD patients, including chronic cortisol levels, the cortisol awakening response (CAR), the salivary alpha amylase awakening response and plasma oxytocin levels - factors which have been linked to gut microbiota alterations in preclinical studies. Finally, in Chapter 7, I report on a longitudinal study assessing the impact of a dietary change involving the consumption of unpasteurised milk and dairy products over 12-weeks, on gut microbiome composition, function and microbial short-chain-fatty-acid production in healthy volunteers. I demonstrate that intake of unpasteurised dairy is associated with significant increases in the

probiotic genus, *Lactobacillus*. Additionally, I show that those participants with high baseline anxiety scores showed a reduction in anxiety levels between pre- and post-course time points.

8.2 The Microbiome-Gut-Brain Axis in SAD; From the laboratory to the clinic

SAD is a complex heterogenous condition that, as I have shown, is associated with alterations across many biological systems, including neuroendocrine, immune and tryptophan-kynurenine pathways. The exact mechanisms by which these abnormalities contribute to the development and persistence of SAD are unclear, and further preclinical exploration of the mechanisms involved, along with larger prospective clinical studies, are required. However, the gut microbiome may represent a unifying factor given that all of these pathways are implicated in bidirectional MGB axis communication.

A major challenge in MGB axis research in psychiatry, and indeed across behavioural neuroscience, is bridging the translational gap between the laboratory and the clinic. As discussed in the introductory chapter, there are many different animal models of depression and anxiety, and a wide variety of behavioural tests that can be used to explore anxiety- and depressive-like behaviours. However, clinical anxiety disorders are diverse, multifaceted, poorly understood conditions, and the construct validity of animal behavioural tests are limited (Garner, 2014; Neumann *et al.*, 2011). However, significant efforts are being made to optimise animal models (Gururajan *et al.*, 2019; Slattery and Cryan, 2014). For instance, while the majority of animal anxiety paradigms assess unspecific/generalised anxiety or fear (e.g., the elevated plus maze, the light-dark box, the open field test) (Kumar, Bhat and Kumar, 2013), a specific animal model of SAD has been developed (Toth, Neumann and Slattery, 2012). This model involves the induction of strong social fear following exposure to a social fear conditioning intervention in mice. Importantly, the induced fear is specific to social stimuli, does not generalise to other non-specific anxiety- and depressive-like behaviours and can be reversed by acute benzodiazepine and chronic antidepressant treatment. Thus, a specific and appropriate model such as this could be an invaluable tool for further investigation of the MGB axis in SAD.

While creating new animal models to more accurately emulate specific psychiatric conditions is useful, another approach to improve animal-human translatability may be to focus on more specific psychopathology domains in human studies. We currently define various aspects of psychopathology in terms of categorical, diagnostic psychiatric classifications (APA, 2013) but moving towards a transdiagnostic, dimensional approach may be more useful for research. Indeed, this has been the objective of the Research Domain Criteria (RDoC), an initiative first proposed by

the National Institute of Mental Health (NIMH) over a decade ago (Insel *et al.*, 2010). It promotes removing the constraints of traditional, diagnostic categories in psychiatric research and analysing groups based on transdiagnostic, dimensional domains such as negative valance, positive valence, cognitive, social processing and arousal/regulatory systems, assessed by various units of analysis ranging from genes to neural circuits to behavioural measures (Cuthbert, 2015). Thus, for example, instead of focussing a study on a cohort of patients with SAD, one might investigate a specific social processing system, such as social communication, across several relevant diagnostic groups such as SAD, ASD or schizophrenia. While our study of the MGB axis in patients with a SAD diagnosis moves the investigation of the microbiome-anxiety link beyond broad non-specific measures of general anxiety, as seen in both animal and human studies to date, taking a RDoC approach would extend further this potential to uncover associations between the microbiota and specific aspects of anxiety symptomatology.

Using the RDoC approach, assessment that is based on objective genetic and neurobiological measures, as well as more subjective clinical symptom observations, would have the advantage of improving the translation of preclinical animal studies to human patient studies, improving the understanding of basic pathophysiology of psychiatric illness and hopefully resulting in more precise, targeted treatments (Kaiser and Feng, 2015). Given the growing body of evidence for the role of the gut microbiota across a range of neuropsychiatric disorders (Butler, Cryan and Dinan, 2019), gut microbiome signatures may represent another transdiagnostic dimension from which to approach the treatment of mental illness (Kelly *et al.*, 2018).

8.3 Gut microbiome manipulation as a therapeutic target in SAD

The key question for clinicians is whether gut microbiome manipulation can be utilised as an effective therapeutic target for mental illness or whether it remains merely an academic pursuit. The microbiome can be altered by several methods (Figure 1). Probiotics, defined as living bacteria that, when administered in adequate amounts, confer a health benefit on the host (Behnsen *et al.*) are easily administered orally. They allow investigation of individual species or bacterial combinations, termed polybiotics, on different parameters in both health and disease states. A less specific, but possibly more effective, method of enhancing specific bacteria is through the use of prebiotics, defined as substrates, usually but not necessarily carbohydrates, which selectively enhance the growth of certain bacteria (Gibson *et al.*, 2017). They can be administered with their preferred bacterial targets for greater efficacy, the combination being referred to as a "synbiotic." (Kolida and Gibson, 2011). Another term widely used in the microbiome arena is the "psychobiotic", which

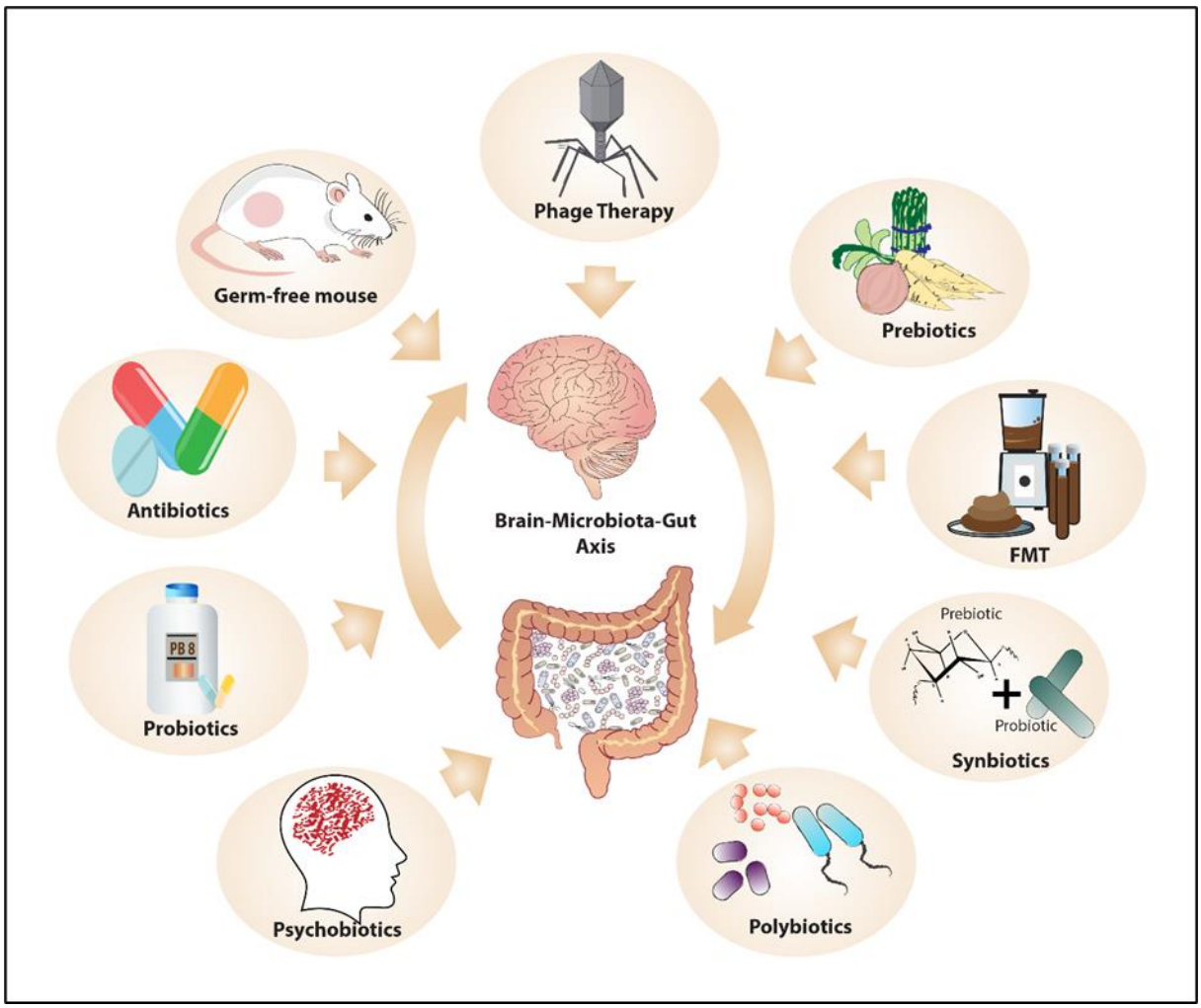
initially referred to probiotics and prebiotics that were shown to confer a specific mental health benefit (Dinan, Stanton and Cryan, 2013), with subsequent expansion of the definition to include anything, including diet and exercise, that exerts a microbiome-mediated psychological effect (Sarkar *et al.*, 2016). Recent meta-analyses have demonstrated a beneficial effect of probiotics for improving mood and reducing anxiety, although the effect sizes are small and the majority of studies have been carried out in healthy populations, and not in clinical samples (Liu, Walsh and Sheehan, 2019). The evidence for antidepressant and anxiolytic properties of prebiotics is less convincing (Liu, Walsh and Sheehan, 2019) and similarly, probiotics, prebiotics or fermented foods do not appear to be beneficial for cognitive outcomes (Marx *et al.*, 2020b). Interestingly, according to a recent meta-analysis involving studies of healthy individuals and patients with a variety of clinical conditions, pro- and prebiotic supplementation has an effect on tryptophan-kynurenine pathway metabolites (Purton *et al.*, 2021) supporting a role for MGB axis manipulation in the treatment of psychiatric disorders. A more drastic means of altering the microbiome is through the use of faecal microbiota transplantation (FMT) which involves the transfer of faecal matter from one individual to another, thereby passing on the donor's microbiota (Pamer, 2014). It has been shown to be highly effective in treating the gastrointestinal infection, *Clostridium difficile* (Bakken *et al.*, 2011) with promising findings also in the long-term treatment in IBS (El-Salhy *et al.*, 2021). This therapeutic potential has extended into the psychiatric domain. Two small studies investigating FMT in the treatment of IBS reported improvements in mood and anxiety symptoms (Kurokawa *et al.*, 2018; Mazzawi *et al.*, 2018) and a small open-label trial demonstrated promising results using FMT as a potential therapy for ASD (Kang *et al.*, 2017) with sustained improvements in gastrointestinal and autism symptoms seen at 2 years (Kang *et al.*, 2019). Several case reports purporting the efficacy of FMT in depressed patients have been published (Xie *et al.*, 2019; Cai *et al.*, 2019) and clinical trials of FMT in both depression (https://www.clinicaltrials.gov/ct2/show/NCT03281044) and bipolar disorder (https://clinicaltrials.gov/ct2/show/NCT03279224) are currently underway. To date, there have been no human FMT studies in clinical anxiety disorder cohorts.

A new and exciting method of altering the microbiome is through the use of phage therapy. Phages, short for bacteriophages, are viruses that infect specific bacteria. Although they have been around for over a century, interest in their use as a method of eliminating pathogenic bacteria largely subsided with the advent of antibiotics. However, renewed curiosity about their therapeutic potential has developed with the emergence of antibiotic resistance (Lin, Koskella and Lin, 2017). The success of FMT in treating resistant gastrointestinal infections such as *Clostridium difficile* is generally attributed to the transfer and colonisation of bacteria. However, it has been shown that the viral component from donor FMT can colonise the recipient gut for up to 12 months and may

play a much greater role than is currently appreciated (Draper *et al.*, 2018). As a modulator of microbiome composition, the use of phage to target the MGB axis is highly plausible, although very much limited to the research domain at present.

Dietary change is another highly effective way of rapidly and reproducibly altering microbiome composition (David *et al.*, 2014). In a society that is becoming increasingly conscious of food choice, using diet as a therapeutic strategy for mental illness is not the pipe dream it might once have been (Marx *et al.*, 2021). Diet quality is poorer in patients with depressive and anxiety disorders (Gibson-Smith *et al.*, 2018). The prophylactic benefits of a Mediterranean diet in protecting against depression are well-recognised (Psaltopoulou *et al.*, 2013) and improving diet quality also appears to have anxiolytic benefits (Firth *et al.*, 2019). Although there have been no dietary interventional studies in clinical anxiety disorders, an innovative RCT has recently expanded the evidence base by demonstrating the efficacy of a Mediterranean-style diet as an effective adjunctive therapy in MDD (Jacka *et al.*, 2017). Interestingly, a potential therapeutic role for the microbiome in SAD is supported by a cross-sectional study which reported that higher intake of fermented, probiotic-containing foods by healthy students, appeared to be protective against developing SAD in those who were at higher genetic risk, as measured by trait neuroticism (Hilimire, DeVylder and Forestell, 2015).

Figure 1: Manipulating the Microbiome; The microbiome can be altered in various ways to investigate the impact on the brain and psychological function (Butler *et al.*, 2019).



8.4 Limitations of the reported studies

Although our studies have generated some novel and exciting insights into the underlying neurobiology of SAD, there are several limitations to be noted. The first is the cross-sectional nature of the data, which obviously limit any definitive conclusions in relation to causation and preclude longitudinal assessment of changes in the gut microbiota and other biological markers as they relate to SAD symptoms. Larger prospective studies are now warranted in this regard. Alternatively, the use of FMT from human SAD donors to animal models with subsequent investigation of the behavioural and physiological consequences, would be an effective way to investigate the potential aetiological role of the MGB axis in SAD, and has been done successfully in other psychiatric conditions, including depression (Kelly *et al.*, 2016; Zheng *et al.*, 2016), ASD (Xiao *et al.*, 2021) and schizophrenia (Zheng *et al.*, 2019). Secondly, although our groups were matched in relation to many

parameters of relevance to the gut microbiome including age, sex, stool consistency, alcohol, smoking status, birth delivery mode and most nutrient groups, they were unmatched for BMI and exercise. Adjusting for BMI did not affect our results in relation to microbiome differences or most biological markers, including our neuroendocrine, immune and kynurenine pathway findings. However, in chapter 3 on intestinal permeability, group differences were lost for zonulin and CRP after adjusting for BMI, and zonulin in particular showed a strong association with BMI. It would of course be preferable to have groups that were balanced in relation to BMI. Similarly, our SAD group had lower exercise levels than the control group, which may also have influenced findings, although I did adjust for exercise throughout. Furthermore, absence of data on menstrual cycle is a significant limitation, in particular with regards to our neuroendocrine findings.

Another limitation is that two-thirds of our SAD sample were taking psychotropic medication, and I acknowledge the potential impact of this on the gut microbiome and other markers. There have been significant efforts in recent years to establish the impact of psychotropic (Cussotto *et al.*, 2019) and other (Vich Vila *et al.*, 2020) medications on the gut microbiome. The majority of our medicated patients were taking SSRIs or SNRIs, which have varying antimicrobial effects (Cussotto *et al.*, 2018; Lukić *et al.*, 2019; Ait Chait *et al.*, 2020), as discussed in detail in Chapter 2. Subgroup analyses between SAD patients who were taking medication and those who were unmedicated did not reveal any differences in terms of the microbiome or intestinal permeability markers. Similarly, apart from elevated plasma KYN levels in medicated compared to unmedicated patients, no differences were found for any other kynurenine pathway metabolites, neuroendocrine or immune parameters. However, given the small sample sizes in these medicated and medication-free subgroups, this must be interpreted with caution. As with other psychiatric disorders, it must be acknowledged that it is difficult to recruit unmedicated anxiety disorder patients, in particular in western countries where, over the past two decades, prescription rates of antidepressant and anxiolytic medications have risen significantly (Noordam *et al.*, 2015; Heald *et al.*, 2020; Rhee and Rosenheck, 2019). Furthermore, excluding patients taking psychotropic medications risks excluding those most severely affected by their psychiatric conditions and thus, those most likely to be of interest in relation to underlying pathophysiology.

A final potential limitation is that some of our SAD patient group had a past history of depression and/or a comorbid anxiety disorder. However, patients with a current depressive episode were excluded, and in all, the primary diagnosis was SAD with any comorbid anxiety disorder representing a secondary diagnosis. Comorbidity amongst clinical anxiety disorders is the rule rather than the exception (Kaufman and Charney, 2000) and trying to isolate cohorts to a pure single diagnosis may

actually make findings less generalisable and exclude patients on the more severe end of the clinical spectrum.

8.5 Perspectives and Conclusions

SAD is a condition which, along with other clinical anxiety disorders, has to date, been relatively neglected in the field of research. This is unfortunate given that it is one of the most common and chronic psychiatric conditions and is associated with significant distress and severe functional disability for many sufferers. Current pharmacological and psychological treatments are only effective for a proportion of patients, and alternative therapeutic approaches are needed, the basis of which will require a greater understanding of the complex underlying neurobiological factors. Given the move towards a more dimensional approach to psychiatric illness, greater elucidation of the aetiology of SAD may aid us in better understanding other fear- and anxiety-based conditions, as well as disorders associated with deficits in social processing systems, such as ASD and schizophrenia.

The meeting of microbiome research and neuroscience represents a new and exciting frontier in psychiatry. We are in great need of a novel paradigm in our approach to mental illness. Depression and anxiety are the tuberculosis and cholera of the 21st century. In 2015, the total estimated number of people living with anxiety disorders in the world was 264 million, an increase of 14.9% from 10 years previously (GBD, 2018). The World Health Organisation ranks anxiety disorders as the sixth largest contributor to non-fatal health loss globally (WHO, 2017). In contrast, development of psychotropic drugs has remained stagnant for many years and there appears to be little current evidence of movement (Hyman, 2013; Tricklebank *et al.*, 2021). Could an aetiological clue, and possibly a new therapeutic answer, to this psychological epidemic reside in our gut? While it seems unlikely that probiotic or prebiotic supplementation alone could significantly impact those with severe psychopathology, it may form part of a comprehensive holistic treatment plan. Similarly, dietary change in those who are motivated may be an alternative way to enhance current treatments, and may be beneficial as a preventative strategy on a wider population-based level (Marx *et al.*, 2017). More drastic microbiome changes, such as those seen with FMT from healthy donors, would seem to be a more plausible way of generating a microbiome change of sufficient magnitude to markedly impact MGB pathways, and therefore potentially lead to symptom improvements in those with significant mental illness. It is an exciting time in microbiome research as we await the upcoming results of current FMT trials in depression and bipolar disorder.

A move toward personalised medicine is an aspiration held by many healthcare professionals and researchers alike, and a microbiome-based approach represents the perfect example of how this new paradigm of individualised healthcare might ultimately be realised. The Human Genome Project, which sequenced and mapped all the genes of the human genome, is undoubtedly one of the greatest achievements of our time (Hood and Rowen, 2013). However, the expectation that it would completely revolutionise medicine, and result in individualised treatments based on the genetic make-up of the patient, has not been realised. This is primarily due to a gross underestimation of the importance of epigenetic phenomena, i.e., the influence of the environment on gene expression. Nonetheless, the development of personalised medicine is an exciting aspiration, especially for psychiatric disorders, which demonstrate extremely high rates of interindividual variability in terms of clinical presentation and treatment response. The first step in precision medicine is the identification of biomarkers. Despite decades of research, we still do not have a reliable biomarker for anxiety disorders or depression, although it appears that using a panel of multiple biomarkers may be of some diagnostic use (Schmidt, Shelton and Duman, 2011; Sharma and Verbeke, 2021). It is certainly plausible that an individual's microbiome fingerprint could be a valuable component of an anxiety or depression biomarker panel and, more importantly, indicate whether microbiome-based treatment may represent a useful part of the therapeutic arsenal. The microbiome represents the perfect target for precision medicine given the significant contribution it makes to interindividual variability in all aspects of health and disease. In addition, the huge amount of genetic material carried by our gut bacteria is readily and easily modifiable, unlike our human genes, thus offering immense potential therapeutic value for a wide range of multifactorial disease states, including anxiety disorders.

The future of psychiatry will likely include microbiome analysis with resultant targeted therapeutic interventions incorporating evidence-based microbiome manipulation through the use of probiotics, prebiotics, specific dietary recommendations and possibly for some, FMT. Although such an approach may appear to some to be a distant dream, the prevailing mood of enthusiasm and excitement among the microbiome research community, and the rapid pace of discovery, mean that patients with debilitating disorders such as SAD may not have so long to wait for a personalised microbiome mental health plan.

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Appendix

Liebowitz Social Anxiety Scale – Self Report (LSAS-SR)

	Fear or Anxiety 0 = None 1 = Mild 2 = Moderate 3 = Severe		Avoidance 0 = Never (0%) 1 = Occasionally (1-33%) 2 = Often (34-66%) 3 = Usually (67-100%)	
Items	Anxiety (S)	Anxiety (P)	Avoid (S)	Avoid (P)
1. Telephoning in public. (P)				
2. Participating in small groups. (P)				
3. Eating in public places. (P)				
4. Drinking with others in public places. (P)				
5. Talking to people in authority. (S)				
6. Acting, performing or giving a talk in front of an audience. (P)				
7. Going to a party. (S)				
8. Working while being observed. (P)				
9. Writing while being observed. (P)				
10. Calling someone you don't know very well. (S)				
11. Talking with people you don't know very well. (S)				
12. Meeting strangers. (S)				
13. Urinating in a public bathroom. (P)				
14. Entering a room when others are already seated. (P)				
15. Being the center of attention. (S)				

16. Speaking up at a meeting. (P)				
17. Taking a test. (P)				
18. Expressing disagreement or disapproval to people you don't know very well. (S)				
19. Looking at people you don't know very well in the eyes. (S)				
20. Giving a report to a group. (P)				
21. Trying to pick up someone. (P)				
22. Returning goods to a store. (S)				
23. Giving a party. (S)				
24. Resisting a high pressure sales person. (S)				
Total Performance (P) Subscore				
Total Social (S) Subscore				
Total Anxiety & Avoidance Subscore				
Total LSAS Score				

SLÁN-06 - FOOD FREQUENCY QUESTIONNAIRE

YOUR DIET OVER THE PAST YEAR

For each food there is an amount shown, either what we think is a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick in the box to indicate how often, **on average**, you have eaten the specified amount of each food, to the nearest whole number **during the past year i.e. from when you receive this questionnaire to the same month the previous year**.

Please estimate your average food use as best you can. Please answer every question, do not leave ANY lines blank.

EXAMPLES:

The following are examples on how to estimate how often and how much bread and potatoes you ate over the past year. Please estimate your food intake for all foodstuffs in the same way.

Potatoes: If you ate a medium serving of potatoes 3 times per week over the past year put a tick in the box "2-4 per week". If you think you usually ate more or less than a medium serving please try to estimate which box suits best.

	AVERAGE USE LAST YEAR								
Potatoes, Rice and Pasta (medium serving)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Boiled, instant or jacket potatoes				√					

For white bread a medium serving is one medium sized slice. Therefore if you usually ate 1 medium slice 4 or 5 times per day, you should put a tick in the column headed "4-5 per day". If you ate 2 medium slices 4 or 5 times per day, then you should put a tick in the column "6+ per day".

	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (One slice or one biscuit)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls (including ciabatta and pannini bread)								√	

Please check that you put a tick (√) on every line

	AVERAGE USE LAST YEAR								
A. MEAT, FISH AND POULTRY (Medium serving – the size of a deck of cards)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Beef roast									
2. Beef: steak									
3. Beef: mince									
4. Beef: stew									
5. Beef burger (1 burger)									
6. Pork: roast									
7. Pork: chops									
8. Pork: slices/escalopes									
9. Lamb: roast									
10. Lamb: chops									
11. Lamb: stew									
12. Chicken portion or other poultry e.g. turkey: roast									
13. Breaded chicken, chicken nuggets, chicken burger									
14. Bacon									
15. Ham									
16. Corned beef, Spam, Luncheon meats									
17. Sausages, Frankfurters (1 sausage)									
18. Savoury pies (e.g. meat pie, pork pie, steak & kidney pie, sausage rolls)									
19. Liver, heart, kidney									
20. Liver paté									
21. Fish fried in batter, as in fish and chips									
22. Fish fried in breadcrumbs									
23. Oven baked/grilled fish (in breadcrumbs or batter)									
24. Fish fingers/fish cakes									
25. Other white fish, fresh or frozen (e.g. cod, haddock, plaice, sole, halibut, coli)									
26. Oily fish, fresh or canned (e.g. mackerel, kippers, tuna, salmon, sardines, herring)									
27. Shellfish (e.g. crab, prawns, mussels)									

	AVERAGE USE LAST YEAR								
B. BREAD AND SAVOURY BISCUITS (One slice or one biscuit)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. White bread and rolls (including ciabatta and pannini bread)									
2. Brown bread and rolls									
3. Wholemeal bread and rolls									
4. Cream crackers, cheese biscuits									
5. Crisp bread, e.g. Ryvita									
6. Pancakes, muffins, oatcakes									

	AVERAGE USE LAST YEAR								
C. CEREALS (One medium sized bowl)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Porridge, Readybrek									
2. All Bran, Weetabix, Shredded Wheat									
3. Branflakes, Bran Buds									
4. Cornflakes, Rice Krispies									
5. Muesli (e.g. Country Store, Alpen, sugar coated)									
6. Sugar Coated Cereals (e.g. Frosties, Crunchy Nut Cornflakes, Crunchy Sugar Coated Muesli)									

	AVERAGE USE LAST YEAR								
D. POTATOES, RICE AND PASTA (Medium serving – about a cupful)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Boiled, instant or jacket potatoes									
2. Mashed potatoes									
3. Chips									
4. Roast potatoes									
5. Potato Salad									
6. White Rice									
7. Brown Rice									

8. White/yellow or green pastas (e.g. spaghetti, macaroni, noodles)									
9. Wholemeal pasta									
10. Lasagne (meat based)									
11. Lasagne (vegetarian)									
12. Moussaka									
13. Pizza									
14. Macaroni Cheese									

	AVERAGE USE LAST YEAR								
E. DAIRY PRODUCTS AND FATS	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Cream (tablespoon)									
2. Full-fat yoghurt or Greekstyle Yoghurt (125g carton)									
3. Dairy desserts (125g carton)									
4. Cheddar cheese (medium serving)									
5. Low-fat cheddar cheese (medium serving)									
6. Eggs as boiled, fried, scrambled, poached (one)									
7. Quiche (medium serving)									
8. Light salad cream or light mayonnaise (tablespoon)									
9. Salad cream, mayonnaise (tablespoon)									
10. French dressing (tablespoon)									
11. Other salad dressing (tablespoon)									
12. The following on bread or vegetables									
13. Butter (teaspoon)									
14. Lite Butter e.g. Dawn Lite, Connacht Gold (teaspoon)									
15. Sunflower margarine e.g. Flora (teaspoon)									
16. Low-fat margarine (e.g. lowlow)									
17. Cholesterol Lowering Spreads e.g. Flora Pro Active, Dairy Gold Heart (teaspoon)									

18. Cream & Vegetable Oil spread e.g. Golden Pasture, Kerrymaid, Dairy Gold – teaspoon									
19. Olive oil spread e.g. Golden Olive (teaspoon)									

	AVERAGE USE LAST YEAR								
F. FRUIT (1 Fruit or medium serving)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Apples									
2. Pears									
3. Oranges, satsumas, mandarins									
4. Grapefruit									
5. Bananas									
6. Grapes									
7. Melon									
8. Peaches, plums									
9. Apricots									
10. Strawberries, raspberries, kiwi fruit									
11. Tinned fruit									
12. Dried fruit e.g. raisins									
13. Frozen fruit									

	AVERAGE USE LAST YEAR								
G. VEGETABLES Fresh, frozen or tinned (Medium Serving – 2 tablespoons)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Carrots									
2. Spinach									
3. Broccoli, spring greens, kale									
4. Brussel sprouts									
5. Cabbage									
6. Peas									
7. Green beans, broad beans, runner beans									
8. Courgettes									
9. Cauliflower									
10. Parsnips, turnips									
11. Leeks									
12. Onions									
13. Garlic									

14. Mushrooms									
15. Sweet peppers									
16. Beansprouts									
17. Green salad, lettuce									
18. Cucumber, celery									
19. Tomatoes									
20. Sweetcorn									
21. Beetroot									
22. Coleslaw									
23. Baked beans									
24. Dried lentils, beans, peas									
25. Tofu, soya meat, TVP, vegeburger									

	AVERAGE USE LAST YEAR								
H. SWEETS AND SNACKS (Medium serving)	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Chocolate coated sweet biscuits e.g. digestive (one)									
2. Plain sweet biscuits e.g. Marietta, digestives, rich tea (one)									
3. Cakes e.g. fruit, sponge									
4. Buns, pastries e.g. croissants, doughnuts									
5. Fruit pies, tarts, crumbles									
6. Sponge puddings									
7. Milk puddings e.g. rice, custard, trifle									
8. Ice cream, choc ices, Frozen desserts									
9. Chocolates, singles or squares									
10. Sweets, toffees, mints									
11. Sugar added to tea coffee, cereal (teaspoon)									
12. Sugar substitute e.g. canderel added to tea coffee, cereal (teaspoon)									
13. Crisps or other packet snacks									
14. Peanuts or other nuts									

	AVERAGE USE LAST YEAR

I. SOUPS, SAUCES AND SPREADS	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Vegetable soups: homemade/fresh (1 bowl)									
2. Vegetable soups: tinned/packet (1 bowl)									
3. Meat or cream soups: homemade/fresh (1 Bowl)									
4. Meat or cream soups: tinned/packet (1 bowl)									
5. Sauces e.g. white sauce, cheese sauce, gravy (tablespoon)									
6. Tomato based sauces e.g. pasta sauces									
7. Curry-type sauces									
8. Pickles, chutney (tablespoon)									
9. Marmite, Bovril (tablespoon)									
10. Jam, marmalade, honey, syrup (teaspoon)									
11. Peanut butter (teaspoon)									

	AVERAGE USE LAST YEAR								
J. DRINKS	Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
1. Tea (cup)									
2. Coffee instant (cup)									
3. Coffee ground (cup)									
4. Coffee, decaffeinated (cup)									
5. Coffee whitner e.g., coffee-mate (teaspoon)									
6. Cocoa, hot chocolate (cup)									
7. Horlicks, Ovaltine (cup)									
8. Wine (glass)									
9. Beer, larger or cider (half-pint)									

10. Alcopops e.g. Bacardi Breezer 11. (Bottle)									
12. Port, Sherry, Vermouth liqueurs (glass)									
13. Spirits e.g. gin, whiskey (single measure)									
14. Low-calorie or diet soft fizzy (glass)									
15. Fizzy soft drinks e.g. Coca Cola (glass)									
16. Pure fruit drinks e.g. orange juice (small glass)									
17. Fruit squash (small glass)									

PARTICIPANT INFORMATION SHEET (Patient Volunteers)

Study Title: Investigating the gut microbiota composition in patients with social anxiety disorder (SAD).

Protocol No.: APC085

Principal Investigator: Prof Timothy Dinan

Site of Investigation: Cork University Hospital (CUH), University College Cork (UCC), & Teagasc Moorepark Food Research, Fermoy, Co. Cork,

Why is this study being run?

Research has shown that the gut bacteria (gut microbiome) can influence our brain function and behaviour in different ways. A previous study in our lab has shown that the gut microbiome is different in people with depression. We are interested in finding out if there are any differences in the gut bacteria of people with Social Anxiety Disorder. We are also interested in looking for other differences involving inflammation, stress hormones and other chemical messengers such as tryptophan. The study will also assess the relationship between early life traumatic experiences, mood, personality, sleep patterns, recent stressful events, physical activity, food intake and the composition of the gut microbiota.There will be two groups taking part in the study; people with Social Anxiety Disorder and healthy controls.

Why do we need patients with Social Anxiety Disorder and healthy control volunteers?

Healthy control volunteers are needed so that we can compare the results from people with Social Anxiety Disorder to people who do not suffer with this disorder. This will allow us to assess differences in gut bacteria, inflammation markers, stress hormones and other chemical messengers between the two groups. Hopefully this will provide us with new insights into what causes Social Anxiety Disorder and clues about possible new treatment options.

Study Procedure:

Patients who are interested in participating in the study will be invited to an interview at their convenience. The interview can take place in Cork University Hospital, University College Cork or even

at a person's home if this is more convenient. This interview will take 1 – 1.5 hours and will involve the following

- Subjects will be requested to read the Participant Information Leaflet and read and sign the informed consent form, and will receive a signed copy.
- They will meet a psychiatrist or research nurse who will ask some questions about psychiatric symptoms. They will then be asked to complete questionnaires regarding social anxiety symptoms, early life traumatic experiences, current levels of general anxiety, stress and depression, recent stressful life events, personality, sociability, sleeping habits, physical activity and diet.
- They will also be asked some general questions about age, race, occupation, education, medical history, family history, current medication use.
- Height and weight will be recorded
- Patients will be asked to provide samples of stool, urine, blood, hair and saliva. If you cannot give the sample at that time you will be given containers to collect the sample at home and the researcher will arrange collection.

What happens if I start the study and change my mind later?

You do not have to take part in the study, participation is entirely voluntary. Refusal to participate, or discontinuing participation at any time, will involve no penalty, loss of benefits or denial of treatment or services by the Cork Teaching Hospital or the participating doctor.

Will I experience any unpleasant effects?

Blood for testing will be obtained usually by a needle in one of the large veins at the front of your elbow region in the arm. During the collection of your blood sample you may experience a slight scratch, which may be uncomfortable for a moment but quickly passes. The risks associated with blood testing are minimal, including bruising at the site of vein puncture, inflammation of the vein and possible infection. This will be minimized by having experienced doctors performing the test and care will be taken to avoid these complications.

If you do not want to give a blood sample you can still participate in the study and provide the other samples.

Funding of trial

There are no cost implications for the Health Services Executive (HSE) or to you. The management of patients and investigative tests will comply with current standards of care. Costs of research tests will be incurred by the Alimentary Pharmabiotic Centre, University College Cork, and Alimentary Health who are collaborators on this study.

Upon completion of the study, you will receive €50 to cover expenses incurred during the course of the study.

Confidentiality

All the information gathered from this study will be stored on a computer and/or paper files and will be treated confidentially. You will be identified only by a participant number. In the event of any publication regarding this study, your identity will not be disclosed.

Insurance

University College Cork is the Sponsor of this research project and has appropriate insurance in place if you are harmed as a result of participation in this trial.

Right of Access and Rectification

You have a right, under the Data Protection Acts 1988 and 2003, to access and/or correct your personal data controlled by UCC. If you wish to avail of this right, you should submit a written request to: *Information Compliance Officer, University College Cork, Western Road, Cork, Phone: (0)21 490 3949, Email: foi@ucc.ie.* If you have any questions regarding your personal data, please contact Dr. Caitríona Long-Smith, c.longsmith@ucc.ie .

What happens if there is anything I do not understand?

If there is anything you are not sure about, the Researcher will be happy to explain in more detail to you. The study will be fully explained to you before you decide if you want to take part.

Lead Investigator: Professor Timothy Dinan

Consultant Psychiatrist, Cork University Hospital (CUH), and

Principal Investigator, APC Microbiome Institute, University College Cork.

Study Researcher: Dr. Mary Butler

Telephone number: 087 0971377

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CONSENT BY PARTICIPANT FOR PARTICIPATION IN A HUMAN OBSERVATIONAL STUDY (Patient Volunteers)

Protocol Number: APC085

Participant Name: _____

Title of protocol: : Investigating the gut microbiota composition in patients with social anxiety disorder (SAD).

Principal Investigator: Prof Timothy Dinan
Study Researcher: Dr Mary Butler
Telephone number 087 0971377

Participation in this study is voluntary and you may withdraw at any time for any reason

The research project and procedure associated with it have been fully explained to me. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and I may withdraw consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. I understand that the investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork University Hospital and University College Cork. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the Doctor listed below. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

I agree that analyses of all samples and information collected will be conducted in the CUH and/or APC Microbiome Institute, UCC. On some occasions the analyses may be done in collaboration with third parties, including commercial partners, which may require samples to be shipped to these organisations. In addition, samples and data will be stored and may be used in other research studies. In all cases, samples and data will be coded with anonymized identifier numbers. Please note that data protection laws in other countries may not be as stringent.

I agree that my biological samples may be used in future research studies.
(***please tick box if you agree***) ☐

I agree that access to samples and/or data will require approval from the Director and/or Executive Management Group pf the APC Microbiome Institute, Cork. I agree that results and bacterial isolates may be used to design a commercial strategy or product for gut health. ☐

I agree that my contact details may be made available for recruitment to further relevant studies ☐

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Participant's Signature: _____ Date: _____

dd mon yy

NAME (BLOCK LETTERS): _____ Time: _____

Investigator's Signature: _____ Date: _____

dd mon yy

NAME (BLOCK LETTERS) _____