An Investigation into the Interaction of the Microbiome-Gut-Brain Axis with Stress

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Abstract

This thesis aimed to investigate whether changes in the gut microbiota and associated biomarkers were associated with stress-induced anxiety-like and depressive-like behaviour.

Two studies used the unpredictable chronic mild stress (UCMS) over 4 or 6 weeks (vs no UCMS, control) in Sprague-Dawley rats. Depressive-like behaviour was measured in female rats using the sucrose preference test, and the Porsolt swim test. Anxiety-like behaviour was measured with the light-dark box test. Faecal corticosterone, caecal microbiota (composition and organic acids), serum gut permeability (lipopolysaccharide-binding protein, LBP) and plasma inflammation (12 cytokines) markers were measured.

Atypical behaviours were observed in female rats following UCMS and no depressive-like behaviours were observed. The circulating concentration of cytokines, but not plasma LBP or caecal organic acids, was higher in UCMS-exposed female rats. Relative abundance of taxa from the Clostridiales order and *Desulfovibrionaceae* family correlated with anxiety-like behaviours and plasma cytokine concentrations, regardless of UCMS.

Studies of these atypical behaviours in female rats confirmed expected patterns of sucrose intake in the sucrose preference test and no decreased depressive-like behaviours in the Porsolt swim test with antidepressant citalopram and imipramine drugs. A further study also showed differences in baseline behaviour in male versus female rats, leading the second UCMS study to be in male rats.

Increased faecal corticosterone and anxiety-like behaviours were observed in male UCMS-exposed and control rats at week 4 of UCMS compared to baseline. Plasma cytokine concentrations were higher in the UCMS group but higher faecal corticosterone concentrations and anxiety behaviours in control rats suggest that they were more stressed than treated rats. Caecal neurotransmitter concentrations did not differ between treatments nor correlate with serum neurotransmitter, cytokines or LBP concentrations or behaviour.

The findings showed an association between the gut microbiota and anxiety-like behaviours, which was not stress dependent. No measured biomarkers explained the observed anxiety-like behaviours. Caecal digesta neurotransmitter profiles were dissimilar to serum profiles indicating it may not be an important influence on serum levels. Despite the atypical behavioural results following the interventions, the results still provided useful and unique information which contributes to the body of Microbiome Gut Brain Axis research.

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Bear, T., Dalziel, J., Coad, J., Butts, C., Parkar, S., Rosendale, D., Roy, N., Gopal, P., 2nd International Mind Mood & Microbes Conference, Amsterdam, The Netherlands, January, 2019. Could Gut Microbiota Influence Stress Resilience? Poster presentation.

Bear, T., Reconnecting – Tūhono – The Nutrition Society Annual Scientific Meeting, December, 2021. The science behind 'Mood Food', the link between diet and depression/anxiety. What we know, what we don't know, and where to next. Online Presentation, Invited Speaker.

Peer-reviewed Articles

Bear, T., Dalziel, J., Coad, J., Roy, N., Butts, C., Gopal, P. The Role of the Gut Microbiota in Dietary Interventions for Depression and Anxiety, Advances in Nutrition, 2020, https://doi.org/10.1093/advances/nmaa016

Bear, T., Dalziel, J., Coad, J., Roy, N., Butts, C., & Gopal, P. (2021). The Microbiome-Gut-Brain Axis and Resilience to Developing Anxiety or Depression under Stress. Microorganisms, 9(4). https://doi.org/10.3390/microorganisms9040723

Published Abstracts

Bear, T. The Science behind 'Mood Food', the Link between Diet and Depression/Anxiety: What We Know, What We Don't Know, and Where to Next, Medical Sciences Forum 9, no. 1: 7, 2022. https://doi.org/10.3390/msf2022009007

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Abbreviations

16S rRNA	16S ribosomal RNA
AA	Arachidonic acid
BDNF	Brain-derived neurotrophic factor
CINC-1	Cytokine-induced neutrophil chemoattractant type-1
DHA	Docosahexaenoic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EPA	Eicosapentaenoic acid
FA	Fatty acid
FFARs	Free fatty acid receptors
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FOS	Fructooligosaccharides
GABA	Gamma-aminobutyric acid
GF	Germ-free
GOS	Galactooligosaccharides
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HFD	High-fat diet
HPA	Hypothalamic-pituitary-adrenal
IDO	Indole-2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
LBP	Lipopolysaccharide binding protein
LDB	Light dark box
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1

MGBA	Microbiome-gut-brain-axis
NOR	Novel object recognition
OFT	Open field test
OUT	Operational taxonomic units
PDX	Polydextrose
PLS	Partial least squares
PRESS	Predicted residual sum of squares
PST	Porsolt swim test
PUFA	Polyunsaturated fatty acid
SCFA	Short chain fatty acid
SFA	Saturated fatty acid
SPF	Specific pathogen-free
SD	Sprague-Dawley
SPT	Sucrose preference test
SI	Sucrose intake
TCT	Three-chamber test for sociability
TNF	Tumor necrosis factor
UCMS	Unpredictable chronic mild stress

Chapter 1. Introduction

Around 15-20% of people will experience mental health disorders such as a depressive episode or anxiety disorder in their lifetime (Julian et al., 2006; Lépine & Briley, 2011), and anxiety and depression are ranked in the top 10 causes of the global burden of disease (Baxter et al., 2014; "Depression Fact Sheet," 2016). Unfortunately, our understanding of the aetiology of these disorders and the ability to effectively treat them is poor. For example, around 30-40% of those with depression do not adequately respond to pharmacological or psychological treatment (DeRubeis et al., 2008). The high impact on individual quality of life and the public health system means that finding new ways of preventing and treating anxiety and depression is a global priority. Consequently, there has been a call for a broader research approach with more interdisciplinary efforts (Marquez & Saxena, 2016; McLaughlin, 2011). With the emergence of the COVID-19 worldwide pandemic, levels of depression, anxiety and stress have increased even further (Lakhan et al., 2020) and there is an even greater need to understand and find new ways for mitigating the effects of stress.

Recent approaches in depression and anxiety research are investigating the links between 1) diet and mood and 2) the influence of gut microbiome on neurobiology and behaviour, termed the microbiome-gut-brain axis (MGBA). There is emerging evidence showing a strong influence of both diet and gut microbiome on emotional behaviour and neurological processes, and because the gut microbiome is strongly affected by diet (Oriach et al., 2016), these two factors are also intertwined. The broad influence of the gut microbiome on human health, including psychiatric health, has begun to be realised and understood over the last decade (Cryan & Dinan, 2012). The gut-brain-axis is the bidirectional communication between the gastrointestinal system and the central nervous system that plays an important role in maintaining neural, hormonal and immunological homeostasis (Carabotti et al., 2015). With emerging evidence showing that the gut microbiome can influence symptoms of depression and anxiety, it is now seen as a key component of this cross-talk between gut and brain, and the term has been extended to MGBA.

Research also suggests that an unhealthy dietary pattern may increase the risk of developing depression or anxiety, while a healthy dietary pattern may decrease it (Bear et al., 2020).

However, this relationship is complex, affected by many confounding variables. It is also likely to be bidirectional, with dietary choices being affected by stress and depression. This complexity is reflected in the data, with sometimes conflicting results among studies. Research is needed in nutritional intervention studies with mood as the primary outcome, and which include the gut microbiota as a variable.

Stress is also likely to be a key variable in MGBA and is a growing area of research. Vulnerability to developing mood disorders depends on a mixture of genetic and environmental factors (Kendler et al., 1992; Tambs et al., 2009; Toomey et al., 2015), and of the environmental factors, stress plays a significant role. Childhood adversity increases susceptibility to developing mood disorders later in life, and around 80% of episodes of major depressive disorder are commonly preceded by psychosocial stress (Mazure, 1998; Newman & Bland, 1994). Stress also alters the gut microbiota (Bailey et al., 2011; Bangsgaard Bendtsen et al., 2012; Galley et al., 2014; Marin et al., 2017; Tannock & Savage, 1974), and the effects of early life stress on microbiota may extend to adulthood (O'Mahony et al., 2009). It is, therefore, plausible that changes in the gut microbiota due to stress at least partially mediate the onset of stress-related depressive or anxious episodes.

This thesis dissertation aimed to investigate the interacting relationships between stress, mood and gut microbiome (Figure 1.1), using animal models.

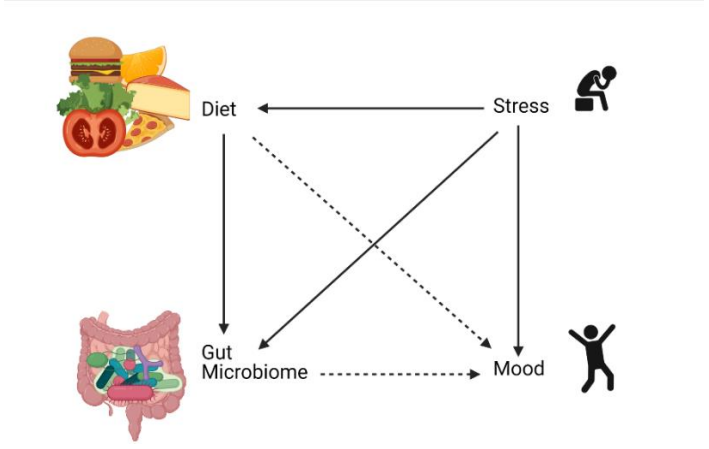

Figure 1.1. *The relationships between the gut microbiome, diet, stress and mood.*

Emerging research shows that diet and the gut microbiome have both been shown to be associated with mood and may influence it. Stress interacts with all these variables and may influence the other relationships. Unbroken lines represent relationships which are known, broken lines represent relationships which have limited and emerging evidence. Figure created with Biorender.com.

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Chapter 2. Literature Review

The aim of the literature review was to identify a focused area within the broader concept of how the diet-mood relationship intersects with the gut microbiota-mood relationship.

My literature review is presented in three sections.

- *Section 2.1 describes the concepts of emotion, mood, depression and anxiety, and then provides a critical review of the research on the relationship between diet, depression/anxiety and the gut microbiota.*
- *Section 2.2 defines stress and stress-resilience and then provides a narrative review on the potential relationship between stress-resilience and the microbiome-gut-brain-axis.*
- *Section 2.3 integrates the concepts discussed in Sections 1 and 2, highlights the gaps in knowledge in this area, and outlines the research aims and hypotheses of this PhD dissertation. A review of the use of animal models in gut-brain-axis stress research is also included.*

Section 2.1. The Role of the Gut Microbiota in Dietary Interventions for Depression and Anxiety

Sections 2.1.2-2.1.7 were published as a part of a review article: Bear, T., Dalziel, J., Coad, J., Roy, N., Butts, C., Gopal, P. The Role of the Gut Microbiota in Dietary Interventions for Depression and Anxiety, Advances in Nutrition, 2020, https://doi.org/10.1093/advances/nmaa016

The abstract and the majority of the introduction of the published review are not replicated in this chapter, as they form part of the abstract and Chapter 1 (introduction). Section 2.1.1 is not part of the published paper. Some changes in the formatting and referencing style from the published paper were made to align with the formatting and referencing style of the thesis.

2.1.1 The Gut Microbiome

The gut microbiota comprises around 0.2 kg of human body weight and has around the same number of cells as human eukaryotic cells (recently revised estimate) (Sender et al., 2016). It also has around 150-fold more genes (Qin et al., 2010). Interest in the gut microbiome has flourished in recent decades due to realisation of its role in production of metabolic and endocrine products, and interactions with the host nervous and immune systems. The commensal microbiota is that which occurs naturally in a healthy individual. Development of an established gut microbiota community though childhood and adolescence is influenced by mode of birth, age, geographic origin, host genotype, and diet (Bäckhed et al., 2012), and becomes relatively stable in adulthood.

Modulation of the gut microbiome community and function occurs though a number of mechanisms. Modulation by the host occurs through the immune system, for example immunoglobin A (IgA) (produced in the gut by GALT tissue) binds to unwanted bacteria so they are removed in the faeces, but leaves commensals alone (Brestoff & Artis, 2013). Endogenous anti-microbial compounds are also produced in the gut (Yano et al., 2015). The microbiota themselves can inhibit the growth of other microbiota through the production of

bacteriocins (proteinaceous toxins), interacting with the host immune system, and altering environmental conditions in the gut such as pH through fermentation products. Commensal bacteria also consume available resources, outcompeting pathogenic bacteria (Brestoff & Artis, 2013). Microbial composition is affected by changes in the gut environment, such as gut motility, pH, and nutrient availability (Macfarlane et al., 1998; Nicholson et al., 2012).

There is huge variance in the population between different individuals, and no "core" microbiome has been identified (Shanahan et al., 2021). Essentially, a healthy microbiome is one which is associated with healthy individuals, and dysbiosis is the term used to describe a microbiome which is associated with disease, and which differs from that of a healthy population (Shanahan et al., 2021; Shreiner et al., 2015). Commensal bacteria are those which populate an individual and, under normal circumstances, live in neutrality or mutualism with the host, although they can become pathogenic under certain circumstances (Tlaskalová-Hogenová et al., 2004). Predominant bacterial species in larger numbers are *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus*, *Bacteroides* and *Fusobacterium*, and in smaller numbers but still common are species such as *Lactobacillus*, *Enterobacter*, and *Escherichia coli* (Savage, 1999; Wilson et al., 2002).

There are however several common traits of a healthy microbiome. Higher alpha diversity (the diversity within an individual), long term stability, and a higher abundance of commensal bacteria with a lower abundance of pathobionts tend to be associated with health (Dikongué & Ségurel, 2017; Frost et al., 2021; Shreiner et al., 2015).

2.1.2 Emotion, Mood, Anxiety and Depression

Emotion and mood are overlapping but distinct concepts. Emotion is a short-lived feeling which reflects the individual's current experience, e.g., elation at good news or fear in a dangerous situation. Mood is a more sustained group of feelings and emotions, and actions, and it does not necessarily reflect the situation the individual is experiencing. Examples of moods include feeling excited, depressed, relaxed, or anxious (Polak et al., 2015; Russell, 2009). Mood disorders such as depression and anxiety disorders are diagnosed when an individual's mood persistently affects a person's life quality and ability to function (American Psychiatric Association, 2013). Emotion, mood, and mood disorders are all discussed in this thesis, depending on the context.

Depression, clinically termed Major Depressive Disorder in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), includes several different, overlapping mood disorders which have the common features of depressed (sad, empty or irritable) mood, as well as cognitive changes and physical symptoms, which in combination, affect a person's life quality and ability to function (American Psychiatric Association, 2013). Depressed mood or anhedonia is required for a diagnosis of depression. Depressed mood includes feelings of sadness, emptiness, hopelessness, or irritability. Anhedonia is a loss of pleasure and/or interest in almost all activities. Other symptoms include an increase or decrease in body weight and/or appetite; alterations in sleeping patterns, psychomotor alteration, fatigue, cognitive changes, feelings of worthlessness, excessive or inappropriate guilt, and somatic symptoms – body aches and pain. There are some differences with age, as hyperphagia and hypersomnia are more likely in children, while psychomotor retardation is more likely in older individuals (American Psychiatric Association, 2013).

There are several neurobiological changes associated with depression that interact with genetics and the environment (See Figure 2.1.1). Reduced expression or increased turnover of monoamine neurotransmitters (including serotonin, dopamine, norepinephrine and epinephrine) is the most well-known aetiology, and most anti-depressant drugs work by increasing the concentration of these in the brain (Willner et al., 2013). Dysfunctions in the balance between excitation and inhibitory signalling of the neuromodulators glutamate and gamma-aminobutyric acid (GABA) also occur, and alterations in the expression of their receptors (Ghosal et al., 2017; Kim et al., 1982; Niewoehner et al., 2007; Tornese et al., 2017). The ratio of neuroprotective metabolites to neurodegenerative metabolites is reduced in depressed patients, which may be due to alterations in tryptophan metabolism, causing less serotonin to be produced, and more quinolinic acid instead via the kynurenine pathway (Myint et al., 2007).

Inflammation is also linked with depression, and studies show that there are increases in the number of pro-inflammatory markers in depressed individuals compared with non-depressed individuals (Kohler et al., 2017; Miller et al., 2009) and immune cells in the brain (microglia) are activated (Song & Wang, 2011; Steiner et al., 2011). Treatment-resistant individuals often have higher levels of the pro-inflammatory cytokines Interleukin (IL)-6 and

Tumour Necrosis Factor (TNF)-α (O'Brien et al., 2007). Inflammation also activates the kynurenine pathway and the hypothalamic-pituitary-adrenal (HPA) axis (Silverman et al., 2005). Significant alterations in the HPA axis are consistently found in depressed individuals, which may directly influence both anxiety and depression or indirectly by failing to downregulate the immune system (Swaab et al., 2005; Zunszain et al., 2011). Other physiological changes are also linked with depression, including possible increased gut permeability (Maes et al., 2008) and sleep architecture (Palagini et al., 2013).

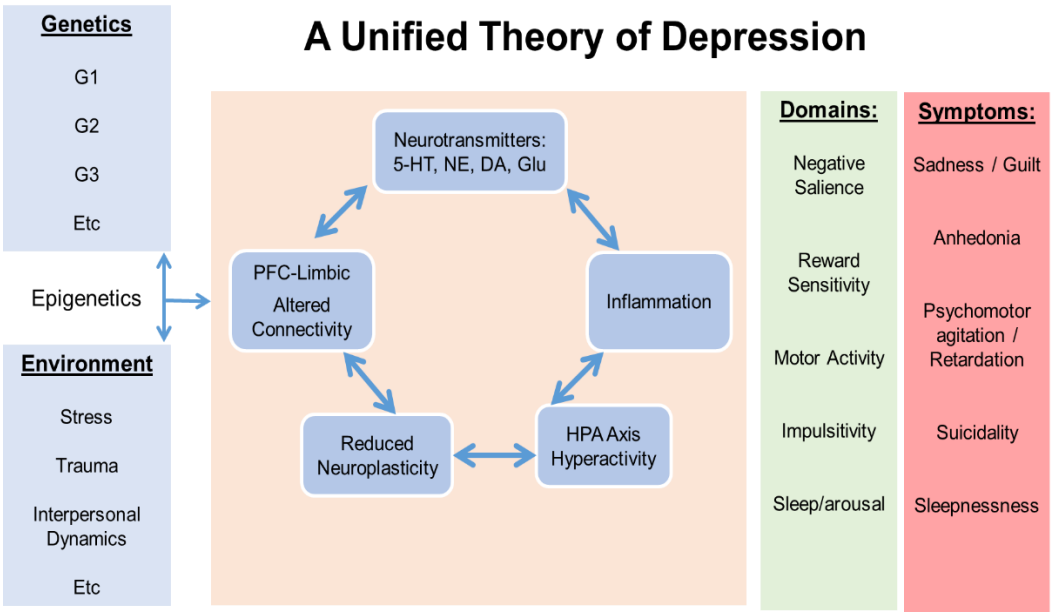

Figure 2.1.1. *Several theories have been proposed for the pathophysiologic mechanisms of depression.*

It is likely that they interact and that multiple mechanism act as triggers. Our understanding of the interactions between the mechanisms is still developing. Abbreviations: 5-HT, 5-Hydroxytryptophan (serotonin); NE, norepinephrine; DA, dopamine; Glu, Glutamate. Figure from Dean and Keshavan (2017), reproduced with permission from Elsevier.

Anxiety is an emotional state which includes subjective feelings of worry, a sense of threat, physiological responses (HPA axis activation, increased heart rate) and behavioural changes, including vigilance, avoidance, and withdrawal from the perceived threat (Gross & Hen, 2004; Weinberger, 2001). Anxiety is an extension of the natural fear response, but fear is an emotional reaction to real danger, whereas anxiety is due to anticipation of danger and may or may not be proportionate to the actual level of threat (Gross & Hen, 2004; Weinberger,

2001). Anxiety is considered a normal experience, but when it becomes extreme and/or persistent and interferes with day-to-day life, it is considered pathological and classed as an anxiety disorder. Six discrete anxiety disorders have been defined (American Psychiatric Association, 2013), which have overlapping symptoms and treatment (Gross & Hen, 2004). State anxiety (in contrast to trait anxiety) measures the acute, current state of anxiety being experienced, and trait anxiety is related to the long-term tendencies to experience high levels of state anxiety (Gross & Hen, 2004; Weinberger, 2001). Alterations in serotonergic and GABA-ergic signalling is implicated as part of the pathophysiology of anxiety (Delli Pizzi et al., 2015; Żmudzka et al., 2018).

Depression and anxiety disorders are commonly comorbid (Hirschfeld, 2001), and there is a strong comorbidity with many other illnesses, including pain (Bair et al., 2003) and gut disorders such as inflammatory bowel disease (Addolorato et al., 1997; Kurina et al., 2001). These findings suggest shared mechanisms. A strong link with the immune system has been shown (Miller et al., 2009). Current treatments for depression and anxiety include psychotherapy, exercise, anti-depressant and anti-anxiety drugs, and electroconvulsive therapy (Gelenberg, 2010).

The network theory of mental disorders is an emergent conceptualisation of the cause of mental distress such as anxiety and depression. Network theory proposes that the individual symptoms of anxiety and depression disorders are actually causal, and part of the pathology, and can be described as nodes in a causal web. For example, avoidance of social situations due to anxiety can create social disconnect which increases social anxiety as well as other "symptoms" or nodes. Therefore, the avoidance is both a result and a cause of the mental distress (Borsboom, 2017; Jones & Robinaugh, 2021). Analysis using network psychometrics has shown physiological markers such as inflammation to operate as "nodes".

2.1.3 The Relationship between Diet and Depression

Research shows that there is a relationship between diet and depression; however, there are conflicting results from studies, and the directionality and mechanism of the relationship are unclear. Many correlative studies in healthy adults show that a lower incidence of depression occurs in those who eat according to "healthy" dietary patterns, characterised by an abundance of vegetables, fruits, cereals, nuts, seeds and pulses, as well as moderate amounts

of dairy, eggs and fish and unsaturated fats (Lai et al., 2014), and with the Mediterranean diet (Crichton et al., 2013; Ford et al., 2013), Japanese diet (Nanri et al., 2010; Suzuki et al., 2013), and Norwegian diet (Jacka et al., 2011). In contrast, a "Western" dietary pattern consisting of sweet and fatty foods, refined grains, fried and processed foods, red meat, high-fat dairy products, and low fruit and vegetable intake is associated with higher depression incidence (Akbaraly et al., 2009; Ford et al., 2013; Ruusunen et al., 2014). However, not all studies show an association, with many showing no association at all (Chocano-Bedoya et al., 2013; Niu et al., 2013; O'Neil et al., 2014; Sugawara et al., 2012) or showing an effect only with a specific food which is not supported by other research (e.g. tomatoes (Niu et al., 2013)).

Conflicting results from studies are potentially due to many factors. There is a possible bias in recall memory due to the use of food-frequency questionnaires and difficulty in controlling for all confounding variables (Sanchez-Villegas & Martínez-González, 2013). The recall bias has not been addressed and may be a specific problem for assessing diet and depression, as depression can affect memory (Burt et al., 1995). Participant and researcher expectation bias is another issue in randomised controlled trials. Since the variables being measured rely on participant reporting, blinding is important to prevent expectancy bias. However, blinding the participants to the hypothesis is difficult. Dieticians, nutritionists and psychologists who deliver the separate arms of the trial should also be blinded to the study hypothesis, but this is rarely done in practice.

Reverse causality is possible. Stress and depression can also alter taste thresholds (Heath et al., 2006), perception of sugary and fatty foods (Noel & Dando, 2015; Platte et al., 2013), and food choices (Frost et al., 1982; Lang et al., 2015; Ouwens et al., 2009). A 10-year longitudinal study in France showed an association between depression incidence and poor diet but found that there was probably reverse causality, with depression increasing the risk of poor eating behaviours (Le Port et al., 2012). A reanalysis of a longitudinal study in Australia showed that those with an existing depressive episode had a poorer diet, but not those with only historical depression (Jacka et al., 2015). The authors suggest that this finding could be due to reverse causality or altered dietary habits after depression treatment. This interpretation is supported by a study showing that 20% of people with depression intentionally improve their diet (Pirotta et al., 2014).

Attempts to determine the direction of causality from prospective studies and randomised controlled trials have shown similar mixed results. A meta-analysis of prospective studies identified that a high-quality diet, regardless of its type and increased fish and vegetable intakes, was associated with a lower incidence of depression, with a dose-type relationship with compliance to the healthy diet. In contrast, this meta-analysis showed that a low-quality diet was not associated with an increased risk of depression, and the results showed a high level of heterogeneity between studies (Molendijk et al., 2018).

A systematic review of randomised controlled trials by Opie et al. (2015) found that study design varied in terms of delivery method, type of intervention, and the study population, and over half of the studies showed no effect from the intervention. The review also showed that few studies have been done in medically healthy people. Interventions that were successful in improving depressive symptoms had a single delivery mode (e.g., face to face), had been delivered by a qualified nutritionist or dietitian, and were more likely to recommend an increase in vegetables as opposed to a cholesterol-lowering diet or a reduction in red meat.

The mixed results and difficulties in research design do not mean that there is no clinically meaningful relationship. There are plausible mechanisms for how diet can affect depression, and bidirectionality is probable. Depression is primarily a psychological illness, and the strength and importance of a diet-depression relationship will vary depending on individual psychological traits, including personality, thought patterns and coping skills. Psychological variables are not usually adequately controlled or accounted for in nutrition studies. The finding that the diet-depression relationship is stronger with a healthy diet than an unhealthy diet could be due to people less susceptible to depression being more resilient to the effects of an unhealthy diet and potentially diluting any measured effect.

Additionally, there are likely to be geographical differences in the strength of the relationship, as variations in micronutrient content in soils, and consequently, in foods, occur (e.g., low selenium content in New Zealand soils (Thomson & Robinson, 1980)). Prospective studies often attempt to determine the direction of causality by excluding those who already have depression from the analysis and then examining the rates of depression developed in the rest of the cohort against the different dietary patterns. The problem with this approach is that not everyone has equal risk of developing depression, as there are strong genetic, epigenetic, and

environmental components to being vulnerable to depression (American Psychiatric Association, 2013). By excluding those who already have depression, the sample is biased towards those who are less vulnerable to developing depression and for whom any link between diet and depression is likely to be much weaker.

Additionally, Molendijk et al. (2018) suggested that controlling for baseline depression severity could cancel out diet-depression effects as the influence of poor diet on depressive symptoms may have begun years earlier. It is also possible that poor diet increases vulnerability to developing depression under stress, rather than directly causing depression. Therefore, a study undertaken in a cohort with low environmental stress may not reach the tipping point in numbers developing depression to reveal the relationship if it exists.

Physiologically, there are multiple plausible mechanisms by which diet can directly influence symptoms of anxiety and depression (see Figure 2.1.2). The aetiology of depression itself is not fully established, but many biological and neurological changes are linked to depressive symptoms. A reduction in monoamine neurotransmitters, especially serotonin, is the most well-known mechanism and the pharmacological target of most antidepressant drugs (Willner et al., 2013). Monoamine deficiency is most likely only a cause of depressive symptoms in a vulnerable population (Ruhé et al., 2007). Other mechanisms found to be linked to depression include a dysfunctional HPA axis (Burke et al., 2005); immune-inflammatory, oxidative, and nitrosative pathways (Arborelius et al., 1999; Kohler et al., 2017; Martin-Subero et al., 2016; Miller et al., 2009; Müller & Schwarz, 2007; Nemeroff et al., 1984; Park et al., 2013; Raison & Miller, 2011; Song et al., 1995; Steiner et al., 2011; Zhang et al., 2016); neuroinflammation (including activated microglial cells) (Steiner et al., 2011); altered vagus nerve tone (Chambers & Allen, 2003); neurotrophic changes, including structural changes such as decreased hippocampus volume (Jayatissa et al., 2008; Videbech & Ravnkilde, 2004), and region-specific changes in brain-derived neurotrophic factor (BDNF) concentrations (Krishnan & Nestler, 2008); an imbalance between neural excitation and inhibitory signalling (Ghosal et al., 2017; Kim et al., 1982; Myint et al., 2007); and alterations in tryptophan metabolism, including the kynurenine pathway (O'Connor et al., 2008).

Several micronutrients are in low levels in people with depression or increased risk of depression, including zinc, magnesium, selenium, iron, and vitamins D, B12, B6, E, and folate

(Alpert & Fava, 1997; Anglin et al., 2013; Gougeon et al., 2016; Jacka et al., 2012; Vulser et al., 2016; Wang et al., 2018). These micronutrients may affect depression risk via effects on the production and activity of monoamine neurotransmitters such as serotonin (Coppen & Bolander-Gouaille, 2005; Dakshinamurti et al., 1990; Hartvig et al., 1995; Partonen, 1998; Paul et al., 2004; Spedding, 2014), alterations to the HPA system (Wang et al., 2018), glutamatergic signalling (Wang et al., 2018), or inflammatory and oxidative stress (Rybka et al., 2013; Wang et al., 2018). A diet high in fruits and vegetables has higher amounts of these micronutrients. Plants also contain effective antioxidant phytochemicals, such as vitamin C, polyphenols and flavonoids, which have been shown to have antidepressant-like or anxiolytic effects (Bouayed, 2010; Chandrasekhar et al., 2017; Hurley et al., 2014; Zhang et al., 2011). Saturated fatty acids and polyunsaturated fatty acids (such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA)), are incorporated into neural tissue and are important for its function (Harauma et al., 2017). The ratio of the different fatty acids affects function; for example, increased SFA decreases cell membrane fluidity and permeability (Ibarguren et al., 2014), and a low ratio of DHA to AA may increase systemic and brain inflammation (Farooqui et al., 2007; Kiecolt-Glaser et al., 2007). Healthy style diets, especially the Mediterranean diet, are anti-inflammatory (Estruch, 2010) and may lower the risk of depression by reducing inflammation.

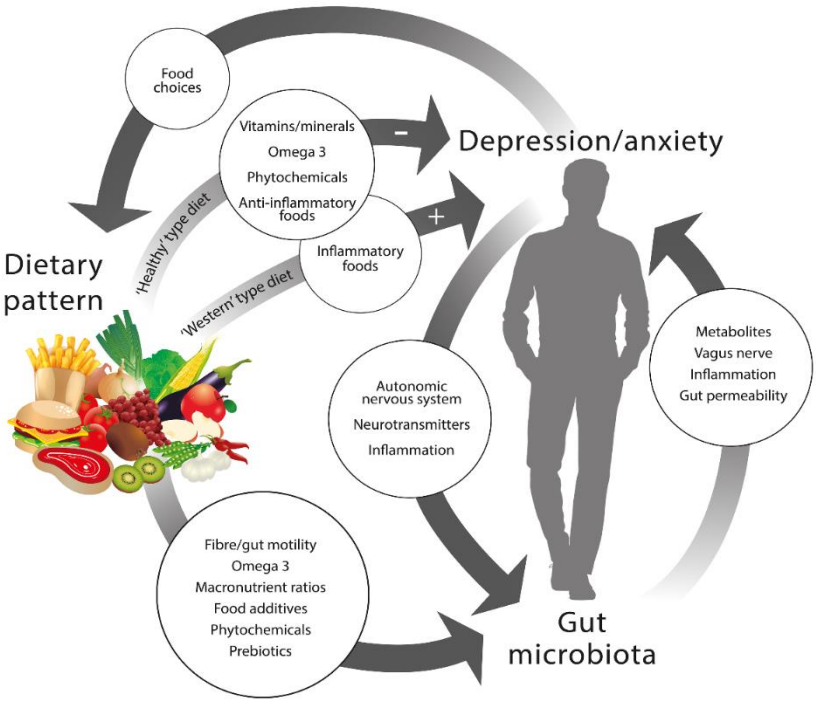

***Figure** 2.1.**2** – Diet has been linked with the risk of developing depression and anxiety.*

There are direct effects from dietary components, which could mediate this relationship. Emerging research also suggests that the gut microbiota is also associated with depression and anxiety in a bidirectional relationship. Since the diet also has a large influence on the gut microbiota, it should be considered a key variable in the diet-depression relationship.

An under-studied aspect in the diet-depression relationship, which could explain some of the inconsistencies, is the relationship between the diet, the gut microbiome and mood. Emerging evidence shows that the gut microbiota is linked to emotional behaviours thought to represent symptoms of both depression and anxiety. The gut microbiome is highly influenced by diet.

2.1.4 The Link between the Gut Microbiota, Depression, and Anxiety

Research into the MGBA began with the observation that there is a high co-morbidity of anxiety and depression in those with inflammatory bowel disease (Addolorato et al., 1997; Kurina et al., 2001) and irritable bowel syndrome (Addolorato et al., 1997; Kurina et al., 2001; Lydiard, 2001; Masand et al., 1995). Additionally, the gut microbiota composition in individuals with anxiety or depression (including those in remission) was different from that in healthy controls (Jiang et al., 2018; Jiang et al., 2015; Naseribafrouei et al., 2014), and animal models of depression show altered gut microbiota composition compared to controls (Park et al., 2013).

Early studies in mice showed that gut infections or chemically-induced colitis caused an increase in patterns of behaviour thought to represent anxiety, including decreased exploration (Lyte et al., 2006) and increased behavioural inhibition (Bercik et al., 2010; Lyte et al., 1998). The direct effect of the gut microbiota on emotional behaviours was shown in studies which identified that anxiety-like behaviours differ between germ-free (GF) rats and mice (born and raised in a microbiota-free environment) and animals with normal, specific pathogen-free (SPF) gut microbiota (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011; Nishino et al., 2013). The colonisation of GF animals with SPF gut microbiota has been shown to ameliorate the behavioural differences (Clarke et al., 2013; Diaz Heijtz et al., 2011; Nishino et al., 2013). Faecal transplants from anxious-type mice into a more resilient strain increased anxiety-like behaviours in the resilient strain, and vice versa (Bercik, Denou, et al., 2011). Probiotic supplementation has also shown promise, with a reduction in anxiety and depression reported in many human and animal studies (Bravo et al., 2011; Desbonnet et al., 2010; Liang et al., 2015; Messaoudi et al., 2011; Rao et al., 2009). Probiotics also seem to be protective against the development of anxiety due to gut infection (Bercik et al., 2010) and immunodeficiency (Smith et al., 2014).

While there is convincing evidence that the gut microbiome can be linked to emotional behaviours, the mechanisms and clinical relevance are not fully understood. Studies in humans are still few and often the results do not translate from animal studies, possibly because they often use healthy people without depressive symptoms (Anglin et al., 2015). Inconsistencies in behavioural changes in animal studies often occur between stress-sensitive and stress-resilient rodent strains (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011; Nishino et al., 2013) and between males and females (Clarke et al., 2013; Neufeld et al., 2011), suggesting that the host-microbiota relationship may depend on the host genotype. There may be critical windows of development during which the gut microbiota have more effect; for example, early life (Diaz Heijtz et al., 2011), adolescence (Clarke et al., 2013; Leclercq et al., 2017; Sudo et al., 2004), or during gestation (Degroote et al., 2016; Tormo-Badia et al., 2014; Zijlmans et al., 2015).

Microbial-mediated mechanisms of mood and neurological processes are still being elucidated (Bercik et al., 2010). At the systemic level, immune modulation has been found in GF mice (Clarke et al., 2013), antibiotic-treated mice (Leclercq et al., 2017), and with probiotic supplementation (Ait-Belgnaoui et al., 2012; Bercik et al., 2010; Clarke et al., 2013; D'Mello et al., 2015; Desbonnet et al., 2009; Desbonnet et al., 2010; Kelly et al., 2017; Leclercq et al., 2017; Liang et al., 2015; Rajkumar et al., 2015). Some studies have shown no evidence of systemic inflammation alongside behavioural changes (Bercik et al., 2010; Kelly et al., 2017) or only a partial effect (Ait-Belgnaoui et al., 2012; D'Mello et al., 2015; Liang et al., 2015); however, even subclinical gut infection without overt inflammation caused behavioural changes in mice (Lyte et al., 1998). Increased HPA axis activation (Messaoudi et al., 2011; Sudo et al., 2004), and alterations to the tryptophan/kynurenine metabolism have also been found (Bercik et al., 2010; Clarke et al., 2013; Desbonnet et al., 2015; Desbonnet et al., 2009; Kelly et al., 2016; Sjögren et al., 2012; Yano et al., 2015). In the gut, changes have been found for short chain fatty acids (SCFAs) (Kelly et al., 2016), motility (Smith et al., 2014) and permeability (Ait-Belgnaoui et al., 2012; Griffiths et al., 2004; Leclercq et al., 2014; Wang et al., 2006). The vagus nerve may be required (Bercik, Park, et al., 2011; Bravo et al., 2011; Sudo et al., 2004; Tanida et al., 2005; Wang et al., 2002), but not always (Bercik, Denou, et al., 2011; Bercik et al., 2010). It is likely that multiple parallel mechanisms are at play, and the mechanisms of the effect of the gut microbiota are specific even to the species level.

Microbial metabolites may play a role, with some bacteria producing the same neuromodulating substances found in the nervous system of animals, including GABA, acetylcholine, dopamine, serotonin, and norepinephrine (Holzer & Farzi, 2014; Lyte, 2011; Roshchina, 2010; Ross et al., 2010; Tsavkelova et al., 2006). GABA, acetylcholine and norepinephrine are also all immunomodulatory (Bjurstöm et al., 2008; de Jonge, 2013). SCFAs, particularly butyrate, contribute to decreased colonic inflammation (Singh et al., 2014) and enhanced gut epithelial integrity (Stilling et al., 2016). They stimulate the secretion of serotonin from enterochromaffin cells in the gut (Fukumoto et al., 2003; Reigstad et al., 2015; Yano et al., 2015), which mainly affects gut motility, but can also activate the vagus nerve and enter the circulation (Fukumoto et al., 2003). SCFAs activate free fatty acid receptors, which seem to have a direct anti-inflammatory effect on microglial activation (Erny et al., 2015), as well as being generally anti-inflammatory, as free fatty acid receptors are present on neutrophils and dendritic cells (Maslowski et al., 2009). No difference in SCFAs was found in the faecal samples of people with depression compared with healthy controls, but when the faecal samples were transplanted into mice, an increase in faecal acetate and total SCFAs was found (Kelly et al., 2016). Gut bacteria are also a significant source of vitamins, including vitamin K2 (menaquinone), and B vitamins niacin (B3), biotin (B7), folate (B9) and pyridoxine (B6) (Burgess et al., 2009; Hill, 1997; LeBlanc et al., 2013; Rosenberg et al., 2017; Sumi et al., 1977). Biotin and niacin are immunomodulatory, and a deficiency could contribute to gut and systemic inflammation (Agrawal et al., 2016; Singh et al., 2014). The concentration of serum folate is lower in those with depression (Bottiglieri et al., 2000) and may be associated with symptom severity (Alpert & Fava, 1997; Wolfersdorf et al., 1993) and responsiveness to anti-depressant treatment (Owen, 2013). Pyridoxine is an essential co-factor in many enzymes in the kynurenine pathway, which is altered in those with depression (Myint et al., 2007). GF rats show increased susceptibility to pyroxidine deficiency (Sumi et al., 1977).

2.1.4.1 Changes in the Gut Microbiota with Depression and Anxiety

The MGBA findings of behavioural effects with probiotic or inflammation-inducing bacteria broadly fit with the microbial profiles associated with positive or negative mental health, although there is no specific gut microbiota composition profile linked to anxiety or depression. Comparisons of the gut microbial changes in humans with depression show various changes compared to healthy controls. However, they show a general pattern of

increases in potentially harmful and inflammatory bacteria such as those from the Proteobacteria phylum, which are normally minor in relative abundance, alongside a decrease in commensal bacteria, which are normally more abundant (Aizawa et al., 2016; Jiang et al., 2015; Kelly et al., 2016; Naseribafrouei et al., 2014; Valles-Colomer et al., 2019; Zheng et al., 2016). In those with Generalised Anxiety Disorder, fewer changes were found, but a similar reduction in commensal bacteria was seen (Jiang et al., 2018). The lack of an identified depression or anxiety 'gut microbiota profile' is likely due to variation in the methods used to evaluate the gut microbiota composition and gene abundance and individual variation in the human gut microbiota. While there are still many unknowns relating to the MGBA and its mechanisms, the emerging evidence, combined with the effect of diet on microbiota, supports its important role in the diet-mood relationship.

2.1.5 Interactions of Diet with the Microbiome-Gut-Brain Axis

2.1.5.1 Whole Diet

There is a paucity of research measuring the effects of the whole diet on the gut microbiota and depressive symptoms, and studies on diet and anxiety in humans are needed. However, dietary patterns associated with a risk of depression are in line with changes in the gut microbial composition and functions, which MGBA research shows can affect emotional behaviour in rodents. Adherence to the Mediterranean diet reduces abundance of inflammatory/pathogenic bacteria such as *Escherichia coli* and increases abundance of key commensal bacteria such as *Bifidobacteria* (Gow & Yadav, 2017), *Clostridium cluster* XVIa and *Faecalibacterium prausnitzii* (Gutiérrez-Díaz et al., 2017). It also increases the concentration of microbial metabolites in faecal samples, including SCFA concentrations (Mitsou et al., 2017), phenolic metabolites, benzoic acid and 3-hydroxyphenylacetic acid (Gutiérrez-Díaz et al., 2017). Vegetarian or entirely plant-based diets have been shown to alter the gut microbial composition (David et al., 2013; Kim et al., 2013; Matijašić et al., 2014) and reduce gut inflammation (Kim et al., 2013). A dietary pattern defined by fast food consumption reduced counts of the *Lactobacilli* genus (Mitsou et al., 2017). A high fat/low carbohydrate diet, regardless of the type of fat, decreased total bacteria abundance (Fava et al., 2012).

Many of the individual dietary elements that are associated with an increased or decreased risk of developing depression also alter the gut microbiota (refer to Table 1). It is plausible

that the effect of a dietary component on the gut microbiota may partially or wholly mediate the effect of that dietary component on mood.

2.1.5.2 Fish and Omega-3 Fatty Acid Intake

While "healthy" dietary patterns containing fish are found to be associated with a lower risk of depression (Chatzi et al., 2011; Ford et al., 2013; Jacka et al., 2010; Le Port et al., 2012; Mamplekou et al., 2010; Rienks et al., 2013; Ruusunen et al., 2014), other studies show fish to be one component of dietary patterns that increases the odds of depression (Nanri et al., 2010) or are inflammatory (Lucas et al., 2014). When looked at in isolation, there is evidence for a decreased risk of depression (Hibbeln, 1998), increased risk (Jacka et al., 2013; Tanskanen et al., 2001; Timonen et al., 2004), or no relationship (Browne et al., 2006; Lucas et al., 2011; Miyake et al., 2006; Strøm et al., 2009). Randomised controlled trials comparing fish oil with olive oil found no difference in mood improvement (Grenyer et al., 2007; Silvers et al., 2005); however, neither group was initially deficient in omega-3 fatty acid (omega-3 FA). Meta-analyses have found that omega-3 FA dietary supplementation (from fish oil or added fish) has a positive effect on mood in those with symptoms of depression but not in healthy controls (Appleton et al., 2010; Grosso et al., 2014). There may be an optimal dose, as some studies have shown a non-linear association between omega-3 FA and depressive symptoms, with the highest doses being less effective than moderate doses (Jacka et al., 2013; Miyake et al., 2006). People with depression have lower levels of omega-3 FA in their red blood cell membranes (Adams et al., 1996; Edwards et al., 1998; Peet et al., 1998), possibly through oxidative damage (Peet et al., 1998) rather than lower omega-3 FA intake. This observation supports emerging evidence that those with depression have higher levels of inflammation and oxidative stress (Maes et al., 2011; Raison et al., 2006).

Lower intake of fish or omega-3 FA may affect depression risk via microbiota-induced inflammation. Research in mice suggests that the anti-inflammatory effect of omega-3 FA may be due to its effect on the gut microbiota (Kaliannan et al., 2015). A diet comprising a high ratio of omega-6 FA: omega-3 FA (~25:1), fed to wild-type mice caused elevated serum levels of the metabolic endotoxemia markers lipopolysaccharide (LPS) and LPS-binding protein, as well as increased gut permeability compared with that in fat-1 transgenic mice, fed the same diet. The fat-1 mice can endogenously produce omega-3 FA from omega-6 FA and therefore

had a lower gut ratio of 4:1. The difference in serum LPS (but not cytokine interleukin (IL)-1β and serum triglycerides) was eliminated when the mice were given antibiotics or when omega-3 FA dietary intake was increased. The mechanism was found to be an omega-3 FA-dependent increase in production of an endogenous anti-microbial peptide intestinal alkaline phosphatase, which suppresses pro-endotoxic bacteria.

Another study in rats showed that supplementation of omega-3 FAs EPA and DHA was associated with restoration of disturbed gut microbiota caused by early life stress (maternal separation) (Pusceddu et al., 2015). In a follow-up study, pregnant mice and their offspring were given diets that were either deficient in omega-3 FAs or supplemented in omega-3 FAs (Robertson et al., 2017). The omega-3 FA deficient diet caused increased fear-induced freezing behaviour, decreased sociability and increased depressive behaviour in the offspring when they had become adults. The diets were, not surprisingly, associated with differences in FA composition in the brain and differences in faecal microbiota compositional profiles. The changes in microbiota numbers showed the Firmicutes:Bacteroidetes ratio was increased by omega-3 FA deficiency. In the omega-3 FA supplemented group, abundance of the *Bifidobacterium* and *Lactobacillus* genera were present in higher numbers in adult mice, the ratio of *Bifidobacteria*/*Enterobacteria* abundance was higher, and *Anaeroplasma genus*, *Clostridium genus*, and *Peptostreptococcaceae* family abundance was lower, in both adolescents and adults.

Fish and omega-3 FA fatty acids may play additional roles to those previously assumed in the link with depression. Rather than it being only a direct relationship between EPA/DPA levels and neural processes, dietary intake of omega-3 FA may also (or only) be important for those with gut dysbiosis-induced systemic inflammation and the ratio of omega-3 FA to omega-3 FA in the diet may also be critical.

2.1.5.3 Micronutrient Intake

Microorganisms require many of the same micronutrients that humans do and obtain many of these micronutrients through the host diet. Subsequently, host micronutrient intake can affect the gut microbiota composition and function. Altered gut microbiota has been found in mice fed a magnesium-deficient diet and was associated with increased depressive-like behaviour (Winther et al., 2015). Whether the change in behaviour was caused by or was additional to a change in gut microbiota is unclear. Vitamin D intake also affects the gut

microbiota; supplementation altered the gut microbiota in faecal samples in patients with Crohn's disease but not healthy controls (Schaffler et al., 2018), or caused a change in that of healthy adults but only in the stomach and duodenal microbiota (Bashir et al., 2016). In infants, the vitamin D status of their mother during pregnancy influenced their gut microbiota at one month old, but an oral supplement given directly to the baby did not, possibly due to different baseline levels of serum vitamin D in the infants (Talsness et al., 2017). The different gut regions sampled (faecal sample versus stomach and duodenum) may explain the differences in these studies. Also, a healthy gut may respond differently than an inflamed gut. Research into whether modulation of the gut microbiota by magnesium and vitamin D supplementation is via immune-modulation effects and whether the vitamin D status affects behaviour is lacking.

Iron is an essential nutrient for many bacteria, and dietary iron intake affects the gut microbiota composition and function (Kortman et al., 2014). Increased colonic iron due to dietary supplementation has been shown to increase gut inflammation and pathogenic bacteria abundance. A diet deficient in iron has been found to increase the relative abundance of *Lactobacilli* (non-siderophilic bacteria, which do not require iron) in mice (reviewed in Kortman et al., 2014). Conversely, the relative abundance of many beneficial bacteria, including butyrate producers, was also reduced in rats fed an iron-deficient diet, and subsequently, SCFA production also decreased (Dostal et al., 2011). Host iron status can influence the ability to fight pathogenic bacteria in the colon, and both high and low iron concentrations are associated with increased pathogen virulence (Kortman et al., 2014). A change in the gut microbial composition can also affect iron absorption due to changes in luminal pH (Kortman et al., 2014). The Western diet contains much more iron than can be absorbed, and so the concentration of iron found in faeces is high (Kortman et al., 2014). Studies with whole diets should examine how micronutrient intake (and absorption) relates to any changes in the gut microbiota.

2.1.5.4 Prebiotic Foods

A 'healthy' dietary pattern contains a larger amount of fruit, vegetables and wholegrains, which contain prebiotics such as fermentable carbohydrates, polyols, and phytochemicals (Gibson et al., 2017). Prebiotic compounds selectively promote the growth and microbial

activity of beneficial bacteria and confer positive health outcomes (Gibson et al., 2017). The higher prebiotic content characteristic of healthy diets may be why the association of depression with diet is stronger for healthy dietary patterns and more variable for poor dietary patterns (Lai et al., 2014; Molendijk et al., 2018; Rienks et al., 2013).

Prebiotic compounds typically have been shown to increase levels of genera *Bifidobacterium* and *Lactobacillus*. However, as microbial research techniques became more sophisticated in the last decades, it is understood that the growth of many other beneficial bacteria is promoted with prebiotics, such as butyrate-producing bacteria. Some dietary fibres are considered prebiotic, but not all. Dietary fibre that promotes the growth of all gut bacteria is not considered a prebiotic because the numbers of pathogenic bacteria are also increased (Gibson et al., 2017). The most well-researched prebiotics are the soluble fibres "fructans" (fructooligosaccharides (FOS) and inulin) and galactans (galactooligosaccharides or GOS). Mannan-oligosaccharides and xylo-oligosaccharides are also considered prebiotic. Phenolics and phytochemicals show prebiotic effects, although some of the health benefits may be from microbially-produced secondary metabolites. Conjugated linoleic acid and PUFAs are also considered candidate prebiotics (Gibson et al., 2017).

Evidence to date for the impact of prebiotics on mood is mixed but mostly positive. Studies in rodents have found reduced baseline and stress-induced anxiety-like and depressive-like behaviours with FOS and GOS (individually or mixed) (Burokas et al., 2017), a mixture of GOS and polydextrose (PDX) (Mika et al., 2017), and the glycoprotein lactoferrin (Mika et al., 2017). In both these studies, the mixed supplements had a stronger effect on these behaviours. In rats, GOS and PDX supplementation improved scores in anxiety and memory tests more than a probiotic supplementation (*Lactobacillus rhamnosus* GG) but less than a synbiotic supplement (*Lactobacillus rhamnosus* GG, PDX and GOS) (McVey Neufeld et al., 2019). In zebrafish, a tendency towards improved behaviours under stress was found with supplementation of mannan-oligosaccharides and glucose (β-glucans) (Forsatkar et al., 2017). A synbiotic (FOS, GOS and inulin with a probiotic mixture containing *Lactobacillus acidophilus* strain T16, *Bifidobacterium bifidum* strain BIA-6, *Bifidobacterium lactis* strain BIA-6, and *Bifidobacterium longum* strain LAF-5) improved depressive symptoms more than the probiotic only in haemodialysis patients (Haghighat et al., 2019). Conversely, increased anxiety-like behaviours

occurred in mice after supplementation with resistant starch (Lyte et al., 2016). In a human study, prebiotic supplementation in healthy volunteers improved results in an emotional bias test with GOS, but not FOS (Schmidt et al., 2015). Prebiotic supplementation altered the gut microbiota in all these studies. In those studies in which positive behavioural results were found, increases in *Lactobacillus* species and decreases in taxa from the phylum Proteobacteria were found, (Burokas et al., 2017; Forsatkar et al., 2017; Mika et al., 2017). Along with increased anxiety-like behaviour with resistant starch supplementation, Lyte et al. (Lyte et al., 2016) found an increase in the phylum Proteobacteria, but interestingly also an increase in *Bifidobacterium* genus.

The biological activity of many phytochemicals has been shown to exert positive health effects, including antidepressant-like or anxiolytic effects (Bouayed, 2010) and a prebiotic effect (Duenas et al., 2015). The actions of phytochemicals may also be due to secondary metabolites created by microbial utilisation (Duenas et al., 2015). Research examining the effect of phytochemicals on both mood and microbiota is lacking.

2.1.5.5 Macronutrients

A large driver of the effect of diet on the composition of the gut microbiota is variation in macronutrient ratios, amounts, and types. Carbohydrate fermentation tends to increase overall microbial fermentation and SCFA production. The amount of fermentation depends on how much reaches the colon, which is influenced by the amount and type of dietary fibre and prebiotic carbohydrates (Scott et al., 2013). A plant-rich diet promotes the phylum Bacteroidetes, specifically the genera *Prevotella* and *Xylanibacter*, which ferment plant fibre. One study found that a reduction in total carbohydrate in the diet reduced the abundance of butyrate producing *Roseburia/Eubacerium rectale* group. Fermentation of protein generates SCFAs, branched-chain fatty acids, sulphides, phenolic and indolic compounds. The sulphides are associated with gut diseases (Scott et al., 2013). The types of bacteria promoted by protein intake are not well established (Scott et al., 2013).

Increased dietary fat alters the gut microbiota composition (Chu et al., 2016; Collins et al., 2016; Daniel et al., 2013; Fava et al., 2012; Fleissner et al., 2010; Ma et al., 2014; Pyndt Jørgensen et al., 2014; Turnbaugh et al., 2009; Volynets et al., 2017; Yang et al., 2017; P. Zhang et al., 2018), possibly via the stimulation of bile secretion and its modulation into secondary bile acid

products (Fava et al., 2012). A high-fat diet (HFD) (72% fat kcal, corn oil and lard) may also affect metabolism, inflammation, and gut permeability via the gut microbiota, likely mediated by LPS and the CD14 receptor. These physiological effects of such diet were able to be reduced with antibiotic treatment (Cani et al., 2008). Changes in the gut microbiota with a HFD (45% kcal fat) compared to a control diet (10%-12% kcal fat) in mice include decreases in beneficial bacteria from the Bacteroidetes phyla (Hildebrandt et al., 2009),and those from the *Akkermansia* (Baboota et al., 2014), *Bifidobacteria* (Baboota et al., 2014) and *Lactobacillus* (Baboota et al., 2014; Hildebrandt et al., 2009) genera, and an increase in Firmicutes phylum, particularly the potentially inflammatory taxa from the Clostridiales order (Baboota et al., 2014; Hildebrandt et al., 2009), *Enterobacteriaceae* family (Baboota et al., 2014) and Proteobacteria phylum (Hildebrandt et al., 2009). Counterintuitively, an increase in the relative abundance of families *Rumunococcaceae* and *Lachnospiraceae* (Pyndt Jørgensen et al., 2014), and the genus *F. prausnitzii* (Fava et al., 2012), which are considered beneficial gut bacteria (Martín et al., 2017), were increased by a 60% kcal HFD.

Some evidence for an effect of macronutrient intake on emotional behaviour has been found in rodent studies, mostly with a HFD. Increased anxiety-like behaviour has been found in mice fed a HFD comprising 60% kcal unspecified unsaturated fatty acids (Buchenauer et al., 2009), 58% kcal hydrogenated coconut oil (Sharma & Fulton, 2013) or 45% kcal lard and soybean oil (Del Rosario et al., 2012), compared with control diets of around 10% kcal fat. Decreased anxiety-like behaviours have also been found with a HFD (Crisco and corn oil, 90% kcal), compared with a diet high in protein (90% kcal) or carbohydrate (90% kcal), which did not alter these behaviours (Prasad & Prasad, 1996). Another study found no change in anxiety-like behaviours with a HFD (60% kcal) but did find alterations in memory and decreased anxiety-like behaviours with a high sucrose diet (70% kcal) (Pyndt Jørgensen et al., 2014). Support for the role of the gut microbiota as a mediator of any behavioural changes with a HFD comes from a study where a faecal transplant from mice fed a HFD (60% kcal fat) into mice with antibiotic-depleted microbiota (fed a normal diet, 13% kcal fat) increased anxiety-like behaviours (Bruce-Keller et al., 2015).

Dietary compounds also interact with each other and may offset their individual effects. Increasing dietary carbohydrate, particularly prebiotic compounds, may reduce some protein

fermentation products because carbohydrate is a preferred substrate (Scott et al., 2013). Increased inflammation and endotoxemia in mice caused by HFD was mitigated by polyphenol supplementation or polyphenol-rich plant extracts (Collins et al., 2016; Gil-Cardoso et al., 2018; Heyman-Linden et al., 2016; Huang et al., 2016; N.-N. Zhang et al., 2018). The polyphenol supplementation was also associated with differences in the gut bacteria, including an increase of the relative abundance of bacteria from the Firmicutes and Verrucomicrobia phyla (N.-N. Zhang et al., 2018). *Akkermansia* spp. (Heyman-Linden et al., 2016; N.-N. Zhang et al., 2018), the *Faecalibacteriaum* genus.(Heyman-Linden et al., 2016), and the *Lacnospiraceae* family, specifically the *Coprococcus* genus (Collins et al., 2016), and a decrease of the relative abundance of bacteria from the Bacteroidetes phylum (N.-N. Zhang et al., 2018) or microbial diversity (Huang et al., 2016). The fat content of these diets varied from 26% kcal (N.-N. Zhang et al., 2018) and 45% kcal (N.-N. Zhang et al., 2018) to 60% kcal (Huang et al., 2016). The fat content in the control diets was low at 0% (N.-N. Zhang et al., 2018) and 4.1% (Huang et al., 2016).

2.1.5.6 Food additives

Western diets include a high proportion of processed foods containing food additives to improve attributes such as shelf life, texture, and palatability. Studies in mice showed that emulsifiers can alter the gut microbiota composition (Chassaing et al., 2015; Holder et al., 2019), increase the pro-inflammatory potential of the gut microbiota (Chassaing et al., 2015), increase microbiota infiltration of the gut mucosa layer (Chassaing et al., 2015), and alter anxiety-like behaviour (Holder et al., 2019). Salt is another food additive that tends to be in high concentration in processed foods. Seck et al. (2018) found that high faecal salinity alters gut microbe composition, including decreased beneficial bacteria *Akkermansia muciniphila* and *Bifidobacterium* spp., specifically *B. longum* and *B. adolescentis*. Maltodextrin reduces mucus production and increases gut inflammation by increasing endoplasmic reticulum stress (Laudisi et al., 2018). The links between a Western diet and depression may include an effect of food additives on the gut microbiota composition. Evidence from human studies is needed.

2.1.5.7 Other Considerations

Fermented foods typically contain strains of *Lactobacillus* and yeasts and are likely to be important because they contain both probiotic microbiota and microbial metabolites (Aslam

et al., 2020). Most studies investigating the effect of fermented foods on the gut microbiota or mood have been undertaken using commercially produced yoghurts with specific microbiota and fall more into the category of probiotic supplements than diet, so are not discussed here. However, there is a need for research into fermented drinks such as wine and kombucha, or foods such as bread, sauerkraut, kimchi, and yoghurt, and their effect on the gut microbiota and mood (Aslam et al., 2020).

Individual variation in response to dietary changes needs to be considered. Differences in physiology and baseline microbiota composition may affect how the gut microbiota responds to dietary changes or disruptions, such as during stress or antibiotic treatment. For example, a high (palmitic) fat diet in mice increased bacteria from Actinobacteria and Firmicutes phyla, with a decrease in those from the Bacteroidetes and Proteobacteria phyla and increasing gut permeability and systemic inflammation in those mice that became obese. In contrast, the mice that were resistant to becoming obese with the same diet did not have the same changes in microbiota or systemic effects (P. Zhang et al., 2018).

Age is a factor that also needs to be taken into account. Dietary changes may be more effective, at least in the short term, in younger subjects. Of a group of urban dwellers who spent 16 days living in a rainforest village and consuming the village diet, the change in gut microbiota composition was greater in the children compared with the adults (Ruggles et al., 2018). Because the gut microbiota composition and stability of the gut microbiota changes with age, different interventions may be more effective at different life stages or conversely, disruption to microbiota may have more of an impact at different ages.

Childhood adversity increases susceptibility to developing mood disorders later in life, and episodes of major depressive disorder are commonly preceded by psychosocial stress (Kendler et al., 1999; Tambs et al., 2009). Stress also alters the gut microbiota (Bailey et al., 2011; Bangsgaard Bendtsen et al., 2012; Galley et al., 2014; Marin et al., 2017; Tannock & Savage, 1974), and the effects of early life stress on the gut microbiota may extend to adulthood (O'Mahony et al., 2009). It is, therefore, plausible that changes in microbiota due to stress at least partially mediate the onset of stress-related depressive or anxious episodes. Dietary intervention during or following stress is a promising area of research (Bangsgaard Bendtsen et al., 2012; Tsilimigras et al., 2018).

Determining which factors help keep a healthy microbiota composition stable or help correct a dysbiosis may be beneficial. The low FODMAP (Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols) diet has a reduced amount of fermentable substrate compared to normal diets. It can alleviate gut symptoms such as pain and bloating in people with irritable bowel syndrome because of reduced gas production by the gut microbiota (Halmos et al., 2015). The diet is followed strictly for 2-6 weeks until gut symptoms resolve, and then foods are then reintroduced to determine individual tolerances. Because of the link between irritable bowel syndrome and depression/anxiety, it is plausible that the low FODMAP diet could correct a dysbiosis related to altered mood. Initial research findings suggest that for those with irritable bowel syndrome symptoms, a low FODMAP diet may reduce symptoms of anxiety (Piacentino et al., 2016). However, the low FODMAP diet reduces total bacterial abundance, which may lower the production of bacterial metabolites such as SCFAs that are important in maintaining gut homeostasis. A decrease in taxa from the *Bifidobacteria* genus and other beneficial bacteria may also occur (Halmos et al., 2015; Staudacher et al., 2012). The diet is designed as a temporary elimination diet for IBS and adhering to it long term is likely to compromise nutritional status. Long-term effects on the microbiota profile are unknown.

There is some evidence that altered gut motility may be associated with mood (Haug et al., 2002) and that the gut microbiota composition is altered by changes in motility and vice versa (Benton et al., 2007). Foods that directly affect factors such as gut motility, e.g., those containing soluble or insoluble fibre, may also affect mood by correcting problems with motility, which could be a confounding variable or could be related to changes in the gut microbiota. More research in this area is needed, and it would be useful for food intervention studies to measure changes in gut function concurrently with assessing changes in mood. Other co-factors usually considered in depression research, such as exercise and sleep, also have independent impacts on the gut microbiota (Kang et al., 2014; Leone et al., 2015), and should be considered when assessing relationships between foods, mood, and the microbiome.

2.1.6 Conclusions

Research shows that there is a link between diet and depression, but conflicting results and limited research mean that we do not yet understand the nature of the relationship. There is likely to be a bidirectional relationship, which may be of more importance in vulnerable individuals. Diet is a large influencer of the gut microbiota composition and function. Changes in the gut microbiota likely contribute to how diet (whole diet and individual components) may affect depression and anxiety. Limited research in this area is sometimes contradictory and mostly in rodents but does show a pattern of results indicating that the gut microbiota may play a significant role and should be considered in dietary intervention studies. Dietary patterns for positive mental health will likely support the growth of commensal microbiota, decrease the growth of pathogenic and colitis-inducing bacteria, and affect gut barrier permeability and inflammation. Additionally, because a change in whole dietary patterns changes the ratio of many dietary components, investigation into these individual components is also important. In dietary studies for depression and anxiety, types and amounts of dietary components (e.g., fat, prebiotics) within the dietary patterns should be identified. The impact on the diet-mood relationship of stress-related changes in diet; and stress-induced changes in the gut bacteria are not well understood and may play an important role.

While examining the changes in the microbial profile is interesting, it is important to remember that it is the collective function and characteristics of the gut microbiota that interact with the host and that more than one microbe can occupy a particular ecological niche within their environment. Therefore, similar functions can be carried out by different microbiota structures, and the same functional outcome could occur with different changes in the gut microbiota. This characteristic particularly supports food as an effective intervention because diet can shift the microbial profile at all taxonomic levels and affect composition and function separately. The type and strength of the effect of diet on the gut microbiota is determined by existing microbiota composition and function and the host phenotype, including interactions with immune function. Research needs to include the study of the gut microbiota function using metabolomics and/or metagenomics techniques so that processes can be identified through gene and metabolite identification. This means dietary interventions

can be targeted at processes (e.g., carbohydrate degradation or butyrate production) rather than specific bacteria.

Research, in both humans and animals, into the mechanisms of the MGBA will continue to help to elucidate the mechanisms by which the gut microbiota affects depression and anxiety symptoms and other psychological and neurological effects. Food interventions have the dual benefit of a direct impact on gut and brain physiology and an indirect effect via the gut microbiota. Continued research investigating these aspects of the MGBA will further our understanding and advance in obtaining a well understood and well guided holistic approach to treating and preventing anxiety and depression.

Table 2.1.1 – *Evidence of associations between diet, depression and the gut microbiota*

Examples of studies (from in vitro*, animal and human studies) showing components within dietary patterns related to depression in humans or emotional behaviours in animals, which directly affect the host and interact with the gut microbiota.*

Dietary component	Effect	Summary	Subject	Ref
Phytochemicals				
Cocoa polyphenols	Affects mood	In a RCT in adults, 500 mg supplement for 30 days increased self-rated calmness and contentedness compared with placebo	Human	(Pase et al., 2013)
	Alters microbial growth	A 6-week diet with 10% cocoa in rats caused a decrease in *Bacteroides*, *Clostridium* and *Staphylococcus* genera in faeces.	Animal	(Massot-Cladera et al., 2012)
		In vitro digestion with 1g cocoa powder/60ml water. 38.6% of phenols were solubilised, and an increase in *Bifidobacteria*, *Lactobacilli*, and butyrate was found	In vitro	(Fogliano et al., 2011)
	Alters immune function	A 6-week diet with10% cocoa in rats caused an altered toll-like receptor pattern and increased gastrointestinal immunoglobin A secretion	Animal	(Massot-Cladera et al., 2012)
Blueberry extract (anthocyanins)	Affects mood and cognition	In a BCT, in children and young adults, a single drink containing 253 mg anthocyanins increased positive but did not change negative affect scores using the 'Positive and Negative Affect Scale' compared with a placebo drink	Human	(Khalid et al., 2017)
		A 5% blueberry drink given to rats for eight weeks protected against cognitive impairment during chronic mild stress	Animal	(Guo et al., 2017)
	Alters host metabolites	Decreased plasma norepinephrine and dopamine concentrations and brain antioxidant compounds concentrations due to 8 weeks of chronic mild stress were attenuated by a 5% blueberry drink	Animal	(Guo et al., 2017)
Fibre (prebiotic)				
Galactooligosaccharide (GOS), polydextrose (PDX), and	Attenuates stress-induced behaviours and mood, and gene	Male rats were fed diets containing GOS + PDX for four weeks and then underwent inescapable stressors. The prebiotic reduced stress-induced exaggerated freezing and deficit in escape latency and attenuated c-fos mRNA in parts of the brain	Animal	(Mika et al., 2017)

fructooligosaccharides (FOS)	expression in the brain			
		Male and female rats underwent early life stress (maternal separation model). Prebiotic supplementation of GOS + FOS for five weeks following the stress attenuated stress-induced deficit in spatial memory and locomotion, but not anxiety-like behaviours	Animal	(McVey Neufeld et al., 2019)
		RCT, patients with depression. Eight weeks' supplementation with 5 g GOS resulted in decreases in scores on the Beck Depression Inventory compared with placebo	Human	(Kazemi et al., 2019)
		Healthy volunteers were given either FOS or GOS daily for three weeks. Salivary cortisol awakening response and emotional bias (attention to negative information) was decreased after GOS but not FOS	Human	(Schmidt et al., 2015)
	Alters the gut microbiota	Prebiotic diet of GOS + FOS increased *Lactobacillus rhamnosus* and also *Lactobacillus* spp.	Animal	(Mika et al., 2017)
		44 elderly subjects, given 5.5 g/day GOS or placebo for 10 weeks in a double-blind, placebo-controlled, crossover study. Increase in *Bifidobacterium* spp., *Lactobacillus, Enterococcus* spp., *Clostridium coccoides–Eubacterium rectale*, and a decrease in *Bacteroides* spp, *Clostridium* histolyticum group, *Escherichia* coli, and *Desulfovibrio* spp.	Human	(Vulevic et al., 2008)
	Alters immune function	Increases in immune function, including reduced pro-inflammatory, and increased anti-inflammatory cytokines, phagocytosis and NK cell activity	Human	(Vulevic et al., 2008)
Wheat arabinoxylan	May counteract effects of high protein diet on the gut microbiota	In pigs fed a 4- week Western-type diet, added soluble fibre (wheat arabinoxylan) increased carbohydrate fermentation and reduced protein fermentation and fermentation products such as ammonia	Animal	(Williams et al., 2016)
Vitamins /minerals				
Vitamin D	Regulates gut physiological processes	Vitamin D receptors in the gut regulate processes, including epithelial barrier function and immune processes	Review	(Barbáchano et al., 2017)
	Associated with changes in the gut microbiota	Plasma 25-hydroxyvitamin D and Vitamin D supplementation in women in their 36th week of pregnancy were measured and compared with faecal samples in their 1- month-old infants. Increased levels of both were associated with decreased *Bifidobacterium* spp. and *Clostridium difficile* and increased *B. fragilis*	Human	(Talsness et al., 2017)

Magnesium	Dietary deficiency alters behaviour	30 mice fed a magnesium restricted diet for six weeks had increased immobility in the forced swim test and increased hippocampal IL-6 compared with mice on a normal diet	Animal	(Winther et al., 2015)
	Associated with changes in the gut microbiota	The caecal gut microbiota was also altered, with cluster analysis showing significant differences between the diets		
Vitamin A	Associated with changes in the gut microbiota and the gut mucosal barrier	A vitamin A-deficient diet in rats increased total bacteria, decreased *Lactobacillus* spp. and increased *Escherichia coli*. Mucin-producing goblet cells were altered, and the expression of toll-like receptors was increased	Animal	(Amit-Romach et al., 2009)
		Vitamin A deficiency in children aged 1-12 months with persistent diarrhoea showed significantly different gut microbiota than in those with normal vitamin A serum levels	Human	(Lv et al.)
Macronutrients				
Omega-3 fatty acids	Immunomodulatory	The metabolic and inflammatory effects in wild-type mice of a diet with a high omega-6 to omega-3 ratio was able to be prevented with antibiotic treatment or by co-housing mice with Fat-1 transgenic mice, which endogenously produce omega-3 fatty acids	Animal	(Kaliannan et al., 2015)
	Increases endogenous anti-microbial defences	Fat-1 mice were found to produce increased intestinal alkaline phosphatase, an endogenous anti-microbial compound, which reduced gut permeability and lipopolysaccharide production	Animal	(Kaliannan et al., 2015)
	Restores gut dysbiosis	Fat-1 transgenic mice were protected against gut dysbiosis and obesity caused by a Western-style diet following early-life antibiotic exposure.	Animal	(Kaliannan et al., 2016)
		Supplementation of 100–250 mg/day omega-3 FA (80% EPA, 20% DHA) for 12 weeks to female rats reversed stress-induced gut dysbiosis	Animal	(Pusceddu et al., 2015)
	Increases gut microbial metabolites (short-chain fatty acids)	An 8-week open-label trial using an EPA/DHA supplement drink or capsule in adult males and females reversibly increased SCFA producing bacteria, including *Bifidobacterium*, *Roseburia* and *Lactobacillus*	Human	(Watson et al., 2018)
	Deficiency affects mood as well as the gut microbiota	An omega-3 FA deficient diet in pregnant mice and their male offspring resulted in an elevated Firmicutes:Bacteroidetes ratio in the offspring, along with altered behaviour and immune function	Animal	(Robertson et al., 2017)

		Increased depressive behaviour (immobility in forced swim test), decreased sociability (TCT), isolation-induced ultrasonic vocalizations in adulthood and decreased memory (NOR) in both adolescence and adulthood. Increased contextual fear conditioning.		
High fat, particularly saturated fat	Alters microbiota composition	A high-fat diet in mice decreased *Ruminococcaceae* and increased *Rikenellaceae* compared with a carbohydrate diet.	Animal	(Daniel et al., 2013)
		Increase in Firmicutes, particularly the family *Erysipelotrichaceae*, and decrease in Bacteroidetes in mice fed a high-fat diet	Animal	(Fleissner et al., 2010)
		Mice fed a low-fat diet who switched to a high-fat diet had a significant shift in microbiome composition within one day. Increased Firmicutes, particularly the *Erysipelotrichi* class, *Bacilli*, and decreased Bacteroidetes.	Animal	(Turnbaugh et al., 2009)
		BALB/c mice fed a high-fat diet showed alterations in the gut microbiota, including an increase in Firmicutes, particularly in the families *Rumunococcaceae* and *Lachnospiraceae*, and a decrease in Bacteroidetes phylum, and a resulting decrease in the Bacteroidetes/ Firmicutes (B/F) ratio	Animal	(Pyndt Jørgensen et al., 2014)
	Alters anxiety-like behaviour	Mice on a high-fat diet displayed less burrowing (anxiety-like) behaviour and displayed reduced memory in the Morris water maze test than mice on a control diet. The diets were not isocaloric, and the mice also gained more weight.	Animal	(Pyndt Jørgensen et al., 2014)
High fat, high sugar diet	Alters microbiota composition	A Western-style diet in humanised mice increased Erysipelotrichi class (mainly *Clostridium innocuum*, *Eubacterium dolichum*, and *Catenibacterium mitsuokai* genera) and *Bacilli* class (mainly Enterococcus spp. genera). The microbial shift occurred after only a single day.	Animal	(Turnbaugh et al., 2009)
High sugar diet	Positive change in behaviour when the gut microbiota not altered	A high-sucrose diet did not alter the gut microbiota in BALB/c mice compared with a control diet and did alter some behaviours, but in a positive direction (increased latency to immobility in the forced swim test, less goal-orientated burrowing, and less anxiety-like behaviour in the triple test).	Animal	(Pyndt Jørgensen et al., 2014)
Red meat	Modifies the gut microbiota composition	A comparison between a diet rich in red meat or whole grains (10-week crossover trial) showed that increased red meat consumption increased the genera *Clostridium* spp. from the phylum Firmicutes.	Human	(Foerster et al., 2014)
	Microbial metabolism of heme-rich meat	Comparison of meat types varying in heme content (beef, pork, chicken) in an in vitro digestion model showed that heme-rich meat caused higher levels of the nitrosoxide compound-derived DNA adduct O-6-carboxymethylguanine (O-6-CMG).	In vitro	(Vanden Bussche et al., 2014)

	increases oxidative compounds			
Food additives				
Emulsifiers carboxylmethylcellulose (CMC) and polysorbate 80 (P80)	Alter the gut microbiota composition	C57Bl/6J mice were given either CMC or P80 emulsifiers at 1% in their drinking water from weaning till three months old. The treatment altered the composition of the gut microbiota. Interestingly the outcomes differed between males and females. In males, Firmicutes phylum and *Oscillospria*, *Coprococcus*, and *rc4_4* genera were reduced, reduced *Dorea* with P80, and reduced *Bacteroides*, *Burkholderia*, *Clostridium*, and *Veillonella* with *CMC*. In females, *Bacteroides*, *Sphingomondales*, *Sphingomonas*, and *Ruminococcus* were reduced, and there was an increase in *Anaeroplasma* with P80, and the Proteobacteria phylum and *Clostridium* and *Burkholderia* genera with CMC.	Animal	(Holder et al., 2019)
	Altered anxiety-like behaviour	Treatment with emulsifiers decreased sociability in the three-chamber test in females only and increased locomotion in the Elevated Plus Maze in males only. No difference found in the Porsolt swim test or light-dark box.		

Abbreviations used: GABA, gamma-Aminobutyric acid; RCT, randomised controlled trial; BCT, blinded crossover trial.

2.1.7 References

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Section 2.2. A Review of the Role of the Gut Microbiota in Resilience to Developing Anxiety or Depression under Stress

Following the critical review on the diet-mood-microbiome relationship, I identified a lack of research on stress as a variable that could explain some of the variances in Microbiome-Gut-Brain-Axis (MGBA) findings and be a key target for dietary intervention. This section explores the current knowledge about stress, stress-resilience and the MGBA.

This review has been accepted for publication by the journal "Microorganisms". Sections 2.2.1.1 and 2.2.1.2 have been added to the thesis and are not part of the review. The abstract and most of the introduction of the published paper are not replicated in this chapter, as they form part of the Abstract and Chapter 1 (Introduction) of this thesis.

2.2.1 Introduction

This narrative review discusses interactions between stress and the MGBA research and examines the evidence and potential mechanisms of how differences in stress-related changes in the gut microbiota may be associated with stress-resilience.

2.2.1.1 Stress

The state of stress occurs when an individual experiences a stimulus or event which is perceived to be a potential threat to their wellbeing (Nesse et al., 2016). The stress response system (SRS) is the biological response to both psychological (perceived threat) and physiological (such as illness, injury) stressors and is mediated by the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) system. The biological responses to psychological and physiological stressors are similar (Del Giudice et al., 2011; Nesse et al., 2016; Pfau & Russo, 2015). Physiological changes caused by the SRS include increased heart rate, respiratory rate, gluconeogenesis, heightened analgesia, decreased appetite (Charmandari et al., 2005) and a transient increase in immune activation (Silverman

& Sternberg, 2012). Cognitive effects include changes in focus and attention, decision making speed processes, and memory (Mendl, 1999; Starcke & Brand, 2012).

When matched appropriately to the type of stress, the SRS increases the individual's ability to deal effectively with the stressor (e.g., win a fight with a group member or run from a predator). In the modern world, an appropriate stress response (sometimes termed eustress) is associated with increased performance in learning, sports, and work (Nesse et al., 2016; Rudland et al., 2020). The SRS is, therefore, an adaptive evolutionary feature and benefits individuals in times of acute stress, particularly in the natural environment. Individual responses to stress are based on conditional adaptation, in that the stress responsivity phenotype is programmed individually (based on life experiences) in a way which is adaptive to their physical and social environment. The individual's perception of the magnitude of the threat influences the level of stress experienced and is based on previous experience of similar threats, balanced against the ability of the individual to cope with that outcome (Del Giudice et al., 2011; Nesse et al., 2016). Early-life stress is associated with higher stress reactivity (Del Giudice et al., 2011).

The SRS is self-regulating, with cortisol, neuropeptide Y (NPY) and the catecholamines, epinephrine and norepinephrine providing negative feedback via activation of glucocorticoid and mineralocorticoid receptors. Immune activation is also down-regulated by the SRS (Silverman & Sternberg, 2012) and the vagus nerve (Pavlov & Tracey, 2012). Under chronic stress or inflammation, with elevated HPA and pro-inflammatory cytokines, glucocorticoid resistance occurs (Pace et al., 2007; Pariante et al., 1999), and the negative feedback of cortisol or catecholamine stress hormones no longer operates effectively. Reduced vagal tone also occurs (Vrijkotte et al., 2000).

2.2.1.2 Stress-resilience

Stress itself is not a disorder, but there is a strong link between the experience of psychosocial stress and changes in mood, including the development of anxiety or depression episodes (Kendler et al., 1999; Mazure, 1998; Newman & Bland, 1994). Not everyone who experiences stress develops a mood disorder. In this thesis, stress-sensitive individuals are defined as those who develop symptoms of anxiety or depression following stress, and stress-resilience

is defined as individuals who experience similar stress but do not develop anxiety or depressive symptoms. Some factors are associated with stress-sensitivity or stress-resilience.

Psychosocial factors which are protective under stress include social support (Ozbay et al., 2007); the use of active rather than passive coping strategies (Franklin et al., 2012); personality factors such as optimism, empathy and tenacity (Schetter & Dolbier, 2011); low emotional reactivity (Smith & Prior, 1995), and psychological flexibility (Kashdan & Rottenberg, 2010). A safe and reliable childhood with strong maternal and caregiver attachment decreases the risk of anxiety and depression later in life (Jaffee, 2007), and prenatal and early-life stress increases the risk (Matthews & Robbins, 2003; Mazure, 1998; McVey Neufeld et al., 2019; Montalvo-Ortiz et al., 2016; Newman & Bland, 1994). However, stress inoculation (brief periods of stress in childhood in the context of an otherwise low-stress childhood) increases resilience (Franklin et al., 2012).

Biological factors are also linked with stress-resilience. The development of stress-induced psychopathology (e.g., anxiety or depressive symptoms) can occur when an individual's SRS is mismatched with the level of stress in the environment (Nesse et al., 2016). Chronic activation of the SRS, a heightened and exaggerated stress response, and/or a delay in returning to baseline following the stress, are associated with reduced stress resilience (Southwick et al., 2004). This effect may be caused by decreased levels of the SRS inhibitor neurochemical NPY, which is released alongside norepinephrine. Alterations in serotonin and dopamine signalling are also related to increased stress-sensitivity (Southwick et al., 2004). Low vagal tone is associated with reduced recovery from stress (Weber et al., 2010). Interestingly, coping style is influenced by the level of biological stress responses and associated neuroendocrine systems (Franklin et al., 2012), and active coping under stress is increased with anti-depressant drugs (Franklin et al., 2012). This finding suggests that interventions which affect the biological side of stress resilience may be a useful adjunct to current treatments and preventative care. These biological factors are affected by genetics, chronic stress, and sex hormones (particularly oestrogen) (Del Giudice et al., 2011; Nesse et al., 2016; Pfau & Russo, 2015; Southwick et al., 2004). Emerging evidence shows that they may also be affected by the gut microbiota.

2.2.2 Stress and the Microbiome–Gut–Brain-Axis

2.2.2.1 The Link between the Gut Microbiota and Behaviour

The suggestion that the gut microbiota is linked with and may influence mood disorders began with the observation of a high co-morbidity of anxiety and depression disorders in people with gut disorders such as inflammatory bowel disease (Addolorato et al., 1997; Kurina et al., 2001) and irritable bowel syndrome (Addolorato et al., 1997; Kurina et al., 2001; Lydiard, 2001; Masand et al., 1995). Correlational studies have shown that faecal microbiota composition in individuals with anxiety or depression (including those in remission) differs from that in healthy controls (Jiang et al., 2018; Jiang et al., 2015; Naseribafrouei et al., 2014). Women with a higher faecal *Prevotella* abundance experienced increased negative emotional response to viewing negative images, and lower brain activity in the hippocampus than those with a higher *Bacteroides* abundance (Tillisch et al., 2017). Several studies in rodents have experimentally shown that changes in the composition of the gut microbiota can alter emotional behaviour. In mice, gut infections or inflammation caused an increase in patterns of behaviour thought to represent anxiety, including decreased exploration (Lyte et al., 2006) and increased behavioural inhibition (Bercik et al., 2010; Lyte et al., 1998).

Germ free (GF) rats and mice (born and raised with no microbiota), and mice with gut microbiota depleted by anti-microbial drugs show either increased or decreased anxiety and depressive-like behaviours compared with counterparts with specific pathogen-free (SPF) gut microbiota (Bercik, Denou, et al., 2011; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Desbonnet et al., 2015; Leclercq et al., 2017; Neufeld et al., 2011; Nishino et al., 2013). Some probiotics also show an effect on mood (Abildgaard et al., 2017; Bercik, Park, et al., 2011; Bercik et al., 2010; Bravo et al., 2011; D'Mello et al., 2015; Desbonnet et al., 2010; Liang et al., 2015; Smith et al., 2014). Psychobiotics are probiotics which have been shown to "confer mental health benefits through interactions with commensal gut bacteria" (Sarkar et al., 2016). Not all probiotics or prebiotics are considered psychobiotics.

2.2.2.2 Inconsistencies and Problems with MGBA Research

The results of MGBA studies do not always agree, and the results from animal studies do not always translate well to human research. This limitation has been a concern with MGBA research. Animal behavioural testing has limitations on how well it reflects anxiety- or

depressive-like symptoms in humans, but there are methodological limitations with human studies due to heterogeneity of lifestyles and because it is difficult (or impossible) to collect certain biological samples such as colon microbiota and host tissues.

Probiotic supplementation has shown mixed effects on emotional behaviour (refer to Table 2.2.1). There are several studies in rodents which show an amelioration of anxiety-like or depressive like behaviour following probiotic supplementation (Abildgaard et al., 2017; Bravo et al., 2011), inflammation-induced behaviour changes (Bercik, Park, et al., 2011; Bercik et al., 2010; D'Mello et al., 2015; Smith et al., 2014), and stress-induced behaviour changes (Desbonnet et al., 2010; Liang et al., 2015). Other studies have found no difference (Barrera-Bugueño et al., 2017; Desbonnet et al., 2009). Translational research has shown variable results. No difference in mood was found in healthy adults (Kelly et al., 2017) or in people with irritable bowel syndrome (Simrén et al., 2010) following a probiotic supplement, but in other studies, depression scores were reduced in people with diagnosed depression (Akkasheh et al., 2016) and healthy adults (Messaoudi, Lalonde, et al., 2011; Messaoudi, Violle, et al., 2011), or emotional reactivity was reduced (Steenbergen et al., 2015). Mixed results include a study where anxiety scores but not depression scores were reduced (Rao et al., 2009), and mood improved only in those who had low baseline mood (Benton et al., 2006).

Another interesting observation is that the direction of change in anxiety-like behaviours in GF rodents (compared with their SPF counterparts) seems to depend on the strain. Stress-sensitive strains (BALB/c mice and Fischer 344 rats) showed increased anxiety-like behaviours, in contrast to decreased anxiety-like behaviours in more resilient strains (NMRI mice, Swiss Webster mice and Wistar rats). Both increased and decreased anxiety-like behaviours occurred in mice when the gut microbiota was depleted with anti-microbial drugs (Bercik, Denou, et al., 2011; Desbonnet et al., 2015; Leclercq et al., 2017).

Table 2.2.1. *Summary of studies testing the effects of probiotic supplements on mood*

Effect on mood is indicated with: + for a positive effect on mood, − == negative effect on mood, and / = no effect on mood. Abbreviations: LDB, Light Dark Box; SDT, Step Down Test; FST, Forced Swim Test; SPT, Sucrose Preference Test; PSS, Perceived Stress Scale; BAI, Beck Depression Inventory; BDI, Beck Anxiety Inventory; HADS, Hospital Anxiety and Depression scale; POMS, Profile of Mood State

Subject, Study Design and Model	Probiotic	Dose and Administration	Treatment Duration	Effect on mood	Effect	Reference
Animal Studies						
Male AKR mice with parasite-induced (Trichuris muris) chronic gut inflammation	*Bifidobacterium longum* NCC3001 and *Lactobacillus rhamnosus* NCC4007	Gavaged daily, dose not specified	10 days	Reduction in anxiety-like behaviours in the LDB	+	(Bercik et al., 2010)
Immunodeficient (B and T cell-deficient) male and female Rag $1^{-/-}$ mice	*L. rhamnosus* R0011 and *L. helveticus* R0052	10^9 CFU/mL in drinking water daily	4 weeks	Probiotic supplement normalised deficits in anxiety in LDB tests	+	(Smith et al., 2014)
Male C57BL/6 mice with liver inflammation-induced sickness behaviour and brain inflammation	Commercial mixture VSL#3*: L. casei, L. plantarum, L. acidophilus and L. delbrueckii subsp. Bulgaricus, B. longum, B. breve and B. infantis, Streptococcus salivarius subsp. Thermophiles*. Strains unspecified	1.7 billion bacteria/day, gavaged daily	10 days	Prevention of a decrease in social interaction	+	(D'Mello et al., 2015)
Male AKR mice with chemically induced colitis	*B. longum* NCC3001 -	100 µL of 1 x E10 CFU	7 days	A probiotic supplementation reduced anxiety-like behaviour in SDT, but only when the vagus nerve was intact	+ /	(Bercik, Park, et al., 2011)
Male Sprague-Dawley (SD) Rats	*B. bifidum* W23*, B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63*, L. casei* W56, *L. salivarius* W24, *L. lactis* W19, *L. lactis* W58	4.5 g (2.5×10^9 CFU/g) of freeze-dried powder in 30 mL of tap water per cage (2 rats) daily	10 weeks	A probiotic mix decreased depressive-like behaviour in FST	+	(Abildgaard et al., 2017)
Male SD rats following maternal separation stress	*B. infantis* 35624	1 $x10^{10}$ live bacterial cells/100 mL drinking water	55 days	A probiotic supplement ameliorated MSS induced depressive-like behaviour in FST	+	(Desbonnet et al., 2010)

A probiotic given alongside 3 weeks of restraint stress in male Sprague-Dawley rats	*L. helveticus* ns8	10^9 CFU/mL live bacteria in drinking water	3 weeks	Probiotic ameliorated stress-induced depressive-like behaviour in SPT, and anxiety like behaviour in EPM	+	(Liang et al., 2015)
Male BALB/c mice	*L. rhamnosus* JB-1	10^9 CFU, gavaged daily	28 days	Decreased anxiety-like behaviours in the EPM	+	(Bravo et al., 2011)
Male SD rats	*L. casei* 54-2-33	10^4 CFU/mL in drinking water	14 days	Increase in anxiety-like behaviour in the OFT and no difference in anxiety-like behaviour in the EPM	–	(Barrera-Bugueño et al., 2017)
Male SD rats	*B. infantis* 35624	1 x10^{10} live bacterial cells/100 mL drinking water	14 days	No decrease in depressive-like behaviours in FST	/	(Desbonnet et al., 2009)
Human Studies						
Healthy adult men	*L. rhamnosus* JB-1	10^9 CFU, probiotic capsule, daily	8 weeks	No reduction in subjective stress measure, depression or anxiety scores on the PSS, BAI or BDI scales or improve cognitive measures	/	(Kelly et al., 2017)
Healthy men and women	*L. helveticus* R0052 and *B. longum* R0175	3 x10^9 CFU probiotic capsule daily	30 days	Reduction in depression and anxiety scores (HADS). In a subset of people with low baseline urinary cortisol, the perceived stress scores were also reduced by the probiotic	+	(Messaoudi, Lalonde, et al., 2011; Messaoudi, Violle, et al., 2011)
Healthy men and women	*B. bifidum* W23, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, and *Lactococcus lactis* (W19 and W58)	2.5 x 10^9 CFU probiotic capsule daily	4 weeks	Reduction in participant's cognitive reactivity to sad mood	+	(Steenbergen et al., 2015)
Men and women with chronic fatigue syndrome	*L. casei* Shirota	8 x 10^9 CFU probiotic capsule daily	2 months	Improved anxiety (BAI) but not depressive (BDI) symptoms	+ /	(Rao et al., 2009)
Healthy men and women	Milk drink containing probiotic *L. casei* Shirota	6.5 x 10^9 CFU in a milk drink	Not specified	Improvement in mood in POMS only in those who already had low mood	+ /	(Benton et al., 2006)

Men and women with irritable bowel syndrome,	Yoghurt containing *L. paracasei*, ssp. *paracasei* F19, *L. acidophilus* La5 and *B. lactis* Bb12 (Cultura; active)	5 x 10^7 cfu ⁄mL x 200 mL milk drink, daily	8 weeks	The probiotic yoghurt drink did not improve mood scores in HADS	/	(Simrén et al., 2010)
Men and women with diagnosed depression	*B. bifidum, L. acidophilus*, and *L. casei* (strains not specified)	*L. acidophilus* (2x10^9 CFU/g), *L.casei* (2x10^9 CFU/g), *B. bifidum* (2x10^9 CFU/g), amount not specified	8 weeks	Reduction in symptoms of depression I BDI, along with fasting plasma insulin, glutathione, and C-reactive protein	+	(Akkasheh et al., 2016)

A faecal transplant from an anxious-type mouse strain into a GF non-anxious strain caused an increase in anxiety behaviours (Bercik, Denou, et al., 2011). The reverse was also observed, with a decrease in anxiety-like behaviour in previously GF mice colonised from a non-anxious mouse strain (Bercik, Denou, et al., 2011).

There are several reasons for the inconsistencies found in MGBA research. Probiotics effects are strain specific, and factors such as variation in survivability, ability to adhere to the gut mucosa, and their capacity to produce bioactive compounds (Pirbaglou et al., 2016). The dose is also important and the efficacious dose may vary between probiotic strains. The variability in the effect on mood in GF rats suggests that it is the interactions of the microbiome with the host which is important, and that variations in host genotype/phenotype may be a key part of whether changes in the gut microbiota impact mood or not. Proposed mechanisms of the MGBA (Figure 2.2.1) are complicated, intertwined, and bidirectional.

Different hosts, with different life experiences (e.g., diet, stress, exercise), mean that the mechanisms for changes in mood could differ between animals and humans, animal strains, and possibly even individuals. Multiple mechanisms could also act in parallel.

2.2.2.3 Stress, the Gut Microbiota, and Behaviour

There is interest developing in the interactions of stress with the MGBA. The gut microbiota composition can be altered under stress, as shown in rodent models of psychological stress (Bailey et al., 2011; Bailey et al., 2010; Bangsgaard Bendtsen et al., 2012; Burokas et al., 2017; Galley, Nelson, et al., 2014; Galley et al., 2017; Galley, Yu, et al., 2014; Gareau et al., 2007; Gautam et al., 2018; Marin et al., 2017; McVey Neufeld et al., 2019; O'Mahony et al., 2009; Tsilimigras et al., 2018; Yang et al., 2017). Stress during pregnancy has also been shown to alter the gut microbiome structure in the dam and mouse offspring (Gur et al., 2017; Jašarević et al., 2017). With the emerging evidence showing that alterations in the gut microbiota can influence mood, it seems plausible that stress-induced changes in the gut microbiota could (at least partially) mediate the development of chronic stress and/or anxiety and depression following a stressful event. Conversely, alleviation of the stress-induced changes in the gut microbiota and/or the physiological effects of this change may help to increase stress resilience.

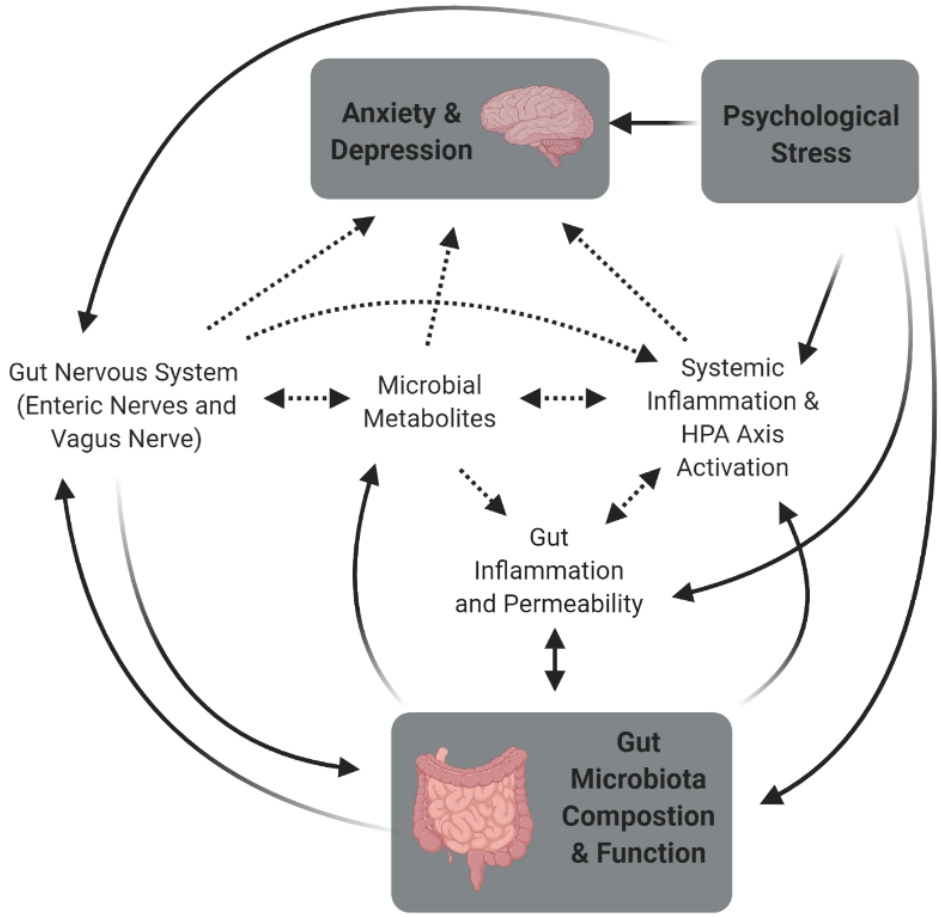

Figure 2.2.1. *The proposed mechanisms of the Microbiome–Gut–Brain-Axis (MGBA)*

The mechanisms are complex and intertwined. Emerging research shows that psychological stress interacts not only directly with the brain and mood, but also with many of the MGBA mechanisms thought to contribute to changes in mood with alteration of the gut microbiota. Solid lines indicate strong evidence of an association, and dotted lines show proposed mechanisms with limited but emerging evidence. Figure prepared using lucidchart online software. Abbreviations: HPA; Hypothalamic-Pituitary-Adrenal.

Compositional changes in the gut microbiota following stress vary between studies, and most of the evidence comes from rodent studies. Changes include a decrease in relative abundance of the genus *Lactobacillus* (Aguilera et al., 2013; Bailey & Coe, 1999; Galley, Nelson, et al., 2014; Marin et al., 2017; Tsilimigras et al., 2018) and an increase in genera containing opportunistic pathogens such as *Odoribacter* (Bangsgaard Bendtsen et al., 2012; Jašarević et al., 2017), *Clostridium* (Bailey et al., 2011; Marin et al., 2017), and *Mucisprillum* (Galley, Yu, et al., 2014; Jašarević et al., 2017). Abundance of the genus *Bifidobacterium* has been shown to decrease under stress (Galley et al., 2017; Marin et al., 2017), and in one study was found to increase under stress in stress-resilient mice only (Yang et al., 2017).

The change in the gut microbiota may differ within different gut niches. For example, restraint stress in male CD-1 mice caused a decrease in the relative abundance of the genus *Lactobacillus* in the gut mucosa-associated microbiota but not the luminal microbiota (Galley, Yu, et al., 2014).

Similarly, recovery of the gut microbiota following stress seems to vary, and changes can be persistent. Galley, Nelson, et al. (2014) found differences in microbial beta diversity (which is comparison of changes in microbiota between samples rather than within samples) after only two hours of social stress in C57BL/6 mice, but a decrease in absolute abundance and relative abundance of *Lactobacillus* spp. after six days (Galley, Nelson, et al., 2014). Infant rhesus monkeys had an altered gut microbiome three days following the stress of being separated from their mothers and placed instead in individual cages near other infant monkeys. Interestingly, five days post-separation, it was restored to the pre-separation composition (Bailey & Coe, 1999). In contrast, differences in faecal microbiota were found in rats seven weeks after maternal separation stress (O'Mahony et al., 2009). Bailey et al. (2011) found that immediately following social stress, the gut microbiome of mice showed reduced alpha diversity and richness, and clustered differently from the control group. However, after 15 h, the separation of the gut microbiota between groups was no longer as clear, with the stress group showing higher variability, suggesting recovery of the gut microbiota following stress may vary between individuals.

Evidence from human studies is sparse. A study looking at the effect of diet and living conditions in (grounded) astronauts found that in faecal samples, the bacterial counts of *Bacteroides fragilis* subsp. *thetaiotaomicron* increased following interpersonal conflict in a confined living situation (Holdeman et al., 1976). Prenatal stress in pregnant women was associated with persistently altered microbiota composition in their infants (Zijlmans et al., 2015). Increased relative abundances of taxa from the Proteobacteria phylum and reduced relative abundance of lactic acid bacteria were found in the infants, which appear to be related to increased reports of gut problems (Zijlmans et al., 2015).

The gut microbiota composition in humans or rodents is probably affected by a shift in the gut environment due to physiological changes in the gut under stress. Stress activates sympathetic pathways in the gut, regulating water absorption by epithelial cells, mucin

production from goblet cells, permeability, and inflammation (increasing mast cell degranulation and cytokine production) (Mayer, 2000). Increased gut motility and mucin secretion occur due to the mast cell degranulation (Castagliuolo et al., 1996; Santos et al., 2001). Stress also causes slowed gastric emptying and decreased whole gut transit time, with increased distal colon motility (Marin et al., 2017; Mayer, 2000).

Bacterial composition and function are also likely to be directly affected by circulating stress hormones (Lyte et al., 2011). Increased growth, virulence, and colonisation of pathogenic bacteria occur with increased concentrations of the catecholamines norepinephrine and epinephrine *in-vitro* and in the lung and gut of stressed animals (reviewed by Lyte, 2014).

Rodent stress models have shown increased colonisation by *Citrobacter rodentium*, a colonic pathogen (Bailey et al., 2010), and increased adherence and penetration of gut bacteria into mucosal cells (Gareau et al., 2007). Dexamethasone (an anti-inflammatory corticosteroid drug with similar actions to cortisol) administration in healthy adult rats caused increased bacterial adherence and increased paracellular gut permeability (discussed in section 3.2 in detail) (Spitz et al., 1994).

2.2.3 Mechanisms Associated with Stress-Induced Changes in the Gut

2.2.3.1 Gut and Systemic Inflammation

Stress-induced gut inflammation could be a key mechanism for changes in emotional behaviour under stress, with the gut microbiome function promoting or decreasing gut tissue and systemic inflammation. Increased plasma inflammatory markers including interleukin (IL)-6 and tumour necrosis factor (TNF)-α have been found in people with anxiety (Vogelzangs et al., 2013) and depression (Kohler et al., 2017; O'Brien et al., 2007), particularly in those who fail to respond to classical treatments (O'Brien et al., 2007). Both depression and anxiety are more prevalent in people with inflammatory bowel disease, and are associated with more frequent flare-ups and more severe symptoms (Mikocka-Walus et al., 2016).

There is some evidence of the ability of the gut microbiome to promote or ameliorate systemic inflammation. Commensal microbiota seems to be associated with decreased inflammation, whereas potentially pathogenic bacteria are associated with inflammatory gut conditions (Bajaj et al., 2012; De Angelis et al., 2015; Shen et al., 2017). In GF mice, the immune response to lipopolysaccharide (LPS) is blunted (Campos et al., 2016; D'Mello et al., 2015; Erny et al.,

2015), suggesting that the gut microbiota primes the immune system. Microglial activation, which causes neural inflammation, seems to be affected by the gut microbiota (Campos et al., 2016; D'Mello et al., 2015; Erny et al., 2015) possibly mediated by free fatty acid receptor 2 in the gut, activated by short-chain fatty acids (SCFAs) produced by the gut microbiota (Erny et al., 2015). Both inflammatory and subclinical microbial gut infections, and chemically-induced colitis increase anxiety and depression behaviours or neurobiochemical markers in rodents (Bercik et al., 2010; Bravo et al., 2011; Gaykema et al., 2004; Gaykema et al., 1998; Goehler et al., 2005; Lyte et al., 2006).

Probiotic supplementation has been shown to attenuate pro-inflammatory markers or responses (Ait-Belgnaoui et al., 2012; D'Mello et al., 2015; Desbonnet et al., 2009; Desbonnet et al., 2010; Liang et al., 2015; Rajkumar et al., 2015), although not always (Bercik et al., 2010; Kelly et al., 2017). Probiotics with anti-inflammatory activity include *Lactobacillus helveticus* ns8 (Liang et al., 2015); *L. salivarius* UBL S22 (Rajkumar et al., 2015); *L. farciminis* (Ait-Belgnaoui et al., 2012); *Bifidobacterium infantis* 35624 (Desbonnet et al., 2009; Desbonnet et al., 2010)) and commercial mix VSL#3 (D'Mello et al., 2015)). *L. rhamnosus* JB-1 (Kelly et al., 2017); *Bifidobacterium longum* NCC3001 and *L. rhamnosus* NCC4007 (Bercik et al., 2010) have shown no anti-inflammatory effect in the reported studies. The probiotic *L. salivarius* UBL S22 also decreased the total faecal *Escherichia coli* count (Rajkumar et al., 2015), a bacteria linked with gut inflammation (Kittana et al., 2018).

Stress-induced changes in the gut microbiota composition, with decreased commensal microbiota and increased opportunistic pathogens, are likely to be inflammatory. Whether it is the cause of stress-induced gut inflammation or mediates emotional behaviour responses is unknown. Stress-induced inflammation also occurs directly via the sympathetic nervous system innervation of lymphoid organs (Mayer, 2000) and activation of mast cells (Castagliuolo et al., 1996; Santos et al., 2001; Soderholm & Perdue, 2001) and dendritic cells (Bharwani et al., 2017). However, antibiotic administration in mice during stress prevented an increase in pro-inflammatory markers, suggesting a key role of the gut microbiota (Bailey et al., 2011). The probiotic *L. rhamnosus* JB-1 was also able to ameliorate stress-induced dendritic cell activation and reduce the effects of stress-related changes in anxiety-like behaviour (Bharwani et al., 2017).

Seven days of repeated restraint stress in mice increased their immune responses to a colonic pathogen (*Citrobacter rodentium*) challenge (Galley et al., 2017). A faecal transplant from the stressed mice into GF mice caused an increased inflammatory response to the colonic pathogen in the GF mice, compared with a faecal transplant from non-stressed mice (Galley et al., 2017). Whether the faecal microbiota itself or other molecules within the faeces caused the increased immune response is unclear, but it is likely due to an interaction between the gut microbiota and the enteric immune system. The gut microbiota plays a fundamental role in the function and maturation of the gut immune system, including CD4 cells (Smith & Garrett, 2011). Susceptibility to colonic inflammation has previously been shown to increase following stress due to sensitisation of CD4(+) lymphocytes, with the increased susceptibility able to be transferred to other rats with intravenous transfer of the CD4(+) lymphocytes (Qiu et al., 1999).

2.2.3.2 Gut Permeability

Increased gut permeability was found in over 40 % of people with depression in one study (Maes et al., 2008), and may be a cause of increased systemic inflammation (Maes, Kubera, Leunis, Berk, et al., 2012). The gut barrier regulates the passage of nutrients and water from gut luminal contents across the gut epithelial membrane while keeping out toxins and bacteria. The barrier is comprised of an epithelial cell layer which works together with a mucus layer and junctional complexes as a physical barrier to control paracellular permeability (in between the epithelial cells) and transcellular permeability (through the epithelial cells). Antimicrobial peptides are also secreted by Paneth cells, and the mucus layer contains the antibody IgA (Schoultz & Keita Å, 2020).

Gram-negative bacteria, such as those from the Proteobacteria phylum (including *E. coli*), have endotoxic LPS chains on their outer cell wall, and with increased gut permeability, translocation of LPS from the gut lumen into the body occurs. LPS interacts with immune cells and induces the expression of several inflammatory molecules such as pro-inflammatory cytokines, nitric oxide, and eicosanoids, which are also found in individuals with depression (Kohler et al., 2017; Miller et al., 2005; Raison et al., 2006). Intravenous administration of LPS has been shown to increase anxiety and depression behaviours in people (Lasselin et al., 2016) and mice (Erny et al., 2015), and can induce neuroinflammation, causing microglial cells to

become activated (Qin et al., 2007; Zhao et al., 2016). Evidence for increased gut permeability and bacterial translocation in people with depression has also been found, with increased concentrations of serum immunoglobin (Ig)A and IgM against LPS (Maes, Kubera, Leunis, & Berk, 2012), and associated increased activation of inflammation, oxidative and nitrosative stress pathways (Maes, Kubera, Leunis, Berk, et al., 2012).

Increased gut permeability occurs under stress (Ait-Belgnaoui et al., 2012; Demaude et al., 2006; Kiliaan et al., 1998; Meddings & Swain, 2000; Saunders et al., 1994; Soderholm et al., 2002), with decreased expression of tight junction proteins (Demaude et al., 2006) and an increase in translocation of large antigenic molecules (Kiliaan et al., 1998; Meddings & Swain, 2000; Saunders et al., 1994). The release of antigens triggers the inflammatory mechanisms such as activation of CD4+ cells resulting in mast cell degranulation, neutrophil infiltration, and increased cytokine interferon (IFN)-γ (Demaude et al., 2006; Soderholm et al., 2002). Increased gut permeability to antigenic molecules is likely to contribute to colonic inflammation due to the reactivation of sensitised CD4(+) cells (Bhatia & Tandon, 2005).

Whether increased gut permeability following stress occurs due to physiological reasons or a change in the gut microbiota is unclear. The increased permeability can be induced by dexamethasone or eliminated by adrenalectomy or glucocorticoid receptor blockade, suggesting physiological mechanisms (Meddings & Swain, 2000). However, the evidence does suggest that promotion of the growth of commensal bacteria with a decrease in LPS-producing bacteria is likely to alleviate stress-induced increases in gut permeability and associated pro-inflammatory immune activation. Dysbiosis, including a decrease in *Bifidobacteria* and an increase in LPS-producing bacteria has been associated with increased gut permeability (Leclercq et al., 2014). Probiotic supplementation (*L. farciminis* (Ait-Belgnaoui et al., 2012); mix of *L. rhamnosus* R0011 and *L. helveticus* R0052 (Gareau et al., 2007); *L. paracasei* NCC2461 (Eutamene et al., 2007)) can attenuate stress-induced gut permeability in rodents. Whether supporting and promoting the growth of commensal bacteria under stress increases psychological resilience is the next level of research needed.

2.2.3.3 Dysbiosis and Hypothalamic-Pituitary-Adrenal Axis Dysfunction

Dysfunctional stress response and HPA-axis may contribute to the development of both anxiety and depression (Arborelius et al., 1999). The gut microbiome may also influence the stress response. The markers of the HPA axis, such as corticosterone, are altered in GF mice compared with SPF counterparts (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011; Sudo et al., 2004). Blunted corticosterone responses have also been found in adult rats exposed to early life stress, and the corticosterone concentrations negatively correlated to faecal *Akkermansia* and *Rikenella* genera (Pusceddu, El Aidy, et al., 2015; Pusceddu, Kelly, et al., 2015). A faecal transplant from depressed people into GF rats caused an increase in the corticosterone response to acute stress alongside increased depression-like and anxiety-like behaviours in the rats (Kelly et al., 2016).

How the gut microbiota affects the stress response is uncertain. Immune activation activates the HPA axis (Silverman et al., 2005; Silverman & Sternberg, 2012; Turnbull & Rivier, 1999), providing an indirect mode of the effect of the gut microbiota on the stress response system. Some bacteria produce catecholamines including norepinephrine, epinephrine and dopamine (Asano et al., 2012; Holzer & Farzi, 2014; Lyte, 2011; Matsumoto et al., 2012; Roshchina, 2010; Ross et al., 2010; Tsavkelova et al., 2006). An increase in gut microbial catecholamines is unlikely to cause a direct increase in concentrations of systemic catecholamines because GF rats have typically been found with increased HPA-axis markers compared to rats with the normal gut microbiota (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011; Sudo et al., 2004). It is plausible however that microbially-produced catecholamines could contribute to baseline concentrations, and therefore HPA-axis programming in early life. Early life dysbiosis could therefore provide artificially high or low basal catecholamine concentrations and cause dysfunctional programming. This hypothesis has not been tested.

The balance between pathogenic and commensal bacteria in the gut is likely to be important. The colonisation of the GF mice prevented elevated plasma Adrenocorticotropic Hormone (ACTH) and corticosterone in GF rats at an early age with *B. infantis* or a mutant strain of *E. coli* lacking the translocated intimin receptor gene, neither of which are internalised into the gut epithelial cells. Wild-type *E. coli*, which do get internalised, did not prevent the heightened stress response (Sudo et al., 2004). Probiotic supplementation during or following stress has

been shown to reduce corticosterone in stressed animals (*L. rhamnosus* JB-1) (Bravo et al., 2011), *L. farciminis* (Ait-Belgnaoui et al., 2012), *L. helveticus* ns8 (Liang et al., 2015), mix of *L. rhamnosus* R0011 and *L. helveticus* R0052 (Gareau et al., 2007) or monoassociation with *B. infantis* (Sudo et al., 2004) or people (probiotic *L. helveticus* R0052 and *B. longum* R0175) (Messaoudi, Lalonde, et al., 2011), but *B. infantis* 35624 did not affect corticosterone in non-stressed animals (Desbonnet et al., 2009). However, no changes in systemic corticosterone concentrations were found following stress-induced changes in the gut microbiota and behaviour in mice (Tsilimigras et al., 2018), or with amelioration of stress-induced behavioural changes in rats following supplementation with *B. infantis* 35624 (Desbonnet et al., 2010). Interestingly, a synbiotic supplement (*L. rhamnosus* GG + polydextrose and galactooligosaccharide) increased plasma corticosterone concentration in rats following acute stress compared to that of control animals, whereas the same probiotic and prebiotic mix given separately did not (McVey Neufeld et al., 2019).

The HPA axis dysregulation in people with depression is not a straightforward relationship, with both high and low levels of cortisol found, and other dysfunction such as delayed return to baseline following acute stress and glucocorticoid resistance. With the gut microbiota able to increase HPA axis activation, stress-induced dysbiosis may increase the physiological stress response higher than that which is required to deal with the stressor effectively. Whether the gut microbiome moderates stress-induced increases in HPA activation in a meaningful way, and whether this affects emotional behaviour is unclear. More research is needed in this area.

2.2.3.4 Metabolites

Microbially derived metabolites include SCFAs, bile acids, choline and phenolic metabolites, indole derivatives, vitamins, polyamines, and lipids (Nicholson et al., 2012). The metabolites are primary secreted signalling molecules (influenced by host-derived signalling molecules) which crosstalk with other microbes, and the host mucosa and immune system, or secondary metabolites (produced through the digestion and metabolism of nutrients, non-food ingested compounds, such as medication, and metabolites from other microbes).

SCFAs activate several receptors in the colon, which have been shown to reduce colonic inflammation (Maslowski et al., 2009; Singh et al., 2014) and microglial neuroinflammation (Erny et al., 2015). These receptors can also increase gut epithelial cell barrier integrity by

increasing the expression of tight junctions (reviewed in Stilling et al., 2016). There is some evidence of altered SCFA production with depression and stress, although the direction of change is conflicting. No difference in SCFA concentrations was found in the faecal samples of people with depression compared with controls in two studies (Kelly et al., 2016; Skonieczna-Zydecka et al., 2018). When faecal samples from depressed people were transplanted into mice, an increase in faecal acetate and total SCFA concentrations was found along with increases in depression-like behaviour (Kelly et al., 2016). Prebiotic supplementation in mice increased SCFA concentrations many of which were negatively correlated with depression-like and anxiety-like behaviours (Burokas et al., 2017).

A decrease in faecal acetate and butyrate, and SCFA-producing bacteria, occurred in mice following psychosocial stress, and these decreases were associated with an increase in gut inflammation (Maltz et al., 2019). A similar study found an increase in caecal acetate, a decrease in propionate, butyrate and valerate, but no change in branched-chain fatty acids (van de Wouw et al., 2018). No increase in systemic LPS was found, despite an increase in gut permeability to FITC-dextran, but this was likely due to the colonic mucus layer being unaffected. Interestingly, only minor changes in the gut microbiota composition at the family and genus levels were observed following the stress intervention (van de Wouw et al., 2018). Functional changes in the gut microbiota have also been seen in mice following stress. KEGG analysis of 16S RNA marker genes in faecal samples predicted reduced pathways for the synthesis and metabolism of neurotransmitter precursors tyrosine and tryptophan, and SCFAs. This finding was positively associated with reduced exploration and sociability in the mice (Bharwani et al., 2016). In contrast, children who had increased self-reported stress showed increased faecal SCFAs (butyrate, valerate, isovalerate and isovalerate), but no increased gut inflammation (based on faecal calprotectin concentrations) was found (Michels et al., 2017). A possible explanation for the increased faecal SCFAs is a stress-induced decrease in gut transit time rather than a change in gut microbiota fermentation. Hair cortisol concentrations (a measure of long-term stress) in the children were not related to SCFAs, but heart rate variability was associated with decreased valerate (Michels et al., 2017). Heart rate variability is a proxy for parasympathetic nervous system activation (Marques et al., 2010), which affects gut transit time (Mayer, 2000). Gut motility likely has bidirectional interactions

with SCFAs and microbial composition. Stress induced dysmotility was able to be reversed in vitro with the application of either propionate or *L. rhamnosus* JB-1 (West et al., 2017).

Supplementation of an SCFA mixture in healthy adult men (174.2 mmol acetate, 13.3 mmol propionate, and 52.4 mmol butyrate), administered daily via the colon, reduced acute corticosterone response to acute stress, and increased serum SCFAs (Dalile et al., 2020). This result was not, however, associated with a change in subjective mood ratings (Dalile et al., 2020). In male mice, daily oral supplementation of SCFAs (67.5 mmol acetate, 25mmol propionate, and 40 mmol butyrate) decreased stress-related increases in anxiety-like behaviours in the open field test, and increased sucrose preference and decreased urine sniffing, both markers of depression-like behaviour. The SCFA supplement was associated with changes in gene expression in the brain related to dopamine receptors, part of the mesolimbic reward pathway which can be altered in depression (van de Wouw et al., 2018). Caecal SCFAs also differ between the stress-sensitive WKY rat strain and the stress-resilient Sprague-Dawley (SD) rat strain (Bassett et al., 2019). Anti-inflammatory effects of an increase in SCFA in some of the populations studied could be contributing to stress resilience.

The gut microbiome is a source of vitamins, including vitamin K and B vitamins niacin, biotin, riboflavin, folate and pyridoxine (Burgess et al., 2009; Hill, 1997; LeBlanc et al., 2013; Rosenberg et al., 2017; Sumi et al., 1977). Serum folate (B9) and pyroxidine (B6) are lower in those with depression or an increased risk of depression (Alpert & Fava, 1997; Gougeon et al., 2016; Jacka et al., 2012; Vulser et al., 2016). Micronutrients may affect depression risk via effects on the production and activity of monoamine neurotransmitters such as serotonin (Coppen & Bolander-Gouaille, 2005; Dakshinamurti et al., 1990; Hartvig et al., 1995; Partonen, 1998; Paul et al., 2004; Spedding, 2014), alterations to the HPA system (Wang et al., 2018), glutamatergic signalling (Wang et al., 2018), or inflammatory and oxidative stress (Rybka et al., 2013; Wang et al., 2018). They also play a role in the gut, for example, niacin is anti-inflammatory in the gut due to activation of the Gpr109a receptor, the same receptor that is activated by the SCFA butyrate (Singh et al., 2014). Folate and biotin are also immunomodulatory (Agrawal et al., 2016; Pfalzer et al., 2014). Pyridoxine (B6), is an essential co-factor for several enzymes in the kynurenine pathway (Myint et al., 2007), and a deficiency increases levels of xanthurenate, a kynurenine metabolite which is an antagonist for glutamate receptors. GF rats show increased

susceptibility to developing B6 deficiency (Sumi et al., 1977), and an accumulation of xanthurenate (Takeuchi & Shibata, 1984). Changes in the gut microbiota could alter the available concentrations of microbially produced vitamins, plausibly contributing to immune and metabolic pathway changes which are related to mood.

Up to 95% of the neurotransmitter serotonin, which has a well-known link to anxiety and depression (Willner et al., 2013), is produced endogenously in the gut mucosa (Gershon, 2013), and secretion of serotonin from enterochromaffin cells is influenced by microbial metabolites (Fukumoto et al., 2003; Reigstad et al., 2015; Sjögren et al., 2012; Yano et al., 2015). It is also produced by the gut microbiota, along with several other neurotransmitters including dopamine, gamma aminobutyric acid (GABA), acetylcholine and norepinephrine (Holzer & Farzi, 2014; Lyte, 2011; Matsumoto et al., 2012; Roshchina, 2010; Ross et al., 2010; Tsavkelova et al., 2006). Neurotransmitter levels and turnover in the brain differ in GF mice (Clarke et al., 2013; Diaz Heijtz et al., 2011; Neufeld et al., 2011) and reduced levels of circulating GABA and serotonin have been found in GF rats (Matsumoto et al., 2013; Wikoff et al., 2009). Whether alterations of these metabolites in the gut (endogenous and microbially produced) are related to those in the brain is uncertain, but there is some evidence that they can cross the blood–brain barrier (e.g. serotonin (Young et al., 2015)), of which the permeability is in itself affected by the gut microbiota (Braniste et al., 2014). Whether gut metabolites can directly reach the brain or not, they can affect the gastric environment and neural signalling. For example, GABA and acetylcholine are immunomodulatory (Bjurstöm et al., 2008; de Jonge, 2013), and GABA and serotonin affect gastric motility and acid secretion via enteric neurons (Fukumoto et al., 2003; Krantis, 2000). Whether stress-related changes in the gut microbiota alter the concentration of microbially produced vitamins or neurotransmitters in the gut is unknown.

Changes in metabolic pathways are another way that neurotransmitters are altered. Chronic stress in mice caused a decrease in the genus *Lactobacillus*, and a correlated increase in depression-like behaviour. Supplementation with the probiotic *L. reuteri* ATCC 23272 decreased the depression-like behaviour, seemingly via the production of H_2O_2 which inhibits the enzyme indoleamine 2, 3-dioxygenase 1 and restores the balance of serotonin/kynurenine pathways (Marin et al., 2017). This enzyme is activated by inflammation and LPS (O'Connor et al., 2008).

2.2.3.5 Gut Nervous System — Enteric Nerves and Vagus Nerve

Autonomic nervous system dysfunction, with increased sympathetic tone and decreased parasympathetic (vagal) tone, is proposed to be a contributing factor in the development of depression (Ondicova et al., 2010). People with depression commonly show decreased heart rate variability, a measure of high sympathetic activity (Agelink et al., 2002), and vagal nerve stimulation may be effective for treatment-resistant depression (e.g., Schlaepfer et al., 2008), although more research is needed (Lv et al., 2019).

The vagus nerve is linked with both the HPA axis and the immune system. Afferent fibres of the vagus nerve innervate the nucleus of the solitary tract, a brain region that directly regulates the HPA axis. Vagal nerve stimulation therapy can normalise the HPA activity (Ondicova et al., 2010). Secondary fibres also innervate brain regions responsible for emotional regulation (Goehler et al., 2000; Ondicova et al., 2010). Vagal sensory endings have receptors for cytokines and relay information about thoracic and gut inflammation to the brain (Goehler et al., 2000). In response, efferent fibres of the vagus nerve influence inflammation via cholinergic signalling, which inhibits cytokine release from LPS-stimulated macrophages. This negative feedback effect is known as the vagal-immune reflex. Low vagal tone is thought to promote systemic inflammation (Pavlov & Tracey, 2012; Tracey, 2002). There may also be an influence on neuroinflammation via receptors located on microglia and astrocytes (Ondicova et al., 2010).

Gut infection or systemic immune challenge with the bacterial endotoxin LPS caused vagal ganglia activation (shown by c-FOS protein immunoreactivity) (Goehler et al., 2005; Lyte et al., 2006). Associated sickness and anxiety-like behaviour were able to be alleviated with vagotomy (Bercik, Park, et al., 2011; Gaykema et al., 1998; Wang et al., 2002), suggesting that vagal signalling can mediate the development of emotional behaviours. Increased vagal activation also occurs with probiotic supplementation (*L. johnsonii* La1 (Tanida et al., 2005); *B. infantis* (Sudo et al., 2004)), and vagotomy prevented the restorative effect of probiotics on anxiety (*B. longum* NCC3001 (Bercik, Park, et al., 2011); *L. rhamnosus* JB-1 (Bravo et al., 2011)). Early life stress in rats increased the cholinergic secretory response of enteric nerves to stimulation (Gareau et al., 2007). While there is strong evidence for the vagus nerve being a key mediator in the gut-brain axis, some studies have found changes in emotional behaviour

in mice due to gut infection (Bercik et al., 2010) or anti-microbial treatment (Bercik, Denou, et al., 2011) despite a vagotomy procedure (Bercik, Denou, et al., 2011; Bercik et al., 2010). The vagus nerve may just be one mechanism of transmitting infection information to the brain.

2.2.4 Early Life Programming

Childhood adversity and stressful life events are both strongly linked with an increased risk of developing depression (Kendler et al., 1999; Mazure, 1998; Newman & Bland, 1994). Early life stress in rodents (during the neonatal period) can cause increased anxiety-like and depressive-like behaviours in adulthood (Matthews & Robbins, 2003; McVey Neufeld et al., 2019; Montalvo-Ortiz et al., 2016; Réus et al., 2011). Stress during adolescence in mice also caused increased anxiety-like behaviours in adulthood (Yohn & Blendy, 2017).

In contrast, a safe and reliable childhood with strong maternal/caregiver attachment decreases the risk of anxiety and depression later in life, even in those with a higher genetic risk (Franklin et al., 2012; Jaffee, 2007). The SRS is the biological response to both psychological and physiological (such as illness, injury) stressors. Both a hyper and hypo-responsive SRS are linked with mood disorders (Daskalakis et al., 2013; Del Giudice et al., 2011; Nesse et al., 2016). The SRS is functionally and epigenetically programmed in early life to match the individual's phenotype to their environment (Heim & Binder, 2012; Schapiro, 1968; Sominsky et al., 2013). Similar epigenetic programming occurs in early post-natal life for the immune system. Early life stress causes an inflammatory immune phenotype characterised by increased circulating pro-inflammatory cytokines (IL-1β, IL-6, tumour necrosis factor-α) which are also associated with depression (Elwenspoek et al., 2017; O'Mahony et al., 2009). Whether the early life stress causes independent epigenetic changes to the immune system or is mediated through epigenetic modulation of the SRS is debated (Elwenspoek et al., 2017). The two systems are interlinked (Silverman et al., 2005; Silverman & Sternberg, 2012; Turnbull & Rivier, 1999); early exposure to high cortisol levels can cause immune dysfunction later in life (Schapiro, 1968).

The gut microbiota may play a role in this early post-natal life programming. The biological response to physiological and psychological stressors are similar, and it is likely that exposure to the increases in inflammation and alterations in HPA-axis signalling due to stress-induced dysbiosis, maybe a significant part of the environmental signalling which causes epigenetic

programming. Injections of LPS in rats in the neonatal period (Sominsky et al., 2013) and in early adolescence (On Wah et al., 2019) caused increased anxiety-like behaviours later in life, and changes in GABA, corticotropin-releasing hormone (CRH) and glucocorticoid receptors in the hippocampus and hypothalamus in adulthood (Sominsky et al., 2013). Maternal Separation Stress (MSS) has been shown to increase gut permeability (Gareau et al., 2007), and therefore systemic LPS exposure. Whether interventions to manipulate the gut microbiota in early life can prevent or reduce the effects of early life stress is still being elucidated.

Probiotic supplementation with *L. rhamnosus* strain R0011 (95%) and *L. helveticus* strain R0052 (5%) during the separation period of MSS was able to prevent the stress-induced increase in serum corticosterone, adherence and penetration of bacteria into colonic mucosal cells, and colon tissue permeability (Gareau et al., 2007). Additionally, increases in anxiety-like behaviour and changes in gene expression in rats following MSS were able to be ameliorated by dietary supplementation of the probiotic *L. rhamnosus* GG alone or in combination with prebiotics polydextrose and galactooligosaccharide (McVey Neufeld et al., 2019). In contrast, supplementation of rats with omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DPA) following MSS restored the gut microbiota (Pusceddu, El Aidy, et al., 2015) and prevented higher levels of corticosterone in response to stress (Pusceddu, Kelly, et al., 2015), but caused reduced anxiety and increased cognitive performance in the non-stressed rats only, with no difference in behaviour in the stressed rats (Pusceddu, Kelly, et al., 2015). These findings may be due to the intervention being after rather than during the stress period, the type of intervention, or an indication that the gut microbiota is not the key mediator of the behavioural effects of early life stress.

2.2.5 The Gut Microbiota Could be Key in Stress-Resilience

The evidence for stress-induced changes in the gut microbiota being a mechanism rather than a covariate of stress-induced changes in mood is limited and sometimes conflicting but has plausible mechanisms. If stress-induced changes in the gut microbiota do reduce stress-resilience, then there could be differences between individuals who are stress-resilient and stress-sensitive and/or correlations of the gut microbiota with mood symptoms should be observed. There is some evidence to support this. Studies investigating links between stress, the gut microbiome and mood are summarised in Table 2.2.2.

A comparison of stress-resilient mice showed increased abundance of *Bifidobacterium spp.* in the stress-resilient mice compared with control mice or stress-sensitive mice (Yang et al., 2017). Some strains of the *Bifidobacterium* genus are considered psychobiotic so it seems straightforward that an increase in their abundance in some of the rats could promote stress resilience. However, the mechanism for how an increase in these strains would occur due to stress was unclear.

Other studies did not compare stress-resilient and stress-sensitive mice, but found some correlations between gut microbiota and behaviour. Marin et al. (2017) found decreased abundance in taxa from the *Lactobacillus* genus in male mice following chronic mild stress and a positive correlation between *Lactobacillus* genus and escape behaviours (active swimming) in the forced swim test (Marin et al., 2017). Bangsgaard Bendtsen et al. (2012) found that in female BALB/c mice exposed to two weeks of grid floor stress, the caecal microbiota differed from that of the control group and was correlated with behaviour. The time spent in the dark compartment of the light/dark box test (considered as anxiety-like behaviour) was positively correlated with an unclassified species from the *Ruminococcaceae*, which was also negatively correlated with the pro-inflammatory cytokine IL-2. Time spent in the closed arm of the elevated plus maze (also considered anxiety-like behaviour) was negatively correlated with the genus *Butyricicoccus* (a butyrate producer). In the same study, risk assessment behaviour (two paws placed in open arms and then retracted) was positively correlated with relative abundance of the *Lachnospiraceae* family, and in the control group only, the relative abundance of the *Bacterioides* genus (a major propionate producer) positively correlated with the number of immobility episodes in the tail suspension test (Bangsgaard Bendtsen et al., 2012). Likewise, a comparison of stress-sensitive WKY rats and stress-resilient SD rats under acute stress showed an increase in the relative abundance of caecal *Lactococcus* genus, a lactic acid producer. Relative abundance of the *Lactococcus* genus was positively correlated with brain and plasma lipid metabolites (Bassett et al., 2019).

The evidence for specific microbiota relating to behaviour is sparse but suggests that changes in SCFAs and inflammation may be key mechanisms. These changes could be linked due to SCFAs being immunomodulatory. Stress-induced decreases in the genus *Lactobacillus* have been found in several animal stress studies (Aguilera et al., 2013; Bailey & Coe, 1999; Bassett

et al., 2019; Galley, Nelson, et al., 2014; Marin et al., 2017; Tsilimigras et al., 2018). It is plausible that individual differences in stress-induced decrease in bacteria from the *Lactobacillus* genus could be the difference between an in individual being stress-resilience or stress-sensitive. Marin et al. (2017) found that kynurenine was increased in stressor-exposed mice alongside the lowered *Lactobacillus* relative abundance and that while a supplement of the probiotic *L. reuteri* ameliorated stress-induced behaviour, it did not work if L-kynurenine was also supplemented alongside. They found that *in vitro*, a reactive oxygen species produced by *Lactobacillus spp.* inhibited the enzyme indoleamine 2,3-Dioxygenase 1 (IDO1), a key enzyme which allows tryptophan to be converted to kynurenine. IDO1 is also activated by inflammation and therefore could be a mechanism.

Not all studies have found associations between the gut microbiota composition and behaviour. Tsilimigras et al. (2018) reported stress-induced changes in behaviour and gut microbiota in mice, but there was no correlation between them. Conflicting results could be due to the site of microbiota sampling: Bangsgaard Bendtsen et al. (2012) found that stress-induced behavioural changes correlated with caecal but not faecal microbiota changes.

The correlation of gut microbiota composition with behaviour does not show causality. It is equally likely that changes in gut motility and inflammatory processes due to different levels of perceived stress in stress-resilient or stress-sensitive individuals are the mediator for changes to the gut microbiota. However, intervention with prebiotic and probiotics have been able to alleviate stress-induced changes in emotional behaviour. People given 30 days of a probiotic mix (*L. helveticus* R0052 and *B. longum* R0175) had reduced anxiety, depression, and perceived stress scores, as well as a decrease in 24h urinary free cortisol from baseline concentrations (Messaoudi, Lalonde, et al., 2011). The baseline anxiety and depression scores of the participants ranged from low to moderately high. In mice, oral supplementation with *Bifidobacterium* spp (LAC-B Granular Powder) increased the number of mice resilient to social defeat stress, and prevented a stress-induced decrease in sucrose intake (Yang et al., 2017). *L. reuteri* 23272 given to male mice during chronic, mild stress decreased despair behaviour in the forced swim test (Marin et al., 2017). A probiotic (*L. rhamnosus* GG), prebiotic mix (polydextrose and galactooligosaccharide) or combined in a synbiotic mix, following maternal separation stress in male and female SD rats reduced stress-induced increases in anxiety-like

behaviour. The synbiotic had the greatest effect and was also able to ameliorate stress-induced memory changes (McVey Neufeld et al., 2019). An increase in stress-induced defecation was able to be prevented by prebiotic (fructooligosaccharides and galactooligosaccharides) supplementation (Burokas et al., 2017).

It is possible that the positive action of probiotic and/or prebiotic supplementation on emotional behaviours may be more effective following stress. SD rats given a probiotic (*B. infantis 35624*) for 40 days following maternal deprivation stress, had reduced stress-induced immobility in the forced swim test (Desbonnet et al., 2010) whereas the same daily dose of the same probiotic (although for only 14 days) without the stress intervention did not affect behaviour (Desbonnet et al., 2009). Similarly, stress-induced increases in anxiety-like and depressive-like behaviours in mice were able to be ameliorated by dietary supplementation with prebiotics fructooligosaccharides and galactooligosaccharides (Burokas et al., 2017). Basal and acute stress-induced corticosterone levels were also reduced, and the prebiotic supplement prevented a stress-induced decrease in the Actinobacteria: Proteobacteria ratio and the relative abundance of bacteria from the *Bifidobacterium* and *Lactobacillus* genera. The decreased Actinobacteria: Proteobacteria ratio may have reflected a decrease in inflammation-reducing bacteria such as those from the *Bifidobacterium* genus and an increase in opportunistic pathogens and bacteria with LPS. This may explain some of the stress-related changes in inflammation and mood. The prebiotic supplementation, given in a prior study with no stress intervention, had a much weaker effect on behaviour (Burokas et al., 2017). Finally, a reduction in anxiety-like behaviour was found in BALB/c stress-sensitive mice following supplementation of *L. rhamnosus* JB-1 (Bravo et al., 2011), but the same probiotic given to healthy men did not alter HPA response or subjective mood or stress measures (Kelly et al., 2017).

It is also possible that stress is one of the reasons why the results of animal intervention studies do not always translate well to human research. Laboratory conditions are neither reflective of real life for humans nor the animals involved in the research. Laboratory conditions can be stressful for animals, for example single housing (Brenes & Fornaguera, 2008). This means that many intervention studies in animal may be effective by alleviating stress-induced changes in

the gut-microbiota and/or physiology. If the same stress-induced changes are not present in human study participants, then there may be no effect.

Table 2.2.2. *Summary of studies investigating links between stress and the gut microbiota.*

Microbiota which show an association (not necessarily causality) with stress-resilience and stress-sensitivity are indicated in the columns labelled SR and SS, respectively. This depends on the study but may mean, e.g., a probiotic supplement which increased stress-resilience, or an increase in relative abundance of the microbiota in stress-resilient individuals. Abbreviations: EPM, Elevated Plus Maze; FST, Forced Swim Test; TST, Tail Suspension Test; HbA1c, Haemoglobin A1c ; IL, Interleukin; IFN-γ, Interferon gamma; LDB, Light-Dark Box; OFT, Open Field Test; NOR, Novel Object Recognition; MSS, Maternal Separation Stress.

Study design (Stress, subjects, intervention)	Results	SR	SS	Reference
• FST • BALB/c mice (M,adult) • Probiotic Lactobacillus rhamnosus JB-1, 28 days prior to FST/Vagotomy	• Increase in anxiety-like (EPM) and depressive like behaviour (FST). Both ameliorated by probiotic • Stress-induced increase in corticosterone ameliorated by probiotic. Stress-induced hyperthermia not affected by probiotic. • Vagotomy prevented the anxiolytic effects of the probiotic. • Changes in gut microbiota not reported	Probiotic: *L. rhamnosus* JB-1	Not applicable	(Bravo et al., 2011)
• Chronic mild stress, 7w • C57BL/6J Mice (M,7w) • Probiotic: *L.* reuteri ATCC 23272, 2w during stress and 2w post-stress	• Increase in depression-like behaviour (FST): prevented by probiotic. • Increase in serum kynurenine following stress. Prevented by probiotic. • Inhibition of the enzyme IDO1 by *Lactobacillus*-produced reactive oxygen species (H_20_2) in vivo • Decreased (faecal) class *Bacillus*, specifically genera *Lactobacillus* and *Turicibacter*	Probiotic: *L. reuteri* ATCC 23272	Decrease in (faecal) *Lactobacillus*	(Marin et al., 2017)
• Grid floor stress, 15d • BALB/c mice (F, 5w) • No intervention	• Increase in anxiety-like (triple test) and depressive-like behaviour (TST) • Lower blood glucose but higher HbA1c were found. Cytokines were reduced in the control mice but not the stressed mice. Correlations were found between IL-6, IFN-γ, and behaviour in the LDB, EPM, and OFT. • Increase in (caecal) *Odoribacter*, *Alistipes* and an unclassified genus from the *Coriobacteriaceae* family. *Lachnospiraceae* correlated to risk assessment behaviours. *Bacterioides* correlated with immobility in TST; *Ruminococccaceae* correlated to entries to closed arms in triple test (anxiety/activity)	Not applicable	*Bacterioides*; *Ruminococccaceae*	(Bangsgaard Bendtsen et al., 2012)
• MSS, 15d • Sprague-Dawley (SD) rats, sex not stated, 4d neonatal	• Behaviour not measured • Increase in serum cortisol and gut permeability. Prevented by probiotic • Decrease in genus *Lactobacillus*. Increase in bacterial adherence and penetration into mucosal cells.	Probiotic: *L. rhamnosus* strain R0011 (95%) and *L. helveticus*	Not applicable	(Gareau et al., 2007)

• Probiotic *L. rhamnosus* strain R0011 (95%) and L helveticus strain R0052 (5%), 15d during stress	• Increases in cortisol, gut permeability, and bacterial adherence/penetration was prevented by probiotic supplementation.	*strain* R0052 (5%)		
• MSS, 15d • SD rats (M, 90d) • Probiotic *Bifidobacterium* infantis 35624, 45 days	• Increase in depressive-like behaviour (FST), ameliorated by probiotic supplementation • No difference in plasma corticosterone, L-kynurenine, tryptophan or kynurenic acid. An increase in IL-6 following stimulation with immune stimulant concanavalin A was prevented by the probiotic • Gut microbiota not measured	Probiotic: *B. infantis* 35624	Not applicable	(Desbonnet et al., 2010).
• MSS, 10d • SD rats (M, 7-8w) • No intervention	• Increased stress-induced faecal boli number in the OFT, but no changes in behaviour • Increased plasma corticosterone and increased systemic immune response in response to in vitro LPS challenge. Decreased pain threshold • Change in microbiota structure (taxa not specified)	Not applicable	Not applicable	(O'Mahony et al., 2009)
• MSS, 1w • Infant rhesus monkeys (M+F, 6-9 m) • No intervention	• Decrease in total abundance of faecal bacteria and *Lactobacillus* by day 3, but back to normal after a week	Not applicable	Not applicable	(Bailey & Coe, 1999)
• Prenatal stress • Human infants • No intervention	• Gastrointestinal symptoms more common in babies from mothers who reported higher stress. Cortisol and stress related questionnaires did not correlate in the mothers. • Increased faecal *Escherichia-enterobacteria* and lower lactic acid bacteria and Actinobacteria	Not applicable	Not applicable	(Zijlmans et al., 2015)
• Prenatal (dam exposed to CMS) • Offspring of pregnant C57BL mice exposed to CMS stress • No intervention	• Behaviour not measured • Increased faecal Rikenellaceae and Odoribacter, Mucispirillum and a decrease in Bacteroides	Not applicable	Not applicable	(Jašarević et al., 2017)
• Prenatal (dam exposed to restraint stress)	• Offspring showed increase in anxiety-like behaviour (EPM, NOR) in adulthood	Not applicable	Not applicable	(Gur et al., 2017)

• Offspring of pregnant C57/B16 mice • No intervention	• Increased plasma IL-1β in placenta and fetal brains but did not persist till adulthood. Decreased BDNF found in maternal placenta and in brains of adult offspring. • Microbial community composition clustered differently in the stress group from control in both pregnant dams and their offspring			
• Restraint Stress (16h/d x 7d) • Swiss Webster & CD-1 mice (M, 6-8w) • Faecal transplant from stressed mice to germ free mice	• Behaviour not measured • Increased inflammatory response to colonic pathogen in germ free mice with faecal transplant from stressor exposed mice. • Increased (faecal) Firmicutes and decreased Actinobacteria and Bifidobacterium.	Not applicable	Not applicable	(Galley et al., 2017)
• Restraint Stress (6h/d x 3w) • SD rats (M, 220-240g) • Probiotic: *L. helveticus* ns8, 26d	• Increased depressive-like behaviour (SPT) and anxiety-like behaviour (EPM, OF). • Body weight was reduced. Increase in plasma corticosterone and pro-inflammatory cytokines TNF-α and IFN-γ and decrease in plasma IL-10. Decreased BDNF in the hippocampus, prevented by probiotic • Stress-induced changes in behaviour, corticosterone, IL-10, BDNF were prevented by the probiotic supplementation • Gut microbiota not measured	Probiotic: *L. helveticus* ns8	Not applicable	(Liang et al., 2015)
• Restraint stress, 12h/dx7d • CD1 mice (M, 8w) • No intervention	• Behaviour not measured • Increased TNF-α gene expression in colonic tissue • Total bacteria and Gram negative bacteria increased in small intestine, caecum, and large intestine. Decrease in bacterial diversity and richness. Reduced family *Porphyromonadaceae*, specifically genus *Tannerella*. Increased colonisation by introduced pathogen *Citrobacter rodentium*	Not applicable	Not applicable	(Bailey et al., 2010)
• Restraint Stress, 15h/d x 7d • CD-1 mice (M, 6-8w) • No intervention	• In the (colonic) mucosca-associated bacteria, a decrease in the families *S24-7* and *Lactobacillaceae* and genera *Lactobacillus* spp., were found, and in an increase in the family *Ruminococcaceae*, and genera *Oscillospira*. In the luminal bacteria, a decrease in the family *S24-7*, as well as genera *Adlercreutzia*, and an unclassified genus in *S24-7* were found	Not applicable	Not applicable	(Galley, Yu, et al., 2014)

• Restraint stress/FST alternated, 19 days • CF-1 mice (M+F, 6w) •	• Distance travelled in EPM and LDB increased, increased rearings in OF. Blood collected after behavioural tests • Males had higher corticosterone levels following acute stress (behavioural testing) • Increase in family *Lachnospiraceae*. Decrease in genus *Sarcina* only in females. *Ruminococcus* gnavus increased in females but decreased in males	Not applicable	Not applicable	(Tsilimigras et al., 2018)
• Social stress: cage in cage aggressor • CD1 and C57BL/6 mice ()M, 6-8w.	• Behaviour not measured • No difference in colonic cytokines • Decrease in relative abundance of families *Porphyromonadaceae* and *Lactobacilliaceae*, and genera *Lactobacillus*, *Parabacteroides*, and an unclassified genus from phylum Firmicutes and unclassified genus from class *Bacilli*. The absolute abundance of *lactobacilli* was also reduced, specifically *L. reuteri*, but only in the outbred CD-1, not the inbred C57BL/6 mice	Not applicable	Not applicable	(Galley, Nelson, et al., 2014)
• Social stress: chronic social defeat • C57BL/6 mice (M, 8w) •	• Decrease in social interaction • Increase in (faecal) genus *Bifidobacterium* in the stress resilient group. Not detected in the control group or stress-sensitive group	*Bifidobacterium*	Not applicable	(Yang et al., 2017)
• Social stress: resident intruder, 6h/d x 10d • C57BL/6J male, juvenile (5-6wk)	• Behaviour not measured • Differed across time points. Key changes were a decrease in phylums Bacteroidetes, Firmicutes, Verrucomicrobia; and genera *Oscilospira* and *Anaeroplasma*, with a trend in decrease in *Lactobacillus*. An increase and decrease in *Akkermansia* were found at different time points. A trend of increase in phylum Proteobacteria was found	Not applicable	Not applicable	(Gautam et al., 2018)
• Social stressor (6d x 2hr/d) • CD1 Mice (M, 8w) • Antibiotics (ampicillin (1 mg/mL), vancomycin (0.5 mg/mL), neomycin sulfate (1 mg/mL), and metronidazole (1 mg/mL))	• Behaviour not measured • Increase in proinflammatory markers, particularly IL-6, prevented in antibiotic group • Immediately after induced stress, the (caecal) microbiome of mice had consistently altered within the group, and clustered separately from the control group, but after 15 h, the separation was no longer as clear, with variation within the stress group. Within genera, decrease in *Bacteroides*,increase in *Clostridium*, trend of decrease in *Lactobacillus*	Not applicable	Not applicable	(Bailey et al., 2011)

	• Stress-induced increases in plasma IL-6 was inversely correlated with relative abundances of genera *Coprococcus*, *Pseudobutyrivibrio* and positively correlated with *Dorea*			
• Water Avoidance Stress (1hr/d x 7d) • C57BL/6N mice (F, 6w) • Antibiotics during stress (Bacitracin A, Neomycin, Amphotericin B)	• Pain related behaviour in response to intracolonic capsaicin increased. Slightly mitigated with antibiotics. • Increased faecal pellet output, plasma corticosterone, and adrenal gland weight. Increased luminal s-IgA levels. In the colon tissue, cannabinoid receptors increased marginally, and tryptophan hydroxylase (TPH1) expression increased by 40% • Antibiotics and stress enhanced bacterial adherence to luminal wall. Faecal *Clostridium coccoideds cluster* XIVa was increased, and *Verrucobactera*, *Lactobacillus* and *Enterococcus* spp. decreased	Not applicable	Not applicable	(Aguilera et al., 2013)

Animal studies do not always translate well to human research, including MGBA research. Stress may be one of the reasons for this. Laboratory conditions are neither reflective of real-life for humans nor the animals involved in the research. Animal behavioural testing has limitations on how well it reflects anxiety- or depressive-like symptoms in humans. Laboratory conditions can also be stressful, for example, the type of housing. Animal stress studies may be comparing stressed to mildly stressed animals. In a similar vein, "stress" is a broad term and defined differently in different studies. External stress is defined by environmental conditions, whereas perceived (internal) stress depends on how the individual feels. It is often assumed that under environmental stress, all the individuals are also experiencing perceived stress. There is also a difference between how individuals experience perceived stress. "Good stress" is termed eustress and can improve performance and mood. "Bad stress" is what is typically considered to be stress and is sometimes defined as distress.

The type of biological processes activated under stress also needs to be differentiated. HPA-axis activation may affect the MGBA differently than sympathetic nervous system activation. It is unclear whether the MGBA increases the risk of chronic perceived stress developing into anxiety and/or depression or increases the risk of an individual experiencing chronic perceived stress under high environmental stress. It may do both.

There is a reported association between diet and depression, but the nature of the relationship is still unclear. Some research indicates that a healthy diet is protective against developing depression, but many studies show no effect [185]. Dietary manipulation of the gut microbiota may be a key variable in the diet-depression relationship, especially in preventing stress-induced changes in the gut microbiota [185]. Taylor et al. [186] found independent relationships between the gut microbiota and mood (stress, anxiety, and depression); dietary factors and mood, with the microbiota-mood associations, were mediated by fibre intake.

2.2.6 Conclusion

Stress-induced changes in the gut microbiota are a key variable which needs to be considered more in mood research. The limited research available suggests that the promotion of commensal bacteria, particularly those considered to be probiotics, is likely to confer some increase in emotional resilience under stress. Whether this is through direct prevention of effects from stress-altered gut microbiota or alleviating physiological consequences of stress-

induced changes, or both, is unknown. More research is needed. The mechanisms are still being elucidated, and which individuals are more likely to respond to microbial support under stress are unknown and whether there are life stages, types of stress, time points in which interventions may be effective. There is strong evidence for the role of many species of the *Lactobacillus* genus, both as a probiotic supplement or as commensal bacteria. Research into probiotics and their mechanisms continue. Dietary manipulation is another key area for research, particularly because diet will affect the gut microbiota composition and function. More physiological and psychological testing is needed to differentiate between environmental, perceived stress and stress-related physiological processes.

2.2.7 References

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Section 3. Knowledge Gaps in the Microbiome-Gut-Brain-Axis Research and Thesis Research Outline

2.3.1 Discussion

A literature review of the diet-mood relationship and its intersection with the MGBA was reported in Section 1, followed in Section 2 with a review of how the MGBA interacts with stress and possibly is a factor in stress-resilience. Outlined here is a summary of findings and how they led to the chosen research direction.

While there is a significant amount of emerging research showing links between diet, mood, stress and the gut microbiota, there is much more that is unknown. The results from different studies often do not align. This discrepancy is likely to be due to the relationships being complicated by multiple variables, as well as being bidirectional. Stress is a significant variable as it interacts with diet, mood, and the gut microbiome. It is plausible that the development of mood disorders following stress is caused by stress-related changes in the gut microbiota, and that these changes may be prevented or increased by dietary factors. These findings in turn may explain some of the variation in research on the diet-mood relationship. Further research examining the effects on mood of a stress-altered gut microbiome is needed, along with the associated mechanisms.

Inflammation stands out as an important mechanism which intersects with mood, diet, stress, and the gut microbiome. People with depression and anxiety have higher levels of systemic inflammation (Kohler et al., 2017; O'Brien et al., 2007; Vogelzangs et al., 2013). Healthy style diets (e.g. the Mediterranean diet) are considered anti-inflammatory (Estruch, 2010; Kim et al., 2013) and unhealthy diets tend to be inflammatory (Cani et al., 2008). Some of the effect of diet on inflammation is likely via interactions with the gut microbiota (Cani et al., 2008; David et al., 2013; Kaliannan et al., 2015; Kim et al., 2013; Matijašić et al., 2014) (reviewed in section 2.1.4). It is possible that the protectiveness found in several studies of a healthy diet against developing mood disorders is because a healthy diet contains multiple components which promote a healthy gut microbiome such as fibre, phytochemicals, and omega-3 fatty acids.

Inflammation also occurs following stress, both directly via neural activation (Bharwani et al., 2017; Castagliuolo et al., 1996; Mayer, 2000; Santos et al., 2001; Soderholm & Perdue, 2001), but also due to undesirable changes in the microbiota, and has been prevented with antibiotic treatment (Bailey et al., 2011). There are several ways in which stress-induced changes in the gut microbiome structure and/or function could contribute to the development of anxiety and depressive symptoms following stress, with inflammation as a mechanism.

1. A decrease in commensal microbes and an increase in opportunistic pathogens can cause gut inflammation and/or systemic inflammation via internalisation into enterocytes.
2. Changes in microbial metabolites could cause increased inflammation through a change in SCFA or vitamin production such as an overall decrease, or a change in the ratios.
3. Gut permeability is increased following stress and is mediated at least partly by the changes in microbial composition. Increased gut permeability allows translocation of gut microbiota into the host where bacterial LPS cause inflammation. A lower abundance of bacteria with LPS may reduce systemic inflammation with increased gut permeability.

Research examining the effect of changes in microbial metabolites on mood is particularly sparse, particularly when stress related. Changes in microbially-produced neurotransmitters could also potentially affect mood, not via inflammation but by directly contributing to the systemic levels, and subsequently, if they are able to cross the blood brain barrier, the levels in the brain. These include neurotransmitters GABA and serotonin which are potentially anxiogenic and antidepressant, or an increase in the stress hormones epinephrine and norepinephrine. It is unlikely that these mechanisms are independent from one another, or that only one or the other would occur. Identifying which stress-induced changes are linked with stress-sensitivity would help in our understanding of the MGBA and its mechanisms. It would also provide direction for testing interventions to manipulate the gut microbiota under stress with the aim of increasing stress-resilience.

The aim of the thesis is to explore whether stress-related changes in the gut microbiota and gut microbial metabolites are linked with inflammation, and whether they play a role in stress resilience. The research approach used in this thesis is to examine the differences between those individuals who thrive under stress (stress-resilient) and those who do not (stress-sensitive), we can identify key mechanisms. Stress-induced changes in the gut microbiome

composition, metabolites (neurotransmitters, SCFAs), gut permeability marker (plasma lipopolysaccharide binding protein), systemic inflammation (plasma cytokines) and faecal corticosterone will be compared between stress-resilient and stress-sensitive individuals.

This correlative study design cannot show causation but depending on the outcome it can provide strong support for or against the hypotheses that the stress-induced change in the gut microbiota and one/some of the associated mechanisms are key for stress-resilience or stress-sensitivity. Any variables which change under stress but differ between stress-resilient and stress-sensitive may be a critical mechanism. Follow-up experimental studies can manipulate that variable to determine if stress-resilience can be increased. In contrast, if stress-induced changes do not differ between the stress-sensitive and stress-resilient groups, then it is more likely that the hypothesis is not supported, and stress-induced changes in the gut microbiome simple vary alongside changes in emotional behaviour.

2.3.2 Research hypotheses

H1: The microbiome structure, SCFAs, caecal neurotransmitters, lipopolysaccharide binding protein and/or systemic inflammation will differ between stress-sensitive and stress-resilient rats following chronic stress

H2: The microbiome structure, and SCFAs, caecal neurotransmitters, lipopolysaccharide binding protein and/or systemic inflammatory markers in the stress-resilient group will be more similar to the structure/concentrations in the control group than the stress-sensitive group.

2.3.3 Methods

A rat model of depression was selected for the research. Most MGBA research is carried out in animals. The heterogeneity of genetics, lifestyle, diet, and life experiences in people makes human studies more difficult, but these factors can be tightly controlled in animals. Animals are not small people, and they differ in their biological make-up, cognition, and psychology from humans. However, subcortical features and many biochemical pathways in mammalian brains and the nervous system are relatively consistent in mammals. Some animals can therefore operate as good models of anxiety and depression, albeit with limitations (Cryan & Holmes, 2005). It is also more useful to use an animal model when investigating mechanisms

because tissue collection (e.g., caecum contents or gut and brain tissue samples) is possible in animals where it is not possible or very difficult in humans.

Unpredictable chronic mild stress (UCMS) is commonly used to create a model of depression in rodents, with reduced sucrose preference typically used to identify rodents who have developed anhedonia following UCMS (Willner, 2017a). The sucrose preference test (SPT) measures the preference of a rodent to drink water containing a low concentration of sucrose over plain water. Rats which have developed the depressive-like behaviour anhedonia will drink less sucrose solution and their sucrose preference will reduce from 80-100% to around 50-60% (Papp et al., 1991). UCMS has been used to test factors involved in stress resilience because a percentage of animals exposed to UCMS are resilient and do not develop depressive-like symptoms. Rates of resilience to developing reduced sucrose preference following UCMS are around 25% to 50% (Bergström et al., 2008; Willner, 2017b).

Completing a range of different behavioural tests when using a new animal model of depression is important as it is uncertain which changes may occur, and each behavioural test targets a different aspect of behaviour. The behavioural tests used in this thesis are summarised in Table 2.3.1. These behaviours have been shown to following exposure to UCMS (Bilkei-Gorzó et al., 1998; Slattery & Cryan, 2012; Wang et al., 2017).

Table 2.3.1. Measures of Emotional Behaviour in Animals

Category of mood disturbance	**Depression or anxiety symptom in humans**	**Animal Behavioural Test**	**Measure indicating increase in anxiety or depression-like symptom**
Depression	Hopelessness	Forced Swim Test	Behavioural Despair, measured by reduced immobility
	Reduced energy and motivation	Coat State	Decreased grooming, measured by an increase in dirty coat score
	Anhedonia	Sucrose Preference Test	Reduced sucrose preference
	Locomotor dynamics	Open Field Test	Reduced locomotion (speed and distance in the open field)
Anxiety	Behavioural inhibition	Light-Dark Box test	Behavioural inhibition, measured by decreased time in the light box
	Behavioural inhibition	Open Field Test	Reduced time in the centre of the open field

Sprague-Dawley rats were chosen to use with the UCMS. Willner (2017b) reported a survey of all laboratories who published studies using UCMS in the previous five years. The survey indicated that outbred rat strains SD and Wistar rats are most commonly used, along with inbred mouse strains C57BL/6 and BALB/c According to Czéh et al. (2016), outbred rat strains are typically preferred by researchers, and most of the behavioural tests (open field test (OFT), Morris water maze and elevated plus maze test) were originally designed for rats as opposed to mice. SD rats are an outbred strain with more genetic variation and more resilient to stress than other strains (e.g., Lopez-Rubalcava & Lucki, 2000; Paré & Tejani-Butt, 1996). This resilience potentially means a higher likelihood of a range of behavioural responses to stress.

2.3.4 Thesis Structure

	Chapter 1	Introduction
Review	*Chapter 2*	Literature Review
Review	*Section 1*	Review of the relationships between diet, mood, and the gut microbiome
Review	*Section 2*	A narrative review on role that the gut microbiome could play in stress resilience
Review	*Section 3*	Summary and research hypotheses
Experimental Chapters	*Chapter 3.*	First chronic stress study in rats
Experimental Chapters	*Study summary:*	Female Sprague-Dawley (SD) rats, n=30. Six-week unpredictable chronic mild stress intervention. • Sucrose preference test • Porsolt swim test • Light-dark box test • Caecal microbiota composition • Caecal short chain fatty acids (SCFAs) • Plasma cytokines • Plasma lipopolysaccharide binding protein
Experimental Chapters	*Chapter 4*	Methodology testing studies
Experimental Chapters	*Study summary:*	Consumption of different sucrose solution concentrations in female SD rats
Experimental Chapters	*Study summary:*	Porsolt swim test in female SD rats with or without antidepressant drug administration
Experimental Chapters	*Study summary:*	Baseline behaviour compared between female and male SD rats. • Sucrose preference • Light-dark box • Open field test • Porsolt swim test
Experimental Chapters	*Chapter 5*	Second chronic stress study in rats
Experimental Chapters	*Subject of study*	Male SD rats, n=30. Four week of unpredictable chronic mild stress intervention. • Sucrose preference test • Open field test • Porsolt swim test • Light-dark box test • Serum cytokines • Serum lipopolysaccharide binding protein • Caecal neurotransmitters • Serum neurotransmitters
	Chapter 6	Discussion, conclusion, and future research perspectives

2.3.5 References

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Chapter 3.

An Investigation into whether Stress-Induced Changes in the Caecal Microbiota, Caecal Organic Acids, Gut Permeability Marker and Plasma Cytokines are Associated with Stress-Sensitivity, using the Unpredictable Chronic Mild Stress Model in Female Sprague-Dawley Rats

This chapter (following modification to a journal manuscript format) is intended to be submitted to the journal Microorganism (impact factor 4.128) in the special issue "Microbiota-Gut-Brain Axis in Health and Disease, and Future Therapies". This special issue includes research which examines Microbiome-Gut-Brain-axis activity in neuropsychiatric disorders and investigates underlying mechanisms.

Abstract

Female Sprague-Dawley (SD) rats were exposed to unpredictable chronic mild stress (UCMS) for six weeks to test differences in several biological markers between stress-resilient rats, stress-sensitive rats and an unstressed control group. Depressive-like behaviour was measured using the sucrose preference test and the Porsolt swim test. Anxiety-like behaviour was measured with the light-dark box test. Faecal corticosterone, caecal microbiota (composition and organic acids), serum gut permeability (lipopolysaccharide-binding protein, LBP) and plasma inflammation (12 cytokines) markers were measured. Atypical behaviours were observed in female rats following UCMS and no depressive-like behaviours were observed. The circulating concentration of cytokines granulocyte-macrophage colony-stimulating factor (GMC-CSF) and cytokine-induced neutrophil chemoattractant 1 (CINC-1) but not plasma LBP or caecal organic acids, was higher in UCMS-exposed female rats. Relative abundance of taxa from the *Clostridiales* order and *Desulfovibrionaceae* family correlated with anxiety-like behaviours and plasma cytokine concentrations, regardless of UCMS.

3.1 Introduction

Depression and anxiety episodes are very commonly preceded by stress. Therefore, identifying factors linked with resilience to developing symptoms of anxiety and/or depression following stress allows the identification of possible avenues of prevention or treatment for depression and anxiety. Several psychosocial factors are known to increase stress resilience (Southwick et al., 2004), but physiological factors are also associated with it as well including exercise (Ozdemir & Akbas Gunes, 2021) and sleep (Arida & Teixeira-Machado, 2021), differences in neurotransmitter levels (Southwick et al., 2004), vagus nerve tone (Weber et al., 2010) and gene expression in the hippocampus (Bergström et al., 2008). The gut microbiome has been shown to affect emotional behaviour in rodents and humans, and it is also affected by stress (Bear et al., 2021). Pre-existing composition of the faecal microbiota has been shown to be associated with stress-sensitivity or stress-resilience in male Sprague-Dawley (SD) rats (Tanelian et al., 2022). Several mechanisms have been proposed by which stress-induced changes in the gut microbiota could affect emotional behaviour. Increased gut permeability and systemic inflammation have been shown to be associated with increased severity of depression symptoms (Maes et al., 2008; Maes et al., 2012). Both gut permeability and inflammation can be increased by stress (Ait-Belgnaoui et al., 2012; Demaude et al., 2006; Kiliaan et al., 1998; Meddings & Swain, 2000; Saunders et al., 1994; Soderholm et al., 2002). Therefore, it is plausible that individual differences in stress-induced changes in the gut microbiota composition and function and associated gut permeability and inflammation changes could contribute to individual differences in stress resilience (Bear et al., 2021).

Depression occurs twice as often in women as it does in men. There is high neurological, hormonal, and genetic variability between males and females and research in male rodents may not translate well to female humans (Beery & Zucker, 2011; Pearse & Young-Pearse, 2019). Some researchers have observed that female rodents are more vulnerable to developing anhedonia in the SPT, reduced open field activity, and decreased serotoninergic activity due to chronic mild stress (Dalla et al., 2005; Palanza, 2001).

3.1.1 Study Aims

The aim of the study was to set up a model of depression and anxiety in rats using UCMS for comparing stress-resilient and stress-sensitive rats, categorising the rats into three groups:

stress-resilient, stress-sensitive rats, and control rats (Figure 3.1). Following the UCMS exposure the caecal microbiota composition, caecal organic acid concentrations, and plasma cytokines and gut permeability marker lipopolysaccharide-binding protein (LBP) concentrations were compared among the groups.

Hypothesis 1: Caecal microbiota, caecal organic acids, plasma lipopolysaccharide-binding protein, and cytokines will differ between stress-resilient and stress-sensitive rats, and the values in the stress-resilient rats will be more similar to the control group.

Hypothesis 2: Anxiety-like and depressive-like behaviours will correlate with any or all of the caecal microbiota, caecal organic acids, plasma lipopolysaccharide-binding protein, and cytokines.

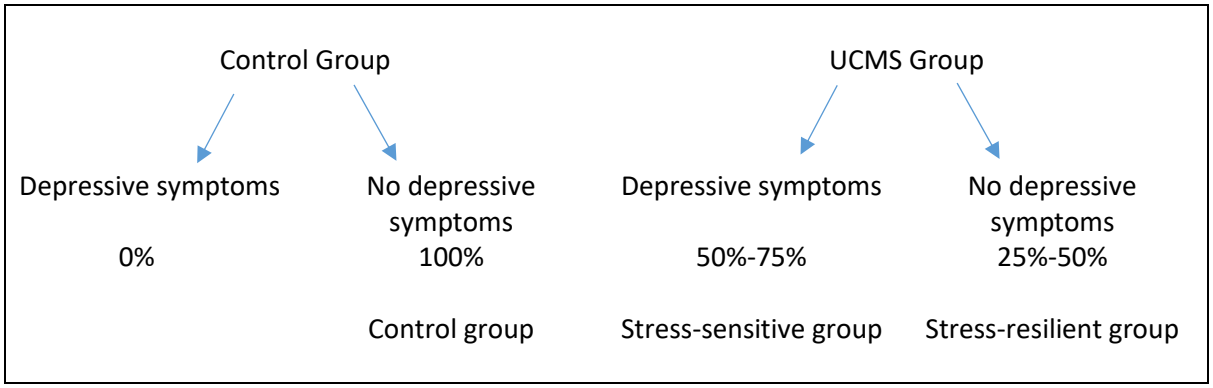

Figure 3.1. *Categories of stress-sensitive and stress-resilient following UCMS*

Depressive symptoms to identify the different groups was defined by a reduction in sucrose preference to around 50-60% in the sucrose preference test. Anticipated percentages of stress-sensitive and stress-resilient groups after unpredictable chronic mild stress based on results from other studies (Bergström et al., 2008; Strekalova et al., 2004; Willner, 2017).

3.2 Method

3.2.1 Design

The study was a two-by-two factorial quasi-experimental design with an experimental group (n=20) exposed to six weeks of chronic stress (UCMS group) and a control group (n=10) not exposed to stress (control group). The study had an experimental group exposed to six weeks of unpredictable chronic mild stress (UCMS group) and a group not exposed to stress (control group). The control group was kept in a separate room to ensure they did not experience any

stress by being in vicinity of experimental procedures. Behavioural tests were conducted throughout and at the end of the UCMS procedure (see Figure 3.2).

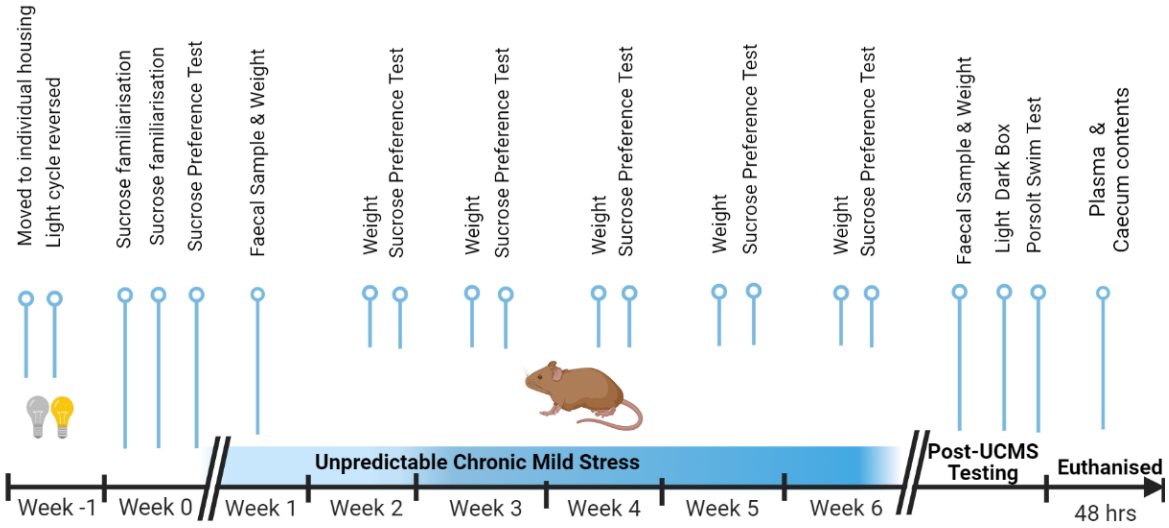

Figure 3.2. Timeline of the study.

3.2.2 Animals

Conventional female SD rats (n = 30) were obtained in house from the animal breeding facility at Plant and Food Research Institute, Palmerston North, New Zealand. The rats were weaned into family groups of six rats at three weeks of age. At seven weeks old, two weeks before the beginning of the study, they were separated into individual housing and the light cycle was reversed to a 12:12 h dark: light cycle (0700/1900 h) so that behavioural tests could be done in their active (dark) phase. They were individually housed in shoebox cages and provided with *ad libitum* food and water. Individual housing is stressful for rats, but it was needed to avoid sharing gut microbiota through coprophagy. The rats weighed 259g $\pm$ 19.4g at the beginning of the study. Procedures were approved by the Grasslands Animal Ethics Committee (approval code 14297) in accordance with the Animal Welfare Act 1999.

3.2.3 Power analysis

A power analysis was completed using the software G*power (version 3.1.9.4). Sucrose preference was the main variable. Based on previous research (Kelly et al., 2016), eight subjects were required per group for a 90% power, an alpha of 5%, and effect size of 0.7 (Cohen's d)

for a repeated measures ANOVA with six timepoints.. Unfortunately, UCMS does not produce the same resilience rate in all laboratories and rates range from 25-50% (Bergström et al., 2008; Strekalova et al., 2004; Willner, 2017). Thus, 16 rats would be needed in the UCMS group if a resilience rate of 50% occurred, and 32 rats if the resilience rate was 25%.

It was more difficult to obtain a power analysis for the other main variables of interest, changes in microbiota composition and organic acids. Comparison to other papers with stress-induced changes in the gut microbiota may not be relevant due to different microbiota compositions in rats in different laboratories. Comparison with microbiota studies from our laboratory was not useful because a stress study had not been done before. Several studies used a sample size of 10-14 rats per treatment for testing (Crumeyrolle-Arias et al., 2014; Fukui et al., 2018; Jašarević et al., 2015).

With these considerations, and for logistical reasons, 30 rats were chosen: 10 rats in the control group and 20 rats in the UCMS group. This sample size was predicted to be sufficient for a resilience rate of 50%.

3.2.4 Procedures

3.2.4.1 Unpredictable Chronic Mild Stress Procedure

Rats were divided into the UCMS and control groups with baseline sucrose preference counterbalanced between the two groups. The stress group was subjected to a UCMS protocol, slightly modified from Varga et al. (2017). Published UCMS protocols differ widely in the suite of stressors used and the intensity of induced stress (Antoniuk et al., 2019). The protocol chosen was intended to be relatively mild, so that it would be more likely that there would be subgroup who were resilient to the stressors. The stress protocol comprised 10 to 12-hour periods of stressors which were rotated in varying order throughout the six weeks. To prevent sharing of faecal microbiota through coprophagia, cage crowding (4-6 rats per cage) was removed from the protocol. It was replaced with individual housing in cages with wire bases, under which used bedding from unfamiliar rats was placed, to provide an olfactory stress. The other stressors were wet bedding (250 mL water poured into bedding), stroboscopic lighting, intermittent lighting (lights on and off every two hours), and loud continuous noise (untuned radio at 90 dB), and cage tilt (45^0). All stressors selected are commonly used in published UCMS protocols (Antoniuk et al., 2019). The control group was exposed only to

normal laboratory practice, with cage enrichment toys rotated every few days, other than weekly SPT, weight and coat state measurement.

3.2.4.2 Sucrose Preference Test

The method used was a 24 hour, two-bottle SPT, using the method of Kelly et al. (2016). In the two-bottle test, one bottle of water and one bottle of sucrose solution were provided. The bottles were stored inverted in the room for at least 6 hours before each test to ensure they were not dripping, and that the solution was at room temperature. The bottles were weighed at the beginning of the test, at 12 hours, and after 24 hours. The side of the cage the bottles were on was switched after 12 hours to reduce effects from side preference. Sucrose preference was defined using the equation: sucrose preference = (sucrose solution consumed / total fluid consumed) x 100 %. The rats were habituated to the taste of sucrose solution by being given two bottles of 1 % sucrose solution for 6 hours, two days before the baseline SPT was undertaken, to prevent neophobia (fear of new food) during the SPTs.

3.2.4.3 Coat State Measurement

Coat state was measured using the method of Ibarguen-Vargas et al. (2008) with seven different body areas scored 0 for groomed coat or 1 for unkempt coat, with the sum of these scores as the coat state measurement. Measurements were taken once before the beginning of the UCMS protocol and at weekly weighing, for six weeks in total.

3.2.4.4 Light-Dark Box

Rats were tested in opaque plastic boxes (50 cm x 50 cm x 50 cm) with black plastic lidded inserts (25 cm x 50 cm x 50 cm). The compartments were connected by a small opening (10 cm x 10 cm). Testing began when the rats were placed in the dark compartment, and the lid was closed. Tests ran for 5 minutes and were filmed. Behavioural measures were manually recorded by a blinded researcher using the footage. The behavioural measures recorded were time spent in the light compartment, latency to enter the light compartment, number of transitions between compartments, number of head pokes from the dark compartment into the light compartment, and time spent in head poking, number of stretch-attend postures from the dark compartment into the light compartment, and time spent stretching, and number of rears and time spent rearing in the light compartment.

3.2.4.5 Porsolt swim test

To measure stress-induced coping behaviours, the PST was performed. The modified PST method (Slattery & Cryan, 2012) was used. However, the method was further altered. On both the habituation day and the following test day, the rats were placed into tanks (50 cm tall and 22 cm in diameter) filled with 30 cm of water at 25 °C. After the PST each day, the rats were removed, dried, and placed back in their home cage. Some rats sank under the water surface at around five minutes on the habituation day, so the habituation time was cut to five minutes for all rats. The test day was also five minutes, and the rats were filmed from the side of the cage. Behaviours were categorised using the footage by a treatment blinded researcher. Swimming, climbing and immobile behaviour were categorised as outlined in Slattery and Cryan (2012). Immobility behaviour is defined as the rat floating in the water and making minimal movements. Swimming behaviour is when the rat is actively moving horizontally around the cylinder with forward momentum. Climbing behaviour is an upward directed movement, often against the side of the cylinder. Diving is when the rat intentionally dives to the bottom of the cylinder and comes back up. Latency to immobility was defined as the first time that immobility behaviour was maintained for $\geq$ 2 seconds as per Kelly et al. (2016).

3.2.4.6 Sample collection

Faecal samples were collected at week 1 and week 6 by placing the rats into clean cages and collecting the first two faecal pellets. They were collected in the morning. If they did not defecate within 5 minutes, their tails were gently lifted to induce defecation. Pellets were collected immediately, placed in sterile tubes and frozen at -80 °C until analysis. Rats were euthanised using CO_2 gas 48 hours after the last behavioural test (PST). Blood was withdrawn using cardiac puncture into a lithium heparin vacuum tube. The tubes were centrifuged, and plasma was separated into aliquots and stored at -80 °C until analysis. Caecal digesta was collected into sterile tubes, snap-frozen in dry ice and then stored at -80 °C until analysis.

3.2.4.7 Organic acid Analysis

Caecum organic acids (formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, hexanoate, heptanoate, lactate, and succinate) were measured. Caecum samples were diluted 5 x with phosphate-buffered saline containing ethyl butyric acid as an internal standard, allowed to defrost and homogenised. They were then centrifuged at 10,000 g for 10 minutes, and an aliquot of 500 μL of the supernatant was removed, and 250 μL HCL and 1000

μL diethyl ether was added. The diethyl ether phase was stored at -80 °C until analysis on gas chromatographer (GC). Samples were again homogenised and centrifuged at 10,000 g for 5 minutes. Next, 100 μL of the upper ether supernatant layer was removed, and 20 μL of derivatising agent (N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA)) was added, and the sample was stored at 80 °C for 20 minutes, and then room temperature for 48 hours. In a capped GC vial, 100 μL of the diethyl ether phase was derivatised with 20 μL MTBSTFA with 1 % tert-butyldimethylchlorosilane (MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) by heating to 80 °C in a water bath for 20 minutes. The samples were left for 48 hours at room temperature before analysis to allow complete derivatisation. Standards containing 2-ethylbutyric acid (5 mM) as an internal standard were prepared for derivatisation alongside the samples.

Analysis was performed on a Shimadzu capillary GC system (GC-2010 Plus, Tokyo, Japan) equipped with a flame ionisation detector (FID) and fitted with a Restek column (SH-Rtx-1, 30 m × 0.25 mm ID × 0.25 μm) (Shimadzu, USA). The carrier gas was helium with a total flow rate of 21.2 mL/minute and a pressure of 131.2 kPa. Makeup gas was nitrogen. The temperature program began at 70 °C, increasing to 115 °C at 6 °C/minute, with a final increase to 300 °C at 60 °C/minute, holding for 3 minutes. Flow control mode was set to linear velocity; 37.5 cm/second. The injector temperature was 260 °C, and the detector temperature was 310 °C. Samples were injected (1 μL) with a split injection (split ratio: 10). The GC instrument was controlled and data processed using Shimadzu GC Work Station LabSolutions Version 5.3. Organic acid quantification was assessed using the peak areas for the formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, hexanoate, heptanoate, lactate, and succinate, relative to ethyl butyric acid. Data acquired provided a final sample result of μmol organic acid/g wet digesta (caecum).

3.2.4.8 Characterisation of the Faecal Microbiota Composition

Overall bacterial community composition of the caecum digesta was analysed by 16S rRNA gene sequencing. Following preparation of the caecum digesta for organic acids, 25 g of the pellet was used for 16S rRNA extraction using the Qiagen DNeasy Powersoil Kit isolation kit (MoBio Laboratories, Carlsbad, CA, USA) in a final volume of 50 μL. Modifications to the manufacturer's instructions included subjecting the samples to 3 × 90-second bead beating

cycles on the FastPrep-24™ 5G (MP Biomedicals, Seven Hills, Australia) at 5.5 m/s with 5 minutes ice rests between cycles. DNA quality and quantity were assessed using a Nanodrop spectrophotometer (ND-1000, ThermoFisher).

The samples were sent for 16S RNA sequencing at Massey Genome Service (Massey University, Palmerston North, New Zealand), where a PCR was run to amplify variable regions V3-V4 of the 16S rRNA gene using barcoded fusion primers 16SF_V3 (5′-AATGATACGGCGACCACCGAGATCTACAC-barcode-TATGGTAATTGGCCTACGGGAGGCAGCAG -3′) and 16SR_V4 (5′-CAAGCAGAAGACGGCATACGAGAT-barcode-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) (Klindworth et al., 2013), which also contain adaptors for downstream Illumina MiSeq sequencing. Each sample was amplified with a pair of unique (8 bases) barcoded primers.

The PCR conditions used were a hold at 95 °C for 2 minutes followed by 30 cycles of 95 °C for 20 seconds, 55 °C for 15 seconds, 72 °C for 5 minutes finishing with a hold at 72 °C for 10 minutes. PCR reagents were Invitrogen AccuPrime™ Pfx SuperMix (Cat—12344-040) (17 μL), 10 μM 16SR_V4 Primer (1 μL), 10 μM 16SF_V3 Primer (1 μL) and 1 μL normalised sample (5 ng/μL). The PCR library clean-up kit used was an Invitrogen SequalPrep Normalisation Plate Kit (Thermo Fisher Scientific, Waltham, MA, USA). Eighteen μL of the PCR product was used in the library clean-up, and the elution volume was 12 μL. A Qubit DNA High Sensitivity assay was used to measure the library concentration, and a Bioanalyser DNA High Sensitivity assay was used for library sizing. The amplicons were pooled in equal molarity, and 16S rRNA gene sequencing was performed on an Illumina MiSeq 2 × 250 base paired-end run.

3.2.4.9 Faecal corticosteroid metabolites

Faecal corticosteroid metabolites are representative of serum corticosteroid from around 14-16 hours earlier and are commonly used as a non-invasive measure for adrenocortical activity in animals (Bamberg et al., 2001; Lepschy et al., 2007). It also avoids the confounding increase in corticosterone from the stress of acute sampling methods and is, therefore, a good way to measure baseline stress levels (Bamberg et al., 2001).

Corticosterone was extracted using the following method. First, faecal samples were defrosted and dried in a freeze drier at 4 °C for 12 hours and then crushed into powder using a metal

spatula. Next, absolute ethanol was added at a concentration of 1 mL ethanol per 0.1 g faecal material (dry weight) and shaken for 30 minutes at 800 mot/minute. Then they were centrifuged at 10,000 g for 15 minutes, and the supernatant was removed and frozen at -80 °C. Faecal corticosterone concentrations in the supernatant were analysed in duplicate using an Eliza kit (ENZO AD1-901-071, Enzo Life Sciences) according to the manufacturer's instructions using a 1:500 dilution of the extracted corticosterone solution. The coefficient of variation (CV %) was accepted at less than 10. Calculated values are reported as nanograms of corticosterone per millilitre.

3.2.4.10 Plasma cytokines and lipopolysaccharide binding protein

Plasma was thawed at room temperature (18 °C). Plasma cytokines interferon-gamma (IFN-γ), cytokine-induced neutrophil chemoattractant type-1 (CINC-1), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-18, IL-12p70, IL-1β, IL-17A, IL-33, IL-1α, and IL-6. The cytokines were measured in duplicate by bead-based LEGENDplex assay (Biolegend 740401) in accordance with the manufacturer's instructions. A dilution of 1:4 was used. Analyses were performed using a BD FACSverse flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analysed via Legendplex V8.0 software (BioLegend) and are presented as pg/mL.

The concentration of LBP was measured in duplicate in plasma using an ELISA kit (ENZO, Cat. #ALX-850-305) in accordance with to the manufacturer's instructions. A dilution of 1:20 was used. Absorbance was measured at 450 nm using FLUOStar Optima® (BMG Labtech, Victoria, Australia). The coefficient of variation (CV %) was accepted at less than 10. Calculated values are reported as nanograms of LBP per millilitre.

3.2.5 Statistical Analysis

Weight, faecal corticosterone, coat state, sucrose preference, and sucrose intake were compared between the UCMS and control groups using two-way factorial repeated measure ANOVAs, with time and group as the independent variables. Sucrose preference scores and faecal corticosterone concentrations were log-transformed before analysis to stabilise the variance. Normality was determined with a visual inspection of residual plots. Differences between individual time points were compared using least significant differences (LSD) at a

level of 5 %. Results of the PST, plasma cytokine concentrations and caecal organic acid concentrations were compared between the UCMS and control groups using a two-tailed independent t-test with a probability value of 5 %. These analyses were conducted using the statistical software package GENSTAT version 19.1.0.21390) (VSN International Ltd).

The microbial sequence data analysis was done using QIIME 2, version 2019.10 (Bolyen et al., 2018). The sequences were quality checked, denoised and chimeric sequences removed by DADA2 step (Callahan et al., 2016) (trimmed by removing 10 bases from the sequences and truncating at base number 235). This output had a total of 572,949 reads and 1685 features (taxa) for the 30 samples, with a minimum frequency (read) of 12,463. Data were used for diversity analysis using phyloseq, with the Bray-Curtis, weighted and unweighted Unifrac distances methods with no rarefaction, and seed set at 12,000 (McMurdie & Holmes, 2014) that was conducted using R (R Core Team, 2015)(version 3.5.0 (2018-04-23)". The taxonomy associated with the SILVA reference database was used (Yilmaz et al., 2014) (v132, 99%, released on Dec 13, 2017). For differential abundance analysis, the operational taxonomic units (OTUs) that have a frequency of less than 0.1 % of the mean sample depth were removed to account for possible Illumina sequencing errors, and taxa not seen more than two times in 10% samples to remove OTU with small mean and large CV were filtered out. Taxa were analysed for differential abundance using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) method using the statistical programme R (Lin & Peddada, 2020).

For partial least squares (PLS) regression, variables with 50 % or more values at a lower or upper threshold value were removed (zero count for microbiota, before conversion to relative abundance; no time spent in behaviour during behavioural tests; or below the detection limit for cytokines, LBP, and organic acids). In addition, variables with a skewness greater than 1.8 or kurtosis great than 3.8 were also removed. PLS regression analysis was firstly undertaken to determine whether there was any relationship between behaviour and physiological variables at the population level. The PLS was run with behaviour as the independent variable, and microbiota relative abundance; plasma cytokines; LBP; and SCFA concentrations as the dependant variables.

Secondly PLS regression was run to determine if there were any population level relationships between microbiota, SCFAs, plasma cytokines, and LBP, and therefore was run with microbiota relative abundance as the independent variable.

Scatterplots were visually checked for a correlational relationship throughout both groups. Correlations were undertaken using Spearman's Rank Coefficient which is less sensitive to outliers. Due to the large number of comparisons, a probability level of 1 % was used, and correction for a false discovery rate of 5 % was done using the Benjamini-Hochberg procedure. All statistics were completed using the statistical software package GENSTAT (version 19.1.0.21390, VSN International Ltd).

3.3 Results

3.3.1 Sucrose Preference Test

Sucrose preference (Figure 3.3a) did not significantly change over time ($F(5,139) = 1.16$, $p = .331$), and there was no difference in sucrose preference between the UCMS and control groups ($F(1,28) = .05$, $p = .826$). Sucrose intake (Figure 3.3b) however, was higher overall in the UCMS group, ($F(1, 28) = 5.75$, $p = .023$). Post hoc analysis using LSD showed this was driven by a significant difference between groups in weeks 4-6. Due to an increase over time in the UCMS group but not in the control group, there was a significant effect of time ($F(5,139) = 9.81$, $p < .001$), and a significant interaction between time and UCMS ($F(5,138) = 4.13$, $p = .004$). Adjusting for weight did not significantly affect the result using analysis of covariance ($F(1,27) = 5.54$, $p = .026$).

3.3.2 Light-Dark Box Test

None of the light-dark box test measures showed significant differences between the UCMS and control groups. However, there was a pattern of decreased behavioural inhibition (decreased anxiety-like behaviour) in the UCMS group. This was shown by non-significant increases in time in the light-box, rearing and stretch-attend posture, as well as decreases in latency to the first transition and head pokes (Figure 3.3 c-d).

3.3.3 Porsolt Swim Test

Results showed no difference between the control and UCMS groups in either immobility ($t(28) = .39$, $p = .700$), swimming ($t(28) = .26$, $p = .798$), climbing ($t(28) = .78$, $p = .442$), or faecal output ($t(28) = .98$, $p = .334$) (Figure 3.3e).

3.3.4 Coat State

There was no significant difference in coat state scores between the UCMS group and the control group ($F(1, 28) = 1.63$, $p = .212$). However, there was a significant effect of time on coat state scores ($F(5, 5) = 11.96$, $p < .001$), with coat state scores decreasing in both groups in weeks 1-3, after the instigation of the UCMS. There was also a treatment x time interaction ($F(5, 5) = 5.73$, $p < .001$) with the pattern of change differing, particularly in week five. Coat state scores are shown in Figure 3.3f.

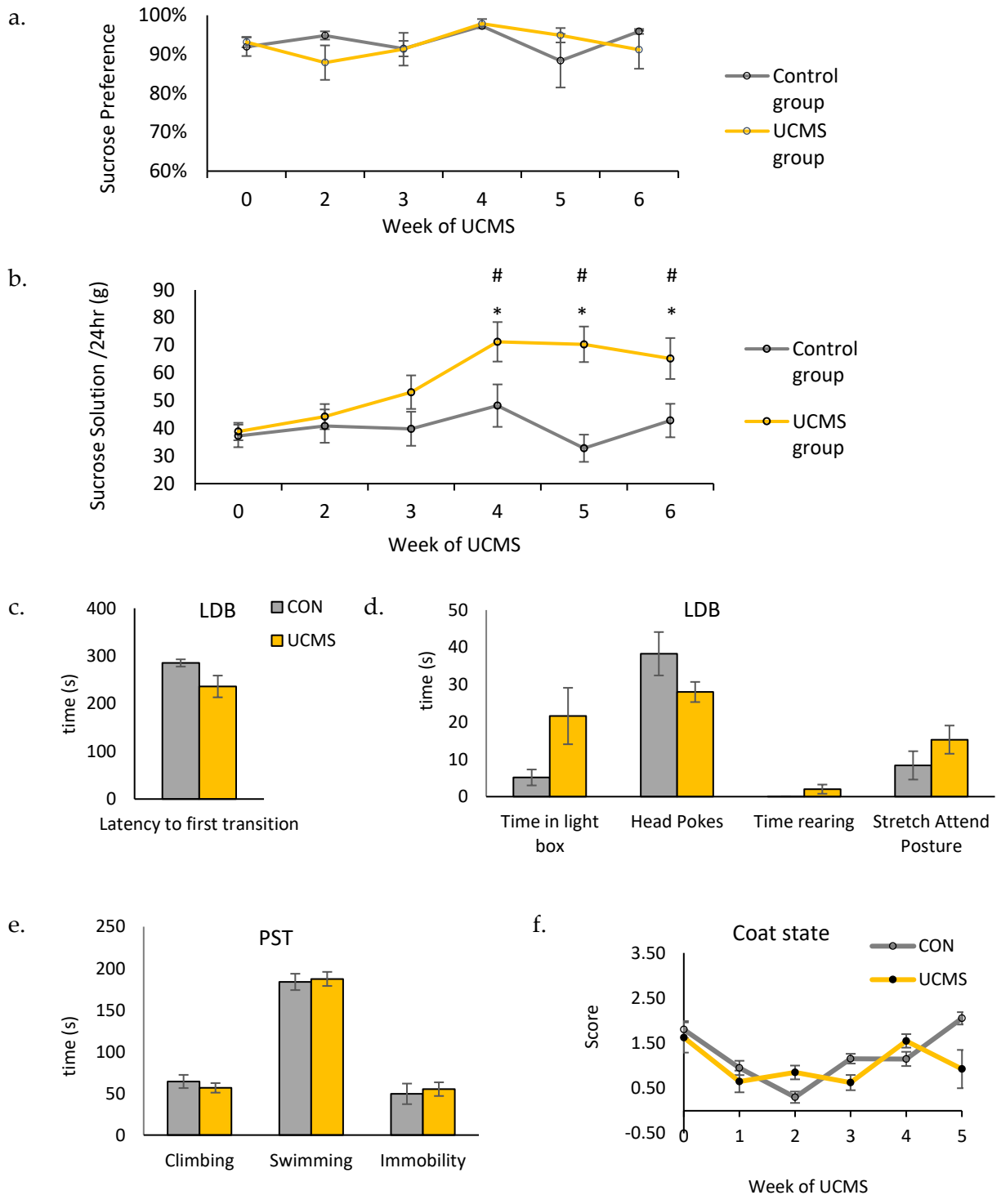

Figure 3.3. Results of behavioural testing. *In Sprague-Dawley rats during and after the six weeks of unpredictable chronic mild stress (UCMS) and control rats, sucrose preference testing prior to and over the UCMS period using the 24-hour sucrose preference test was not altered by UCMS (a), but total sucrose solution intake increased in the UCMS group (b). No significant differences in individual scores in the light-dark box test were found (c and d), but there was an overall pattern of decreased behavioural inhibition in the UCMS group. No differences were found in the Porsolt swim test or coat state test between groups (e and f). Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20 .*P<.05 between UCMS group and control group. #P<.05 between time-point and baseline.*

3.3.5 Weight

There was no difference in weight between groups at the beginning of the study. Weight showed an effect of time, as both groups increased in weight over the six weeks of the study ($F(6, 168) = 29.6$, $p < .001$). There was no overall treatment effect on weight ($F(1,28) = .31$, $p = .580$), nor a treatment x time interaction ($F(6,168) = .4$, $p = .670$).

3.3.6 Faecal Corticosterone

Faecal corticosterone (Figure 3.4b) differed between the UCMS group and the control group ($F(1,28) = 21.40$, $p < .001$). There was also a significant effect of time $F(1,28) = 9.10$, $p = .005$), and a time x intervention interaction $F(1,28) = 4.88$, $p = .035$). Post hoc testing using LSD (5%) showed the lower faecal corticosterone in the UCMS group in the first week of the intervention period. In the sixth week of the intervention there was no longer a significant difference between groups. The faecal corticosterone concentrations of the UCMS group increased from week one to week six, and there was no difference over time in the control group.

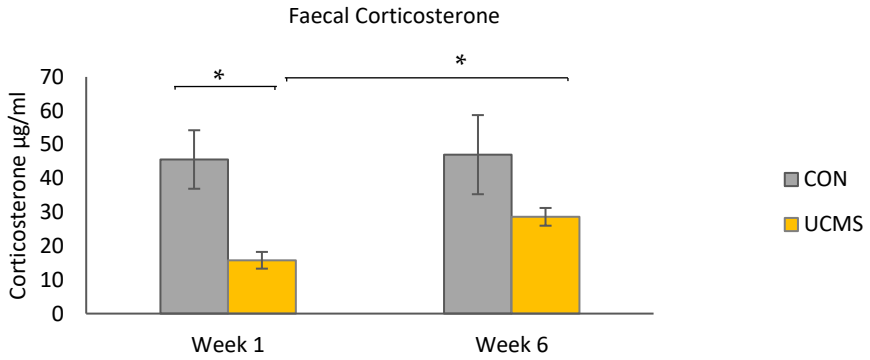

Figure 3.4. F*aecal corticosterone concentrations in Sprague-Dawley rats over the six weeks of unpredictable chronic mild stress (UCMS) and control rats. Comparison with ANOVA showed no difference between groups in weight. ANOVA and post hoc testing with least significant difference (5%) showed UCMS to have a lower faecal corticosterone concentration than the control group in week one, and to increase over time. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20*

3.3.7 Plasma Lipopolysaccharide-Binding Protein

There was no difference in plasma LBP between the UCMS group (mean 0.273 $\pm$ 0.013 SEM) and control group (0.244 $\pm$ 0.019 SEM).

3.3.8 Cytokines

There was an overall pattern of increased plasma cytokine concentrations in the UCMS group compared with the control group (Figure 3.5b-l). Significantly increased cytokines were CINC-1 ($t(28) = 2.25$, $p = .033$), and GMC-CSF ($t(28) = 2.67$, $p = .013$). IL-1β was below the detection limit in 29 out of 30 samples.

3.3.9 Organic Acids

No difference was found in concentration in individual organic acids between the control and UCMS groups (Table 3.1).

Table 3.1. Organic acid concentrations in caecal digesta. There was no difference between concentrations in female Sprague-Dawley rats following six weeks of unpredictable chronic mild stress (UCMS) or the unexposed control group. Concentrations are in µmol Organic Acid/g wet weight. Mean and standard error reported.

	Control			UCMS		
Organic acid	Mean		SEM	Mean		SEM
Formate	0.30	+	0.00	0.30	+	0.00
Acetate	80.17	+	6.28	79.26	+	2.75
Propionate	10.92	+	0.54	9.68	+	0.34
Isobutyrate	0.52	+	0.07	0.44	+	0.04
Butyrate	31.64	+	3.34	34.44	+	1.38
Isovalerate	0.26	+	0.05	0.22	+	0.02
Valerate	1.08	+	0.03	1.02	+	0.03
Hexanoate	1.41	+	0.11	1.51	+	0.08
Heptanoate	0.10	+	0.00	0.10	+	0.00
Lactate	0.33	+	0.08	0.31	+	0.03
Succinate	0.38	+	0.11	0.30	+	0.02

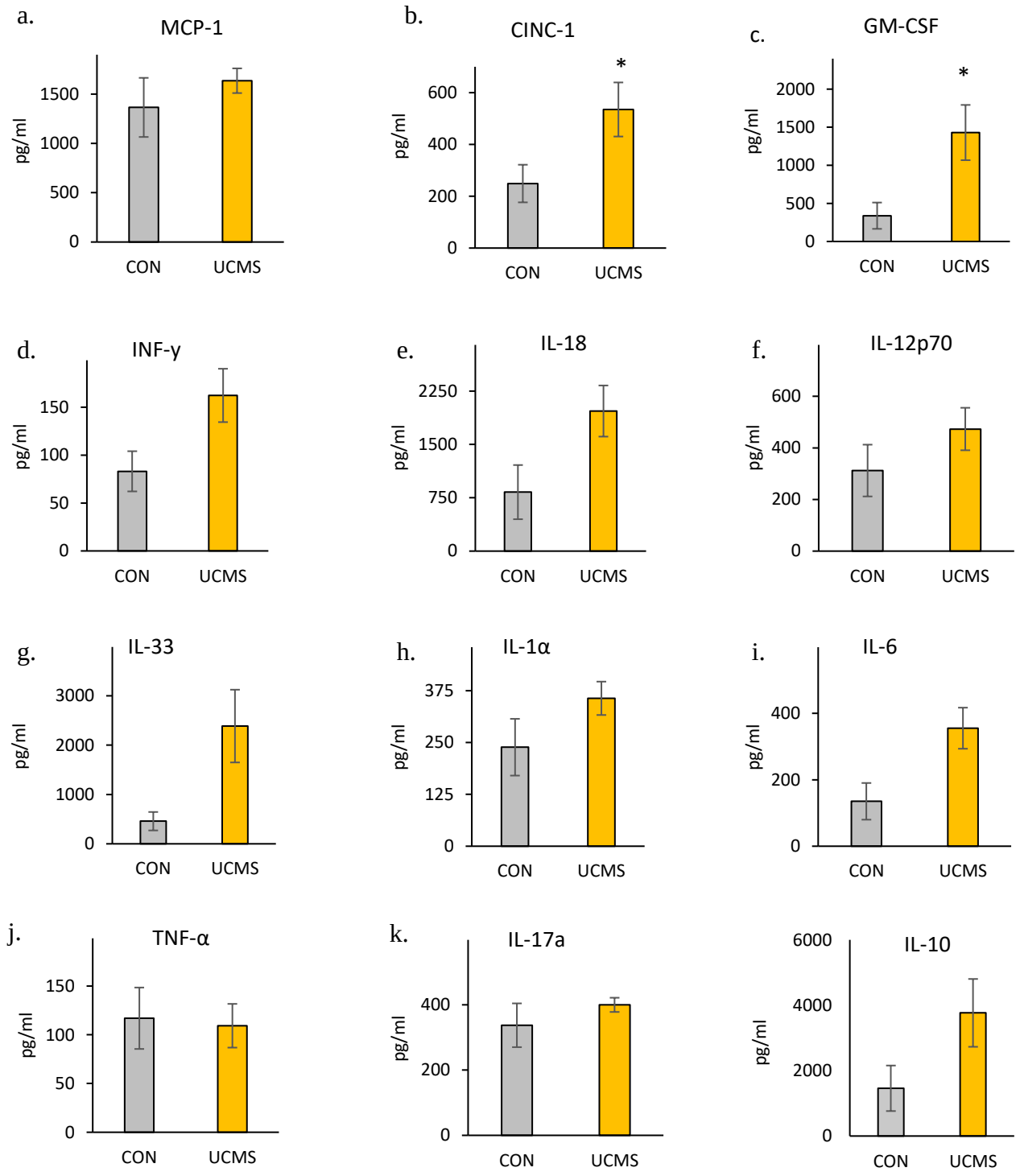

Figure 3.5. *Plasma cytokine concentrations. In female Sprague-Dawley rats, following six weeks of unpredictable chronic mild stress (UCMS), a pattern of increased pro- and anti-inflammatory markers was observed compared with the control group (CON). * indicates a significantly different value from the control group at P<.05. Cytokines include interferon-gamma (IFN-γ), cytokine-induced neutrophil chemoattractant type-1 (CINC-1), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-18, IL-12p70, IL-1ß, IL-17A, IL-33, IL-1α, and IL-6. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20*

3.3.10 Caecal Microbiota Composition

Alpha-diversity (Observed, Chao1, Shannon, and Simpson) was not significantly different between the UCMS group and the control group (Figure 3.6). Beta-diversity differed between the UCMS and control groups (Figure 3.7) when assessed using the Bray-Curtis distance, weighted and unweighted Unifrac distances.

Several taxa differed between the UCMS and control groups (Figure 3.8). The phylum *Cyanobacteria* (w = 2.536, p = .011) showed a lower relative abundance, and the phylum Patescibacteria (w = 3.318, p = .001) showed a higher relative abundance in the UCMS group compared with the control group. The relative abundance of the families *Saccharimonadaceae* (w = 2.986, p = .003) and *Rikenellaceae* (w = 3.029, p = .002), and that of the species *Lachnospiraceae bacterium* DW59 (w = 4.570, p < .001) were also higher in the UCMS group. There were no changes for any genera. Species within *Lactobacillus* and *Bifidobacterium* genera with known association with emotional behaviour showed no significant difference between groups.

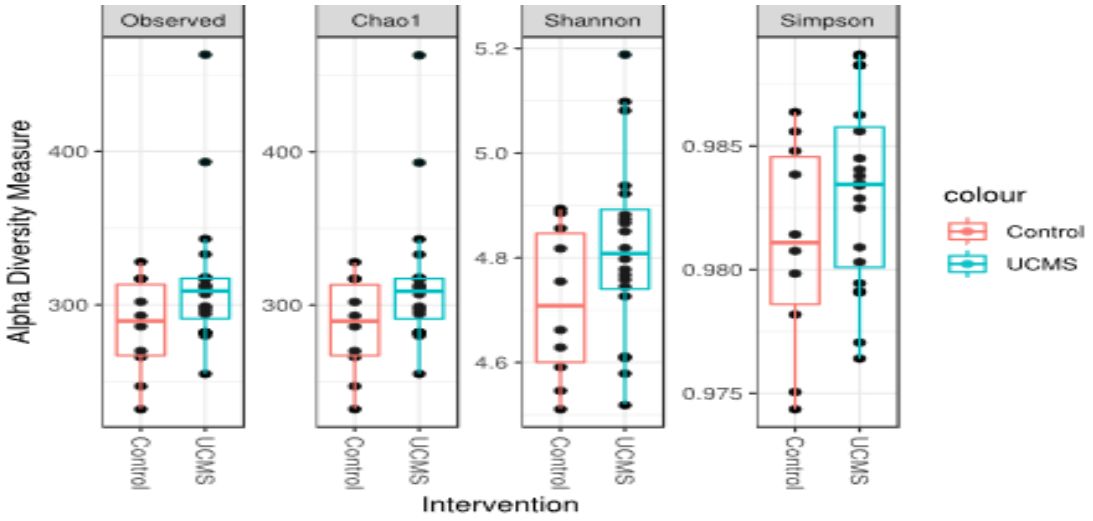

***Figure 3.6**. Alpha-diversity (measured by four indexes: Observed, Chao1, Shannon, and Simpson) of the caecal microbiota in Sprague-Dawley rats after six week exposure to unpredictable chronic mild stress (UCMS) and control rats. No significant difference was found between stress-exposed group (UCMS group) and the control group. Data are presented as median +/- 1 SD, control group n = 10, UCMS group n=20*

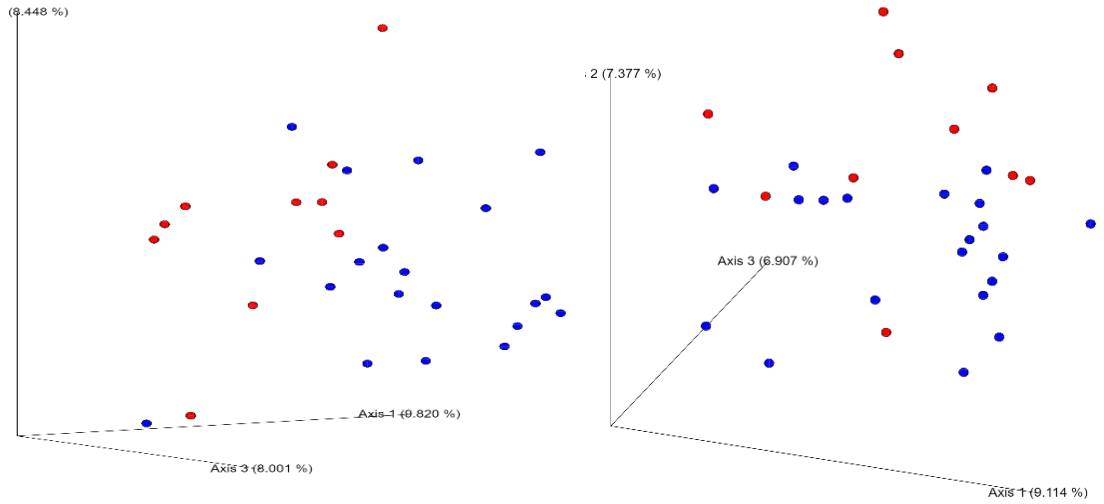

***Figure 3.7**. Beta-diversity PCA plots of the caecal microbiota in Sprague-Dawley rats after six-week exposure to unpredictable chronic mild stress (shown in blue) and control rats (shown in red).PCA plots show Bray-Curtis distance (a), weighted unifrac distances (b), and unweighted unifrac distances (c). Significance level is p = .001*

3.3.11 Assessment of Stress-Resilience

The SPT is the typical measure used to define stress sensitivity. None of the rats exposed to the UCMS protocol developed lowered sucrose preference (anhedonia), and therefore they could not be classified as stress-resilient or stress-sensitive.

A factor analysis was considered for the behavioural results to classify the meaning of increased sucrose intake in the context of the other behaviours. However, a Kaiser Mayer Olkin test (KMO = .373) and Bartlett's Sphericity test ($\chi 2$ (55) = 227.43 $p < .001$) showed the behavioural data were unsuitable for factor analysis due to high covariance. Instead, a Spearmans rank non-parametric correlation matrix was created between all behavioural variables. No significant correlations were observed between sucrose intake and any other measures. Because of the lack of clear meaning of sucrose intake, it is invalid to use it to classify stress-resilient and stress-sensitive groups. Instead, the results were compared using the multi-variate regression model partial least squares, which groups both the dependent and independent variables according to covariance. This analysis allowed any associations between patterns of variables, such as high or low sucrose intake, and other variables to be identified. This analysis works well for the explanatory variables caecal organic acids, caecal

microbiota and plasma cytokines, as any covariant groups which are associated with the dependent variables will be identified.

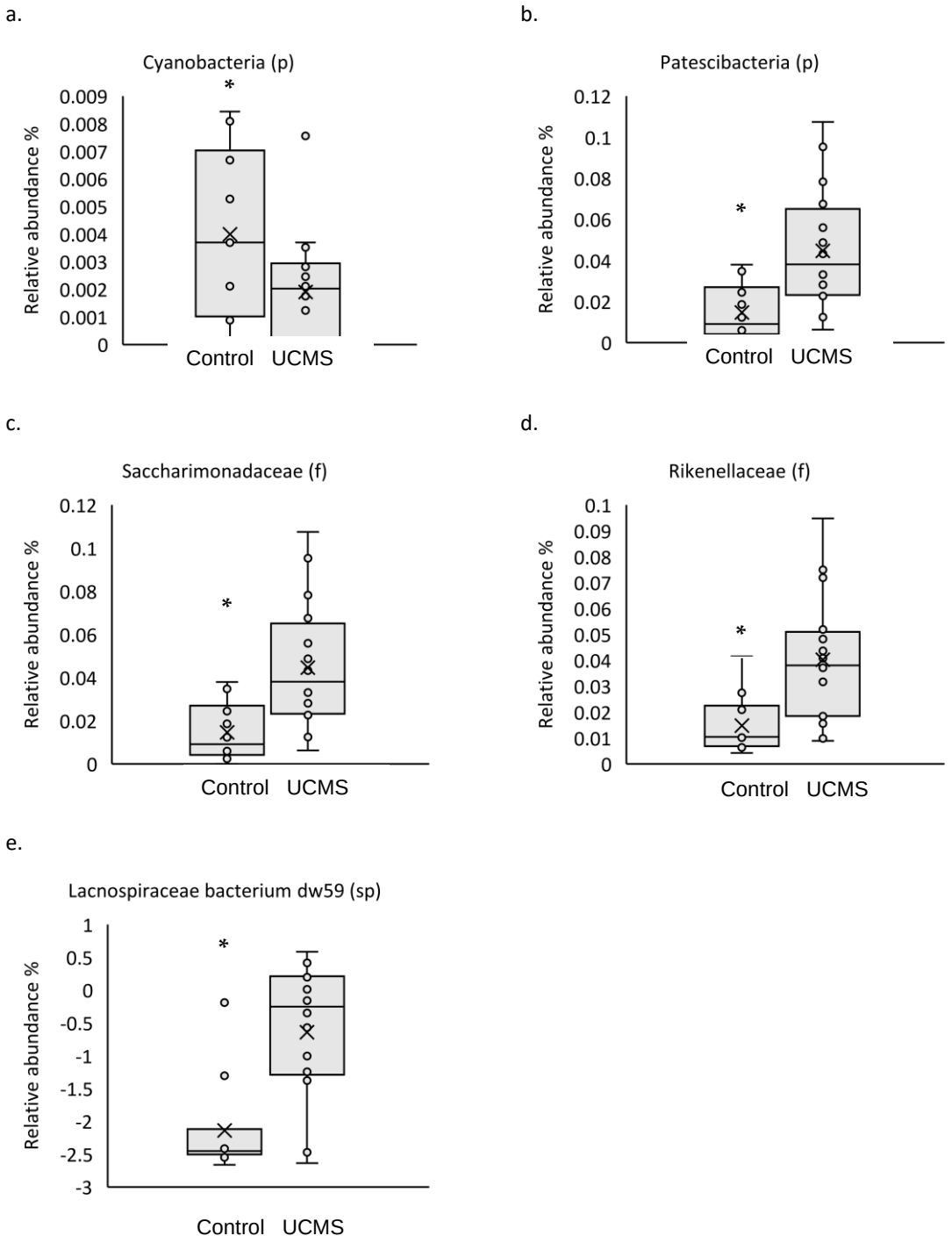

***Figure 3.8**. Relative abundance of caecal microbiota taxa identified by 16S next gene sequencing. Female Sprague-Dawley rats exposed to six weeks of unpredictable chronic mild stress (UCMS) showed differences in several taxa (a-e) compared with the control group. Box and whisker plots show relative abundance of the microbial taxa for each rat, with the minimum value, first quartile, median, third quartile and maximum value of each group shown. p = phylum, f = family, sp = species. * indicates significant difference at p value <.05. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20*

3.3.12 Associations between Behaviour and Physiological Measurements

Measurements were compared using Spearman's rank correlation and partial least squares multiple regression to identify any associations between variables. A visual analysis of scatterplots followed this analysis.

3.3.12.1 Associations between Behaviour and Caecal Microbiota and Corticosterone, Caecal Organic Acids, Plasma Cytokines and Lipopolysaccharide Binding Protein

Behavioural variable scores from all rats were compared with concentrations of caecal organic acids, plasma cytokines, faecal corticosterone, and plasma LBP, and with the relative abundance of caecal microbiota at the species level. Over 200 correlations were identified (Appendix A, Table A.1,) between behaviour and organic acids, cytokines, and caecal microbiota. However, when the significance p value was corrected for a false discovery rate of 5%, just five correlations remained (Table 3.2). When the values from all the rats (regardless of group) were used, an unclassified species from the family *Family XIII* was significantly, negatively correlated with two behaviours in the light-dark box test: time in the light box (see Figure 3.10a) and number of transitions (see Figure 3.10b). An unspecified species in the *Ruminococcus* genus also correlated negatively with the number of faecal pellets excreted in the PST (see Figure 3.10c).

When the correlation analysis was repeated with only the control or UCMS group, a negative correlation was identified in the UCMS group between an unspecified bacterium in the species *Lachnospiraceae NK4A136 group* and time spent in stretch-attend posture (Figure 3.10c). A positive correlation was observed in the UCMS group between the number of head pokes in the light-dark box test and the species *Oscillibacter sp. 1-3* (Figure 3.10d). All the bacteria which were found to correlate with behaviour were in the order *Clostridiales* of the Firmicutes phylum.

No relationship between behaviours and any biological variables was found with PLS regression analysis. Significance testing of dimensions using Osten's F-test showed the predicted residual sum of squares (PRESS) for the PLS models was higher than the original data, indicating over-fitting. This result indicates that there was not a population level association between these variables.

3.3.12.2 Associations between Caecal Microbiota, Gut Permeability Index and Inflammation Markers

One dimension was identified using partial least squares regression, but significance testing of the dimension using 10 cross-validation groups showed inconsistent results when run multiple times. This result suggests that there may be some individual relationships but not a strong relationship overall between the caecal microbiota, plasma LBP, and plasma cytokines. An arrow biplot from the partial least squares correlation is shown in Figure 3.9. Of the taxa which showed a significant correlation with cytokines, Taxa 51 (unspecified species in the genus *Acetatifactor*), Taxa 90 (unspecified species in the family *Lachnospiraceae)* and Taxa 122 (unspecified species in the genus *Ruminococcaceae NK4A214 group)* show the strongest loadings in similar directions (positive correlation) to plasma cytokines.

Analysis of individual variable using Spearman's rank correlation showed many correlations between LBP and the caecal microbiota, between plasma cytokines and caecal organic acids, and between plasma cytokines and caecal microbiota, but not plasma LBP and cytokines (r values are presented in Appendix A, Table A.2). Following correction for a 5% FDR, several significant correlations remained between the caecal microbiota and plasma cytokines. Correlations r values are presented in Table 3.2.

Strong positive correlations were found in the control group between an uncultured bacterium in the genus *Ruminiclostridium group* (taxa 117) with plasma cytokines IL-6 and CINC-1 (Figure 3.10f-g). Moderate positive correlations were found in the UCMS group between and uncultured bacterium in the genus *Ruminococcaceae NK4A214 group* (taxa 122) with plasma IL-1α and MCP-1 (see Figure 3.10h-i). Taxa 122 also showed up strongly in PLS analysis. All the bacteria which positively correlated with plasma cytokines were in the order Clostridiales of the Firmucutes phylum.

Strong positive correlations were found in the control group between an uncultured bacterium in the *Desulfovibrionaceae* family of the Proteobacteria phylum and plasma cytokines GM-CSF, IL-1α, IL-6, IL-10, and IL-18 (Figure 3.10 j-n).

No significant correlations remained between plasma LBP and the caecal microbiota or plasma cytokines. This result was backed up by the PLS analysis which also showed a loading of LBP of close to zero on the dimension identified by PLS (Figure 3.9a).

3.3.12.3 Association between Organic acids, Gut Permeability and Inflammation markers

Correlation analysis was carried out to determine whether caecal organic acid concentrations may be linked with plasma inflammatory markers. One dimension was identified using PLS regression with SCFA as the independent variable, and was found to be significant using Osten's F-test ($F(5,145) = 4.75$, $p < .001$). An arrow plot (Figure 3.9b) showed butyrate to be loaded with similar directionality as plasma cytokines. The initial individual correlation analysis showed numerous weak correlations between caecal organic acids and plasma cytokines (Table A.2, Appendix A) but no individual significant correlations were identified following FDR correction.

No correlation was found between any caecal organic acids and plasma LBP even before correction with the FDR. This result is supported by a very weak loading of LBP in the identified dimension from PLS regression.

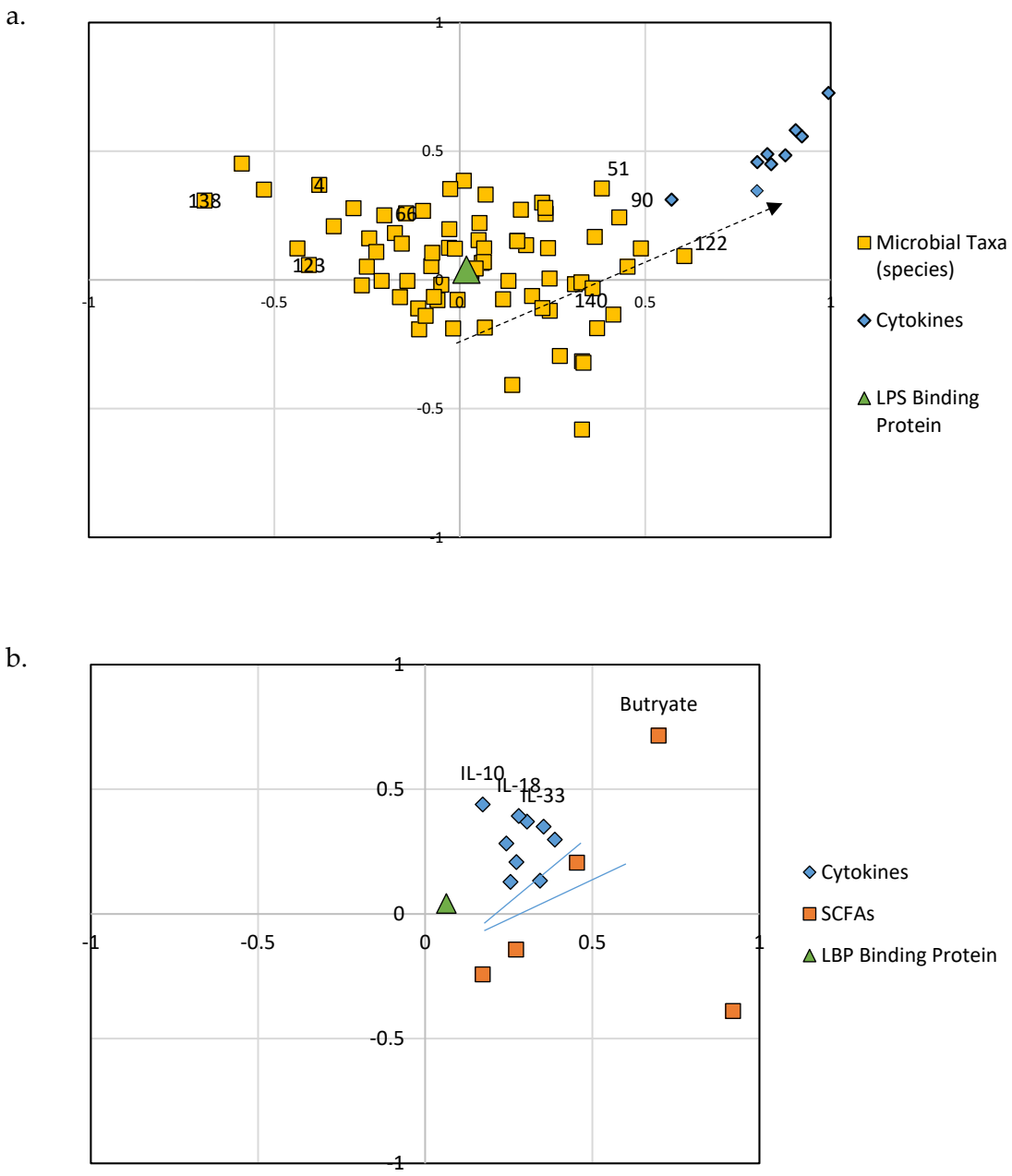

Figure 3.9. *Arrow Biplot*s of results from *Partial Least Squares correlation analysis* of *caecal microbiota, plasma cytokines, and plasma lipopolysaccharide (a), or plasma cytokines, plasma lipopolysaccharide and caecal organic acids. In a grouped analysis of female Sprague-Dawley rats exposed or unexposed to unpredictable chronic mild stress for four weeks (a), the biplot* shows a similar direction from the centre of cytokines with several taxa. These include taxa 51 (uncultured bacterium in *Acetatifactor genus); taxa 90 (Lachnospiraceae spp.); and taxa 122 (uncultured bacterium in Ruminococcaceae NK4A214 group genus), all in the Clostridiales order. A similar analysis shows a moderate relationship between caecal butyrate and several cytokines (b).*

Table 3.2. *Table of r values of faecal microbiota species which significantly correlated with behaviours or plasma cytokines. Correlation analysis was undertaken in female Sprague-Dawley rats, in those exposed to unpredictable chronic mild stress (UCMS), those kept as controls, and a third analysis with all rats combined. Significant correlations were those with p<.01 following correction for a false discovery rate of 5 %.*

Phylum	Class	Order	Family	Genus	Species		Time in the light box test	Number of Transitions in the LDB test	Faecal pellets expressed in the PST	Time in stretch attend posture	Number of head pokes	Cytokine GM-CSF	Cytokine IL-10	Cytokine IL-18	Cytokine IL-1α	Cytokine IL-6	Cytokine CINC-1	Cytokine MCP-1	Cytokine TNF-α
Firmicutes																			
	Clostridia																		
		Clostridiales																	
			Family XIII																
				Unspecified															
					Unspecified	Taxa 47	-0.63 (all)	-0.65 (all)											
			Lachnospiraceae																
				Lachnospiraceae NK4A136 group															
					Uncultured bacterium	Taxa 69				-0.77 (ucms)									
			Ruminococcaceae																
				Ruminiclostridium															
					Uncultured bacterium	Taxa 117										0.94 (con)	0.92 (con)		
				Oscillibacter															
					Oscillibacter sp. 1-3	Taxa 108					0.74 (ucms)								
				Ruminococcaceae NK4A214 group															
					Uncultured bacterium	Taxa 122									0.75 (ucms)			0.76 (ucms)	
				Ruminococcus															
					Unspecified	Taxa 142			-0.647 (all)										
Proteobacteria																			
	Deltaproteobacteria																		
		Desulfovibrionales																	
			Desulfovibrionaceae																
				Uncultured															
					Uncultured bacterium	Taxa 164						0.93 (con)	0.93 (con)	0.95 (con)	0.95 (con)	0.92(con)			

Note: GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL; Interleukin; CINC-1, Cytokine-induced neutrophil chemoattractant 1; MCP-1, Monocyte Chemoattractant Protein-1; TNF, Tumour Necrosis Factor.

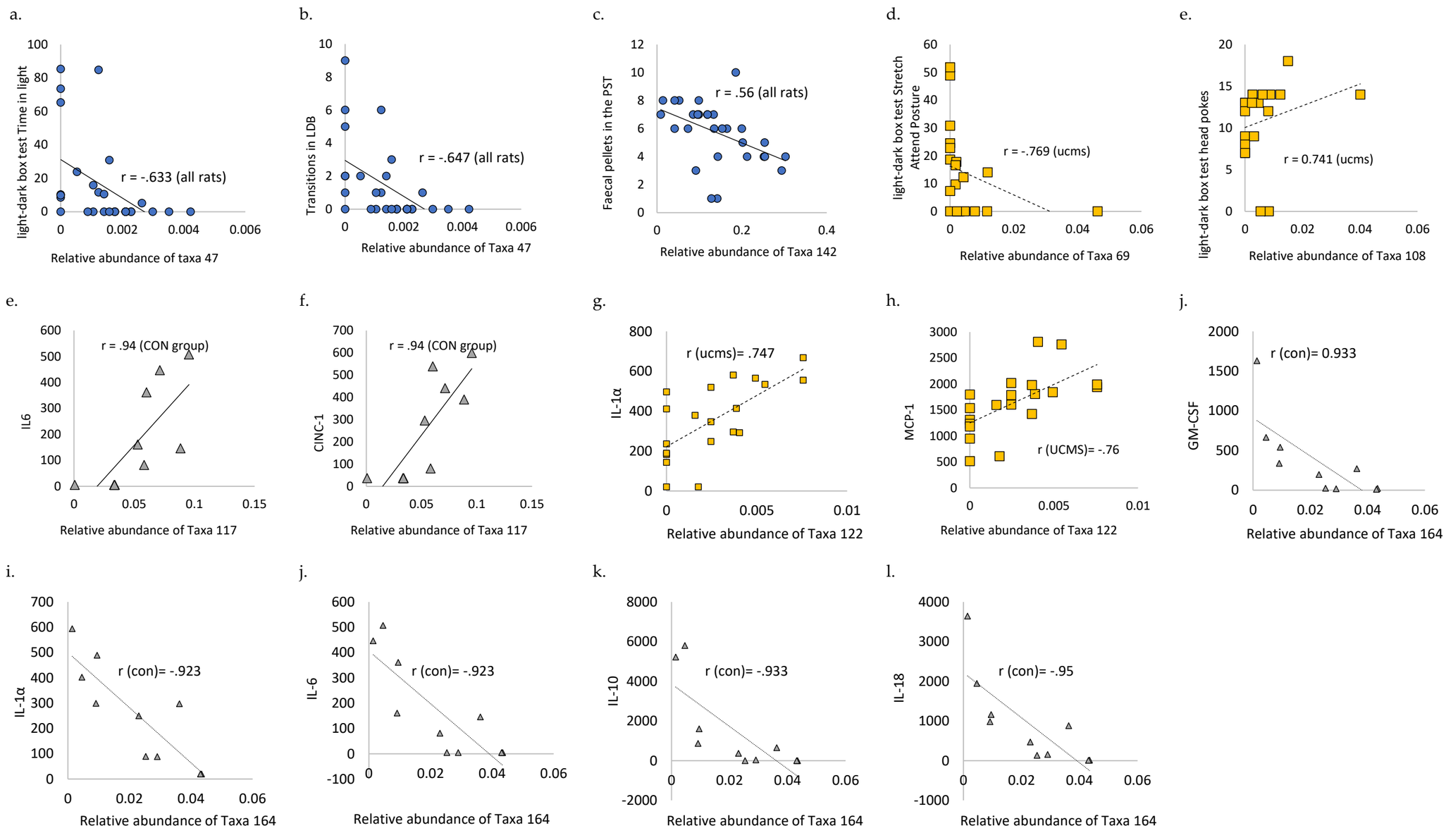

Figure 3.10*. Scatterplots showing correlations between the caecal microbiota, behavioural variables, and plasma cytokines in Sprague-Dawley rats exposed to unpredictable chronic mild stress (UCMS) and kept as controls (CON).*

3.4 Discussion

UCMS was used to induce depression-like and anxiety-like symptoms in female SD rats. The aim of the study was to test if stress-induced changes in the caecal microbiota composition, caecal organic acids, and systemic gut permeability and inflammation markers differed between rats that were stress-resilient, stress-sensitive, and non-stressed (controls).

3.4.1 Stress Resulted in Atypical Behavioural Changes

In contrast to typical results using this model, the UCMS exposed rats did not show depression-like behaviour develop in the SPT over time, or in the PST and coat state measurement following UCMS compared with the control group. Instead, they showed an increase in sucrose solution intake over time, and a pattern of lower anxiety-like behaviour in the light-dark box test compared to the control group (but no differences in individual measures).

The meaning of an increase in sucrose intake in the SPT is unclear. Several other rat studies have found similar changes in behaviour following stressor exposure (Kanarik et al., 2011; Konkle et al., 2003; Nowacka-Chmielewska et al., 2017; van de Wouw et al., 2018; Van den Hove et al., 2014). Kanarik et al. (2011) observed an increase in in sucrose intake (1% solution) in male Wistar rats following social defeat stress, only in those rats who initially had a high sucrose intake. In male Wistar rats, an increase in sucrose intake (1% solution) over time was observed with or without stressor exposure. Sucrose intake also differed depending on sex, and whether the rats had been exposed to prenatal stress and/or chronic mild stress (Van den Hove et al., 2014). Another study reported that, in female SD rats, sucrose intake increased along with an increase in anxiety-like behaviour (decreased exploration in the open field test and a trend of decreased time in the centre) following four weeks of chronic mild stress. In addition, the rats had increased adrenal gland weight, supporting that they had been stressed (Nowacka-Chmielewska et al., 2017).

Neurologically, sucrose intake has been linked with both dopamine and serotonin. In male Wistar rats, systemic (intraperitoneal) administration of the drug parachloroamphetamine which is selectively neurotoxic to serotoninergic neurons and reduces serotonin neurotransmission, caused reduced serotonin in the frontal cortex, hippocampus, and septum. Increased sucrose intake, reduced social interactions and increased immobility were found in

the PST. These behavioural changes mirrored those of a separate group which experienced 14 days of chronic mild stress (Harro et al., 2001). In a follow-up study, daily injections of citalopram reduced sucrose intake in rats that had received parachloroamphetamine or had undergone 20 days of chronic mild stress (Tõnissaar et al., 2008).

Similarly, both male and female SD rats showed a decrease in intake of sucrose solution (4% concentration) following a neurotoxic-drug induced (6-hydroxydopamine) reduction of dopamine in the left ventromedial medial pre-frontal cortex. (Sullivan et al., 2014). Sucrose intake of a 1% solution by rats was found to be a stable individual trait and positively correlated with increased dopamine D2 receptor function (measured by GTPγS binding assay), but only in the dark phase (Tõnissaar et al., 2006). Rats with high baseline sucrose intake have also shown higher baseline energy metabolism in the brain, which reduced after stress (Kanarik et al., 2011).

Increased sucrose intake could be a different expression of anhedonia. A reduction in anhedonia does not automatically cause a decrease in a certain behaviour, simply that the hedonic pleasure associated with that behaviour is reduced. Inter-individual differences dictate whether the individual decreases that behaviour due to the lack of enjoyment or increases that behaviour to obtain the same level of enjoyment previously experienced. Anhedonia has, for example, been associated with binge eating in people (Keränen et al., 2010). Numerous studies use different methods of measuring anhedonia, e.g., intracranial stimulation, which shows a decrease in anhedonia with stress (Willner, 2005). Another study that observed increased sucrose intake following chronic mild stress, although in male C57Bl/6J mice (not rats), also found a decrease in female urine sniffing. Female urine sniffing is another measure of hedonic behaviour in rats, and a reduction indicates anhedonia. No changes in memory or anxiety- and depressive-like behaviour occurred (van de Wouw et al., 2018).

A different but plausible reason is that the increased sucrose intake in the UCMS rats could be due to increased time awake during the light phase, when they would usually spend most of their time sleeping, because of a disrupted circadian rhythm. A disruption in circadian rhythm and associated activity times and levels can be caused by stress (Gorka et al., 1996). The UCMS procedure included altering the light schedule (lights on during the dark phase or

alternating light and dark every two hours during the dark phase) which is also likely to have caused circadian disruption.

The other behavioural tests which measure depressive-like behaviour, the PST and coat state, did not show any difference between groups. These two tests might have been inadequate to capture changes in behaviour under stress. The PST was modified with only a five-minute swim on the first day (typically a 15-minute swim), and this period might not be long enough to induce behavioural despair or any other coping mechanisms on the second day. Coat state scores have been found to increase in rats exposed to UCMS (Wang et al., 2017) and be restored with anti-depressant drugs (Mutlu et al., 2012). However, rats can also display an increase in grooming under stress, and an assessment of changes in grooming patterns may have been a better measure (Kalueff & Tuohimaa, 2005).

3.4.1.1 Stress in the UCMS Group and Possible Stress in the Control Group

The atypical behavioural results, and the faecal corticosterone concentration not being higher in the UCMS group than the control group gives the possibility that the UCMS protocol did not cause stress in the UCMS-exposed rats. The UCMS protocol in this study used stressors at the milder end of what many protocols are reported to use (Antoniuk et al., 2019). Previous studies in male and female SD rats in which depressive-like behaviours developed in the rats, more intensive stressors such as restraint stress, cold stress, and overcrowding, swim stress and food and water deprivation were used (Rai et al., 2020; Walker et al., 2022; Zhang et al., 2019). It is possible that the stressors used might have been too mild allowing the UCMS rats to become habituated to the UCMS stressor and experience a low level or no chronic stress. This is not supported however by the corticosterone results which show an increase in faecal corticosterone from week one to week six in the UCMS group. The relationship between stress and corticosterone is not always straightforward in chronic stress, and several studies have found reduced or no difference in baseline blood corticosterone in rats following chronic mild stress (Bielajew et al., 2002; Grønli et al., 2004; José Jaime et al., 2016; Nowacka-Chmielewska et al., 2017; Retana-Márquez et al., 2003). However the mechanisms of this include downregulation of stress-induced corticosterone and/or corticosterone receptors (Sapolsky et al., 1984). An increase over time is unlikely to be indicative of anything but increased stress. Faecal corticosterone is an effective way to measure baseline corticosterone as it is the

representative of corticosterone from the previous 6 to 20 hours, and therefore is not affected by changes in blood levels due to sampling (Bamberg et al., 2001; Touma et al., 2004). The increase in faecal corticosterone from week one to week six in the UCMS group, and the increase in sucrose intake over time, suggests that the UCMS rats were stressed and experienced an atypical response to the UCMS stressor.

The possibility that the control group experienced chronic stress, and at a higher level than the UCMS group, is suggested by the higher faecal corticosterone levels and the pattern of higher levels of behavioural inhibition (anxiety-like) behaviour displayed by the control group compared with the UCMS group. The reason for the probable stress in the control group is unclear. The rats were kept in different rooms, and there may have been something in their room which caused a higher level of stress. There are several variables in animal care which could contribute to stress including differences in sound, smell, temperature, handling, and lighting (Balcombe et al., 2004; Morgan & Tromborg, 2007).

The control group may have experienced isolation stress due to single housing. It is well known that single housing causes isolation stress and can increase anxiety-like and depressive-like behaviour (Prager et al., 2011). However, the UCMS group was also singly housed. It is possible that the isolation stress of single housing was ameliorated in the UCMS group due to the novel stressors of the UCMS causing either psychological adaptation (reduced stress during acute behavioural test stressors) or physiological adaptation (periodic acute increases in corticosterone allowing negative feedback to occur). The reason for using singly housed rats for both the control and UCMS groups was to prevent the exchange of faecal microbiota through coprophagy. The cohousing of genetically modified mice with conventional mice has been shown to cause transmission of faecal metabolites that can influence physiology in conventional mice (Kaliannan et al., 2015). There are numerous studies that have successfully used singly housed rats as a control group for a stress intervention (Bergström et al., 2007; Guo et al., 2017; Jayatissa et al., 2006; Varga et al., 2017; Zhang et al., 2017). Even if there had been a low level of stress in the control group, a comparison between the groups would have been possible. The finding that the control group may have experienced higher stress than the UCMS group was unexpected.

The difference in behavioural results could also be due to habituation to handling and novel stressors by the UCMS group, meaning that they experienced less stress during the behavioural tests than the control group. This possibility does not mean that the UCMS group did not experience any acute stress, just less than the control group.

In conclusion, it seems likely that the rats exposed to UCMS developed an atypical response to stress, with increased sucrose intake being the key behavioural expression. Whether they showed a lower level of stress compared to the control group is uncertain. Although the behavioural effects of the UCMS did not match that expected, the findings do indicate that broad physiological changes occurred that were consistent with exposure to stress. It is still meaningful to investigate associations between observed behaviour and physiology.

3.4.2 Caecal microbiota, Caecal Organic Acids, Stress, and Behaviour

The caecal microbiota composition differed between the control and UCMS groups. Beta-diversity (measure of diversity between the samples) was altered following UCMS, but alpha-diversity (measure of richness and evenness of microbiome within a sample) was not. The current study showed lower relative abundance of the phylum Cyanobacteria, and higher relative abundance of the phylum Patescibacteria, families *Saccharimonadaceae* and *Rikenellaceae*, and the species *Lachnospiraceae bacterium DW59* in the UCMS group compared with the control group. Caecal organic acids did not differ from the control group, suggesting changes to microbiota structure did not result in major changes to the metabolic activity of the microbiome.

The change in the caecal microbiota is likely to be due to exposure to the UCMS. Differences in food intake could cause a difference in the gut microbiota, but while food intake was not measured, there was no difference in weight or weight gain between the groups, and therefore was likely a similar intake. Differences in sugar intake could also alter the gut microbiota; a high sugar diet caused a difference in the faecal microbiota in mice (Magnusson et al., 2015). However, the fat or sugar in this that study were 42% and 70% respectively of the diet. In the current study sucrose was only 3.25% of the rats' diet and the difference in sucrose intake between groups due to the difference in sucrose preference test results was around 5-10% of their weekly dietary sucrose intake. The change in the caecal microbiota was more likely to be a mixture of long-term effects from the chronic UCMS stressor and response to acute stress.

The caecum samples were obtained at euthanasia, one-two days following the end of the UCMS procedure, and exposure to the PST, an acute stressor. The timeframe of the effect of stress on the caecal microbiota varies, and studies have shown an effect of stressors on the caecal and faecal microbiota within two hours to a week, lasting anywhere between 15 hours and seven weeks (see section 2.1.2.3 of this thesis for a summary).

3.4.2.1 Differences between groups

Changes in microbial taxa had similarities and differences from previous rodent stress studies. Many bacteria which were altered between the UCMS and control groups have been found to differ in stress studies in rats or mice, alongside changes in behaviour. The abundance of the phylum Patescibacteria has been found to be reduced in mice following exposure to chronic mild stress alongside reduced sucrose preference and increased mobility. It also positively correlated with levels of serotonin and antioxidant GLP-1 in the brain (Zhang et al., 2022). A change in the family *Saccharimonadaceae* has been seen in mice exposed to both restraint stress and UCMS, but not when exposed to one type of stress (Qiao et al., 2020). The stress was associated with reduced levels of serotonin in the brain, increased serum corticosterone, reduced serum superoxide dismutase, and increased immobility in the PST and TST, and reduced sucrose preference.

Increases in the abundance of the family *Rikenellaceae* have been found in NIH Swiss mice following 30 minutes of restraint stress, with no difference in corticosterone following this acute stress (Desbonnet et al., 2015). The family *Rikenellaceae* was also increased in mice exposed to dark stress (continuous darkness), compared with mice exposed to light stress (continuous light) or a normal 12 hour cycle (Kim et al., 2019). *Alistipes*, a genus of the family *Rikenellaceae,* also showed increased abundance in BALB/c mice following two weeks of grid floor stress (Bangsgaard Bendtsen et al., 2012). Exposure to the stressor was associated with increased depression-like behaviour in the Tail Suspension Test, but no differences in anxiety-like measures in the Triple Test, a behaviour test, or plasma cytokine concentrations. A reduction in the family *Rikenellaceae* following antibiotic treatment in BALB/c and NIH Swiss mice occurred alongside a decrease in anxiety-like behaviour (Desbonnet et al., 2015; Leclercq et al., 2017). The BALB/c mice also showed decreased sociability and increased aggressiveness in the male but not female mice. Both were prevented with supplementation of probiotic

Lactobacillus rhamnosus JB-1 (Leclercq et al., 2017). A reduction in olfactory and recognition memory were also found in the NIH Swiss mice (Desbonnet et al., 2015). The family *Rikenellaceae* has also been associated with emotionality in people. In children, an undefined genus in the family *Rikenellaceae* was positively correlated with a high rating of activity and/or high-intensity pleasure in boys and positively associated with fear in girls (Christian et al., 2015). The genus *Alistipes* belonging to that family has been associated with depression (Jiang et al., 2015; Naseribafrouei et al., 2014).

There is minimal research on the species *Lachnospiraceae bacterium* DW59 in rats, and no studies have reported this bacterium as being altered by stress. However, the family *Lachnospiraceae,* which the species *Lachnospiraceae bacterium* DW59 is classified under, was correlated with risk assessment behaviours in mice following stress (Bangsgaard Bendtsen et al., 2012). Furthermore, the abundance of the family *Lachnospiraceae* has been negatively correlated with depression (Naseribafrouei et al., 2014) and decreased in people with depression (Jiang et al., 2015).

The particular stress-induced changes in abundance of caecal bacteria are novel. The phylum Cyanobacteria has not been found to differ in abundance in other stress studies including maternal separation stress in rats (Gareau et al., 2007; McVey Neufeld et al., 2019; O'Mahony et al., 2009) or in mice exposed to acute social stress (Galley, Nelson, et al., 2014), acute restraint stress (Desbonnet et al., 2015), chronic restraint stress (Bailey et al., 2010; Galley et al., 2017; Galley, Yu, et al., 2014; Tsilimigras et al., 2018), chronic grid floor stress (Bangsgaard Bendtsen et al., 2012), chronic mild stress (Bailey et al., 2011; Gautam et al., 2018; Yang et al., 2017), chronic mild stress (Marin et al., 2017), or five weeks of behavioural testing (Burokas et al., 2017). Taxa from the phylum Cyanobacteria are photosynthesising bacteria, mostly known for their environmental presence as nitrogen fixers in soil and as blue-green algae in water. They can be toxic to people and animals (He et al., 2016). However, administration of mass cultured Cyanobacteria was shown to have a hypoglycaemic effect when administered to diabetic rats (Pandurangan & Kim, 2016) which suggests that stress-induced changes in the caecal microbiota of this phylum could have a physiological effect.

The implication of similar bacteria being altered by stress in the current study and previous research without concomitant behavioural changes shown in previous studies is that bacterial

and behavioural changes due to stress may be independent and not a causal relationship. It is also plausible that these bacteria can induce depressive-like behaviour in rodents, but did not in this study because there was no increase in gut permeability to LPS. This has been found previously. A study in adult male C57Bl/6J mice reported increased sucrose intake and minor changes in their faecal microbiota at the family and genus level following three weeks of psychosocial stress, but not change in depressive-like behaviour. While the stressor increased gut permeability to FITC-dextran, low plasma LBP levels showed that bacterial translocation did not occur due to maintenance of the mucus layer (van de Wouw et al., 2018).

Other studies showing an increase in depressive-like behaviour in rodents following stress have shown changes in the gut microbiota (Bangsgaard Bendtsen et al., 2012; Marin et al., 2017; Qiao et al., 2020) that differ from that in the current study. These changes were decreased abundance in *Bifidobacterium* and/or *Lactobacillus* genera. There is some evidence that strains of *Bifidobacterium* and *Lactobacillus* genera may be protective against stress-induced changes in mood in both rodents and people (reviewed in Bear et al., 2021). Most of the evidence of this link is from probiotic studies, but stress-induced decreases in taxa from the *Lactobacillus* genus have been found in several rodent sub-chronic and chronic stress studies. A decrease in the relative abundance of caecal taxa from the *Lactobacillus* genus was observed in male rats following 11 days of behavioural tests in Wistar Kyoto but not SD rats (Bassett et al., 2019). In six-nine-month-old male and female rhesus monkeys, cell counts of *Lactobacillus* were increased on day one and then decreased on day two of maternal separation. Cell counts were restored to baseline levels by day seven (Bailey & Coe, 1999). Reduced absolute abundance of *Lactobacillus* occurred in male c57BL/6 mice following six days of two-hour social disruption stress sessions but was not apparent on day one (Galley, Nelson, et al., 2014). Decreased faecal *Lactobacillus* following exposure to five weeks of UCMS in male mice was associated with increased immobility in the PST (Marin et al., 2017) and decreased colonic *Lactobacillus* in male mice following three weeks of UCMS was associated with increased anxiety-like behaviour in the OFT, and depressive-like behaviour in the SPT, PST and TST (Qiao et al., 2020). An increase in *Bifidobacterium* abundance following 10 days of social defeat stress was found in mice resilient to developing decreased social behaviours (Yang et al., 2017), and administration of the probiotic *Bifidobacterium infantis* 35624 can prevent the development of stress-induced depressive-like behaviours in rats (Desbonnet et al., 2010).

Similar *Lactobacillus* or *Bifidobacterium* genera abundances in the UCMS and control groups in the current study could be a key factor in the lack of development of depressive-like or anxiety-like behaviours. This finding could also be linked with the lack of increase in gut permeability to LPS (as shown by no change in LBP), as different strains of *Lactobacillus* given as a probiotic supplement have been found to promote gut integrity in rats following stress, liver injury, dysbiosis, or chemical colitis, as well as in healthy and ill people and in gut epithelial cells (Reviewed in Ahrne & Johansson Hagslatt, 2011).

3.4.2.2 Correlations between individual caecal microbes and behaviours

Correlations between several individual gut microbiota and behaviours were identified, Negative correlations were found between exploratory/ behavioural activation behaviours (time in light box, transitions between light and dark, and time in stretch-attend posture) and unspecified species in the family *Family XIII* and genus *Lachnospiraceae NK4A136 group.* A positive correlation was found between exploratory behaviour (head pokes) and the species *Oscillibacter sp. 1-3*. An unspecified bacterium in the *Ruminococcus* genus was also negatively associated with the number of faecal pellets expressed in the PST.

The individual correlations between several caecal bacteria and behaviour indicate that there was an association with emotional behaviour. However, partial least squares analysis, which compares the data as a group rather than the individual measures, did not show the same correlation. This indicates that the role of individual bacteria may be more important than the overall composition.

Similar associations between emotional behaviours and individual faecal microbes have been observed in other studies. The same three families (*Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales Family_XIII*) which were found to be associated with changes in anxiety-like behaviour showed a higher relative abundance in tail biting pigs compared with non-tail biters (Verbeek et al., 2021). The abundance of taxa from the family *Ruminococcaceae* was also higher in hens which had high rates of feather pecking (van der Eijk et al., 2019). Both tail biting in pigs and feather pecking in chickens are stress-induced behaviours (van der Eijk et al., 2019; Verbeek et al., 2021). In both studies, lower relative abundances of Lactobacillus genus was also found. Altered faecal short chain fatty acid (SCFA) concentrations were also found in chickens with high levels of feather pecking (Meyer et al., 2013).

The gut microbiota play an important role in the production of serotonin in the gut via short chain fatty acids (Reigstad et al., 2015; Yano et al., 2015). *Clostridium* species may be key microbes which have this effect (Luna et al., 2017; Yano et al., 2015). The families *Ruminococcaceae* and *Lachnospiraceae* have been associated with carbohydrate metabolism (Daneshzad et al., 2020) and produce SCFAs (Vital et al., 2017). If these bacteria do promote serotonin production in the gut, then the negative correlations observed with exploratory (in contrast with anxiety-like) behaviours seem counterintuitive because anxiety behaviour is typically linked with low serotonin. However, high levels of serotonin in the brain have been linked with increased anxiety in people (Frick et al., 2015; Marcinkiewcz et al., 2016). Whether the serotonin levels in the gut contribute to serotonin levels in the brain is not yet clear.

An unspecified bacterium in the family *Family XIII* was negatively correlated with time in the light box in the light-dark box test. Reduced time in the light box is considered anxiety-like behaviour. A species in the family *Family XIII* UCG 001, was shown to be decreased in rats susceptible to developing pain-induced anhedonia. The increased depression-like behaviours were able to be transferred from the susceptible mice into antibiotic-induced pseudo germ-free mice following a faecal microbial transplant, indicating that the microbiota were causative in the depressive-like behaviours (Yang et al., 2019). In rats bred for an anxious phenotype, treatment of antibiotic minocycline caused an increase in the abundance of the *Family XIII*, alongside decreased depressive-like behaviour (immobility in the FST) and microglial numbers, but no difference in anxiety-like behaviours (Schmidtner et al., 2019). In contrast, the abundance of *Family XIII* was positively associated with anxiety scores in people suffering from mood disorders (Rhee et al., 2021).

A negative association between time in the stretch-attend posture and an unspecified bacterium in the species *Lachnospiraceae NK4A136 group* was found in the current study. Stretch-attend posture has been suggested to be an ambivalent behaviour, reflecting approach-avoid conflict (Van Der Poel, 1979) and is considered a risk assessment behaviour (Blanchard et al., 1990; Choleris et al., 2001). Whether it increases or decreases under stress is unclear and needs to be considered in the context of the situation. Blanchard et al. (1990) found that risk assessment behaviours of rats in a high stress environment (cat exposure) were increased with anxiolytic drug diazepam, but in a moderate stress environment (cat odour

exposure) diazepam decreased risk assessment behaviours. In the current study stretch-attend posture followed the same pattern as time in the light box so is assumed to represent behavioural activation. The gut microbial species *Lachnospiraceae NK4A136 group* seems to be associated with lower gut inflammation. It has been found to be decreased in mice with dextran sulphate sodium or LPS-induced colitis, and increased in those mice following butyrate (Dou et al., 2020) or glycine (Zhang et al., 2021) supplementation.

The relative abundance of the species *Oscillibacter sp. 1-3* was positively associated with the number of head pokes. Head pokes showed the opposite pattern to time in the light box in the current study, so are likely to represent anxiety-like behaviour. In prairie voles, the abundance of *Oscillibacter sp. 1-3* was decreased in males but increased in females following social isolation (Donovan et al., 2020).

The negative correlation between the relative abundance of *Ruminococcus spp*. and the number of faecal pellets excreted by rats during the PST, representative of stress-induced changes in lower gut motility, indicates an association with the sympathetic nervous system. In contrast, no correlation was found between any caecal bacteria abundances and faecal corticosterone concentrations, which is controlled by the HPA axis. Another species in the *Ruminococcus* genus, *Ruminococcus flavefaciens*, when administered to mice, was able to prevent the anti-depressant effect of the medication duloxetine (Lukić et al., 2019). A mechanism was not determined. It is possible that the *Ruminococcuss spp*. can influence the sympathetic nervous system.

The correlations between anxiety-like behaviour and the caecal bacteria abundance do not prove causality. Neither the bacteria or behaviours found to correlate were altered by stress, but it is possible that stress levels were too mild to affect bacterial abundances or behaviours between groups. The lack of change in caecal organic acid concentrations with the UCMS intervention could also be a contributing factor to the lack of typical stress-induced behavioural changes, particularly if these bacteria can affect serotonin production through organic acid production.

3.4.3 Plasma Inflammation Markers and Gut Permeability

In the current study, plasma CINC-1 and GM-CSF concentrations were elevated in the UCMS group compared with the control group. A pattern of increase was observed in several other

cytokines. There was however no difference in plasma concentration of LBP, a marker of gut permeability, nor correlations between plasma cytokines, plasma LBP or behaviours in the UCMS group. However, in the UCMS group, plasma MCP-1 and IL1α concentrations were positively associated with unspecified bacterium in the *Ruminococcaceae NK4A214 group* genus. In the control group, an unspecified bacterium in the *Ruminiclostridium* genus was positively correlated with plasma IL-6 and CINC-1 levels. An uncultured bacterium in the *Desulfovibrionaceae* family was positively correlated in the control group only with plasma GM-CSF, IL-10, IL-18, IL-1α, and IL-6 concentrations.

Whether the caecal microbes affected inflammation or vice versa (or both changed by a third variable) is uncertain. Some research suggests that changes in systemic immune function can alter the gut microbiota (Wei et al., 2021). Taxa from the family *Desulfovibrionaceae* are endotoxin producing bacteria, which is inflammatory, so this is an expected result. Interestingly, all the bacteria except those from the family *Desulfovibrionaceae* which correlated with plasma cytokine concentrations was in the order Clostridiales, just as those which correlated with behaviour. However, there was no association between behaviour and inflammation. This result suggests that the increase in plasma cytokines may have been influenced by the gut microbiota (or conversely, the gut microbiota structure was influenced by inflammation) but was not mediated via increased gut permeability.

Inflammation as a potential mediator of depression is not well understood. A recent meta-analysis of human studies found elevated peripheral concentrations of IL-6, TNF- α, IL-10, the soluble IL-2 receptor, C-C chemokine ligand 2, IL-13, IL-18, IL-12, the IL-1 receptor antagonist, and the soluble TNF receptor 2 in people with depression, whereas the IFN-γ concentration was decreased (Kohler et al., 2017). However, they also indicated that results from studies are heterogeneous, and not all studies find the same increase in plasma cytokines (Kohler et al., 2017).

Alteration of tryptophan metabolism through the kynurenine pathway is one possible mechanism linking systemic inflammation and depression via the MGBA (Kennedy et al., 2017). Key enzymes in the kynurenine pathway divert the metabolism of tryptophan towards more neurotoxic/excitatory neuro-molecules. Some of these enzymes (e.g., indole-2,3-dioxygenase (IDO)) are stimulated by LPS, IFNs, TNF- α, IL-1β, and IL-6 (Campbell et al.,

2014). In the current study, the concentration of LBP (a proxy for LPS levels) and TNF-α was not increased and that of IFN-y, IL-6 showed only a small non-significant increase, and IL-1β was below the detection limit. Therefore, it is possible that the lack of typical depression-like behaviour in the UCMS rats was because the inflammation profile as assessed by the LBP and cytokine levels did not cause alteration to the kynurenine pathway.

3.4.4 Strengths and Limitations

A strength of this study is that due to it being an animal study, the rats all had the same diet and environment, removing many confounding variables that occur in human studies.

There are however limitations. Consistent patterns of decreased behavioural inhibition in the light-dark box test and increased plasma cytokines were seen, of which few individual measures reached significance. Post-hoc power analyses showed the study was underpowered for showing differences in these measures. This is likely due to a lack of strong difference in stress levels between the groups. In addition, hormone fluctuations in females due to oestrous cycles should be considered and measured. Testing in males removes this extra set of confounding variables.

The lack of a baseline faecal corticosterone measurement (before UCMS began) made it more difficult to interpret the faecal corticosterone results. Having baseline measurements of faecal corticosterone, would be more useful, as well as baseline measurements for all the behaviour tests. Ideally, the gut microbiota would be measured before and after the stress as well.

3.5 Conclusion

Following six weeks of UCMS, atypical behavioural changes were observed in female SD rats. There is a possibility that the UCMS group did not experience perceived stress and/or the control group experienced a higher level of stress than the UCMS group. The main evidence suggesting that the rats showed an atypical response to stress was an increase in sucrose preference over time in the UCMS group only. This finding has been seen in some other studies following stress. As more research is undertaken in the MGBA and provides added context, the implication of these results may become clearer. However, while their interpretation is not straightforward, there are some key new findings.

The lack of decreased relative abundance of taxa from the *Lactobacillus* and *Bifidobacterium* genera following UCMS, alongside a lack of development of depressive-like and anxiety-like symptoms in these rats, supports previous evidence of a protective role of these bacteria in emotional experience.

The inflammatory markers had an overall pattern of increased inflammation which was not associated with increased anxiety-like or depressive-like behaviours. However, an increase in IL-10 and a lack of increase in TNF-α support the role of these specific inflammatory molecules as being respectively protective and inducing of depression and/or anxiety, possibly via alterations in the kynurenine pathway.

Overall, the implication of the results of this study supports the hypothesis that gut microbiota are associated with anxiety, and suggest that bacteria in the order Clostridiales could be important in this relationship.

3.5.1.1 Future Research

There are several possibilities for future research leading on from this study. For example, a different rat genotype (e.g., males instead of females, or a different species) may show a more typical response to stress, allowing the relationship between the gut microbiome, physiological markers, and depressive symptoms to be assessed. Alternatively, a different stress protocol could be tested, such as chronic restraint stress or maternal separation stress. Measuring neurotransmitter metabolites such as serotonin in the plasma and gut contents, would be useful to determine if they change alongside behaviour and if it is associated with bacteria in the *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales Family_XIII* families. Finally, measuring changes in the tryptophan/kynurenine pathway would be worthwhile to assess if stress-induced changes in inflammation are altering this pathway.

3.6 References

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Chapter 4.

Investigation into Baseline Behavioural Characteristics in Sprague-Dawley rats

4.1 Abstract

This chapter reports the results of three studies designed to investigate possible reasons for the atypical behavioural results in the chronic stress study in female Sprague-Dawley (SD) rats reported in Chapter 3. Three aspects of behaviour were investigated: typical intake of sucrose solution in female SD rats; a validation test of female SD rats in the Porsolt swim test by assessing behaviour with and without antidepressant drugs imipramine and fluoxetine; and baseline behaviour in female SD rats compared with male SD rats. Results showed that female SD rats had a u-shaped curve of sucrose solution intake at different concentrations, which is a typical pattern. Behaviour did not change in the Porsolt Swim test with either antidepressant drug. Behaviour measured in the male and female SD rats showed differences between the groups in behaviours in the light-dark box test and the open field test, indicating that males show more anxiety-like behaviour in response to acute stress. In summary, these results, in combination with the results reported in Chapter 3 suggest that the female SD rats may be less responsive to both acute and chronic stress in our laboratory. Male SD rats may show a more typical response to chronic stress and be able to be separated into stress-sensitive and stress-resilient rats.

4.2 Introduction

The study reported in Chapter 3 showed atypical behaviour in female Sprague-Dawley (SD) rats following exposure to the unpredictable chronic mild stress (UCMS). This included increased sucrose solution intake in the sucrose preference test over time, and a pattern of decreased anxiety-like behaviours in the lightbox and no difference in immobility in the Porsolt swim test compared with the control group. While it is an interesting result, the aim

of this thesis is to use a model in which the rats can be divided into stress-sensitive and stress-resilient using typical behavioural results. Therefore, three studies were undertaken to investigate possible reasons for the atypical behavioural results, and to determine whether the method used in the study reported in Chapter 3 should be altered. The first study, reported in Section 4.3 investigated the intake of sucrose solution in female SD rats at a range of concentrations. The second study, reported in Section 4.4 was a validation test of the Porsolt swim test, and in Section 4.5 results are reported of a comparison of behaviour in different behavioural tests between male and female SD rats.

4.3 Study 1. Sucrose Concentration

The sucrose concentration that is effective in the sucrose preference test (SPT) differs between rat strains, laboratories, and even the same rat strain from different suppliers (Forbes et al., 1996). For example, the sucrose consumption test was first used as a model of anhedonia in Hooded Lister and Wistar rats, where sucrose concentration was set at 1% w/w. This finding was based on a measured sucrose solution intake at different concentrations and was set at the midpoint of the ascending curve to identify either an increased or decreased preference (Willner et al., 1992). When these researchers moved to a new laboratory and used rats from a different breeding facility, the test was ineffective with 1 % w/v sucrose solution. They changed the concentration to 2 %, and the SPT was again effective (Willner, 1997).

The varying results from different laboratories suggest that measuring the baseline sucrose intake of the rat strain and sex to be used in each laboratory may be needed to increase the likelihood of successfully measuring reduced sucrose preference following a stress protocol.

4.3.1.1 Study aim

The aim of this study was to test the sucrose solution intake at different concentrations in female SD rats undergoing the SPT and to identify whether a 1% solution (as used in the UCMS study reported in Chapter 3) is an effective concentration.

4.3.2 Method

4.3.2.1 Animals

Twenty female SD rats, 13 weeks old and weighing 310 $\pm$ 31 g were used. A power analysis was not completed because the study was observational. The number of rats was chosen to allow each concentration of sucrose to be given to two rats during each SPT (10 concentrations

x 2 rats). The rats were obtained in-house from the Plant & Food Research Small Animal Facility. The rats were maintained on a 12:12 h light: dark cycle (0700/1900 h), individually housed in shoebox cages (230 mm x 440 mm) and provided with *ad libitum* food and water. Procedures were approved by the Grasslands Animal Ethics Committee in accordance with the Animal Welfare Act 1999 (Application number 14239).

4.3.2.2 Design

The rats were given a series of 24 hour two-bottle SPTs with concentrations of 0%, 0.2%, 0.5%, 0.75%, 1%, 2%, 4%, 8%, 16%, and 32% w/w to get the ascending and descending part of the sucrose intake curve. The rats were initially exposed to all concentrations except 0% in an order specified by a Williams modified Latin square (Table 4.1). Technical difficulties were found, with labelling difficult to read in the dark and dripping bottles. These issues were sorted, and the study was repeated. Since the rats had already been exposed to all the sucrose concentrations, they were given the different concentrations in ascending order. Ascending order was used to reduce the possibility that the rats would increase their sucrose intake to obtain the same reward value of the higher concentration from the previous test. Each 24-hour test was separated by a day where the rats were given only water.

Table 4.1. *Design of original sucrose exposure using Williams modified Latin Square.*

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
Day 1	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%	3.5%	4.0%	4.5%	5.0%
Day 3	2.5%	0.5%	1.0%	1.5%	2.0%	5.0%	3.0%	3.5%	4.0%	4.5%
Day 5	1.0%	1.5%	2.0%	2.5%	0.5%	3.5%	4.0%	4.5%	5.0%	3.0%
Day 7	2.0%	2.5%	0.5%	1.0%	1.5%	4.5%	5.0%	3.0%	3.5%	4.0%
Day 9	1.5%	2.0%	2.5%	0.5%	1.0%	4.0%	4.5%	5.0%	3.0%	3.5%
Day 11	3.0%	3.5%	4.0%	4.5%	5.0%	0.5%	1.0%	1.5%	2.0%	2.5%
Day 12	3.5%	4.0%	4.5%	5.0%	3.0%	1.0%	1.5%	2.0%	2.5%	0.5%
Day 13	5.0%	3.0%	3.5%	4.0%	4.5%	2.5%	0.5%	1.0%	1.5%	2.0%
Day 14	4.0%	4.5%	5.0%	3.0%	3.5%	1.5%	2.0%	2.5%	0.5%	1.0%
Day 15	4.5%	5.0%	3.0%	3.5%	4.0%	2.0%	2.5%	0.5%	1.0%	1.5%

4.3.2.3 Sucrose preference test

The SPT, light-dark box test and PST behaviour assessments were completed as previously described in Chapter 3 Section 3.1. In addition, no sucrose solution was given prior to the SPTs for habituation, so any variation in behaviour with exposure to different initial solutions could be observed.

4.3.3 Results

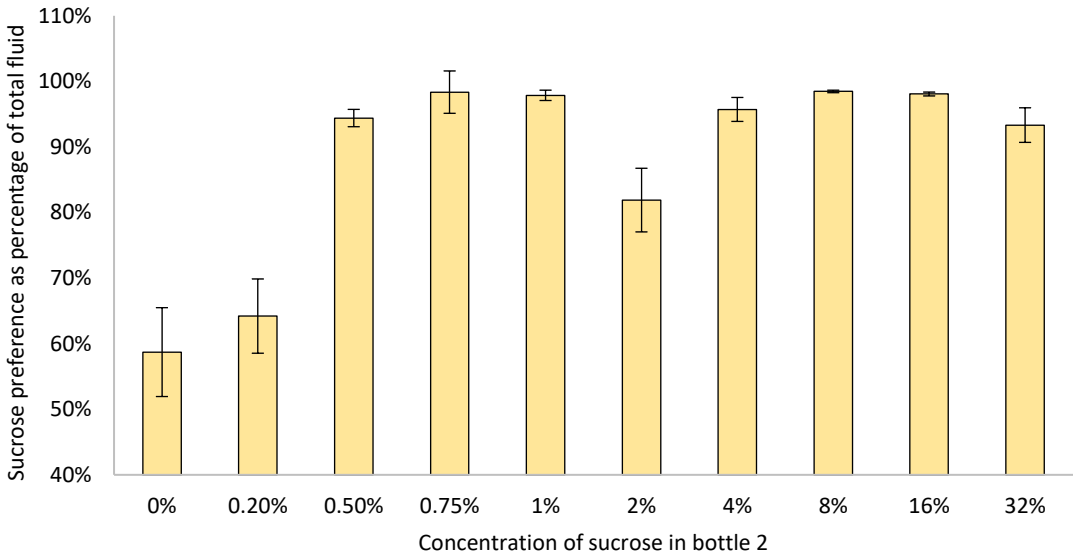
The sucrose preference at different sucrose concentrations is shown in Figure 4.1a. A sharp increase in sucrose preference occurred at a 0.5% concentration and remained above 90% sucrose preference for most concentrations. The total intake of sucrose solution and water are shown in Figure 4.1b. The rats consumed more sucrose solution than water at all concentrations. There was a pattern of increased sucrose solution intake with increased sucrose concentration, until a peak at 8% sucrose solution concentration. Sucrose solution intake then began to decrease at higher concentrations. A similar pattern was seen in total fluid consumption, which comprised mostly sucrose solution.

4.3.4 Discussion

Study 1, assessing sucrose intake, showed a bell-shaped curve (Figure 4.1b) similar to that found previously (Collier & Bolles, 1968). A 1% concentration was halfway up the ascending curve and not far past the plateau of increased sucrose preference, beginning at 0.5%. In other studies, at higher concentrations, the amount of sucrose chosen to be consumed is not due to its reward value but is influenced by total dietary energy intake (Collier & Bolles, 1968; Willner et al., 1992). This finding is likely to explain the drop in sucrose intake and total fluid at 16% sucrose and higher.

Based on these results, 1 % sucrose seems to be an appropriate concentration. It fits the same logic used by the researchers who originally developed the method (Willner et al., 1992), as it allows either an increase or decrease in sucrose preference to be measured. Based on these results, it does not seem likely that the 1% sucrose concentration used in the UCMS study reported in Chapter 3 was the reason for the increased sucrose intake following UCMS exposure.

a.



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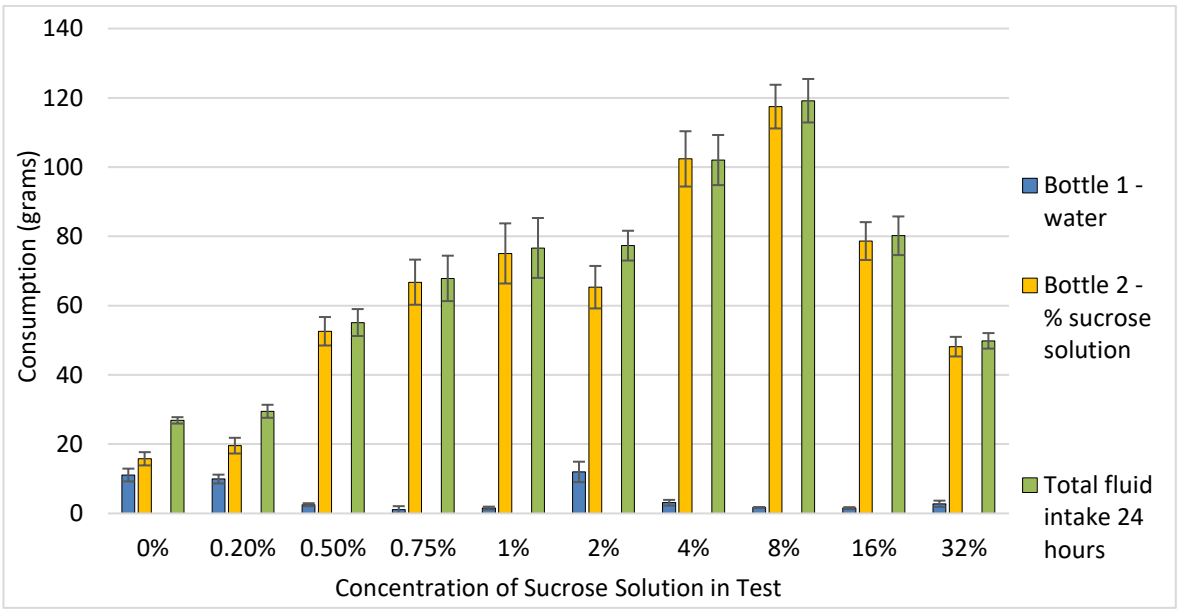

Figure 4.1. *Sucrose preference test results at different sucrose solution concentrations. Two bottle sucrose preference tests were undertaken in female Sprague-Dawley rats (n=10) at a range of sucrose concentrations from 0% to 32% sucrose. Results showed increasing sucrose preference with increased sucrose concentration at lower concentrations only (a) and a u-shaped curve of water, sucrose and total fluid intake with increasing concentrations (b). Results shown are mean + standard error. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20, Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

4.4 Study 2. Porsolt Swim Test Validation

It is unclear if the reported lack of effect of the UCMS procedure on behaviour in the PST carried out in the study reported in Chapter 3 was due to the UCMS procedure not being sufficiently stressful or the rat phenotype responding differently to stress. Several factors can affect results in the PST, and it can be validated using the anti-depressant drugs fluoxetine and imipramine (Slattery & Cryan, 2012). Fluoxetine is a selective serotonin reuptake inhibitor, which increases the concentration of serotonin in the brain. It tends to increase swimming behaviours in the PST. Imipramine is a tricyclic anti-depressant drug, increasing serotonin and norepinephrine and climbing behaviours in the PST (Slattery & Cryan, 2012).

4.4.1.1 Study aim

The aim of this study was to determine if the female SD rats in our laboratory responded to the PST as is typical in other laboratories which use it. The PST was undertaken in a cohort of female SD rats along with administration of imipramine and fluoxetine. Expected results in the PST validation were that both the anti-depressant drugs reduced the time immobile, and that fluoxetine increases time swimming while imipramine increases time climbing.

4.4.2 Method

4.4.2.1 Animals

Twenty-four female SD rats were used, aged 14 weeks (312-359 grams). Power analysis was undertaken using G*Power software based on the variable immobility. Based on previously reported results in Sprague-Dawley rats (Cryan & Lucki, 2000), eight rats were required per group for a 95% power, an alpha of 5%, and an effect size of 1.4 (Cohen's D) for the variables of immobility and swimming. Animals were kept as described in section 4.3.2.1. Procedures were approved by the Grasslands Animal Ethics Committee in accordance with the Animal Welfare Act 1999 (Application number 14504).

4.4.2.2 Experimental Design

The rats were divided into three groups of eight rats. One group was administered the selective serotonin reuptake inhibitor anti-depressant drug fluoxetine at a dose of 20 mg kg^{-1}, and a second group was administered tricyclic anti-depressant drug imipramine at a dose of 20 mg kg^{-1}. The control group (n=8) received only the saline vehicle. The drugs were

administered 23.5 hours, 5 hours, and 1 hour before the PST by subcutaneous injection into the thigh. The timeline is shown in Figure 4.2.

4.4.2.3 Drugs

Selective serotonin reuptake inhibitor anti-depressant drugs fluoxetine (Fluoxetine Hydrochloride, Sigma Aldrich) and tricyclic anti-depressant drug imipramine (Imipramine Hydrochloride, Sigma Aldrich) were resuspended in a solution of sterile saline/ethanol solution (14% v/v pure ethanol) on the same day of use and stored at 4 ^{0}C until use.

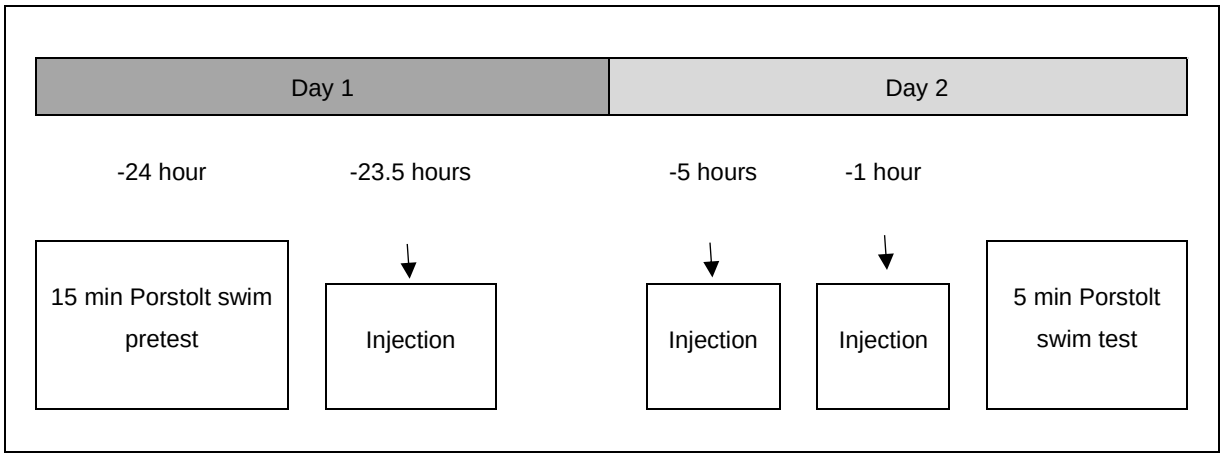

Figure 4.2. *Timeline of Porsolt swim test validation study. Female Sprague-Dawley rats (n=8) were given injections of antidepressant drugs Imipramine or Fluoxetine, or saline vehicle at three timepoints in between the 15 minute pre-test and the 5 minute test.*

4.4.2.4 Porsolt Swim Test

The PST was completed with a 15 minute pre-test on day 1 and a five minute test on day 2, as per the published protocol (Slattery & Cryan, 2012). The rats remained in their home cage and had access to food and water until the time of the test.

4.4.2.5 Statistical analysis

Statistical analysis was conducted using the statistical programme Gentstat v.20.1.2.24528. Individual behaviours in the PST under different interventions were compared using one-way ANOVA with post-hoc comparison using the least significant difference at 5 %. Data are presented as mean $\pm$ standard error.

4.4.3 Results

Behaviour results are shown in Figure 4.3. No significant differences were observed between the three groups for immobility ($F(2, 21) = .9617$, $p = .398$), latency to immobility ($F(2, 21) = .4484$, $p = .645$), climbing ($F(2, 21) = .0743$, $p = .929$), diving ($F(2,21) = .2704$, $p = .766$), swimming ($F(2, 21) = 2.1305$, $p = .496$ or number of faecal pellets ($F(2, 21) = 2.2364$ $p = .132$).

A non-significant increase in climbing and decrease in swimming with imipramine, were observed. Post-hoc power calculations showed that the study was underpowered to detect if this is a true difference and required 36 and 72 rats respectively for a power of 0.95 and alpha of 0.05.

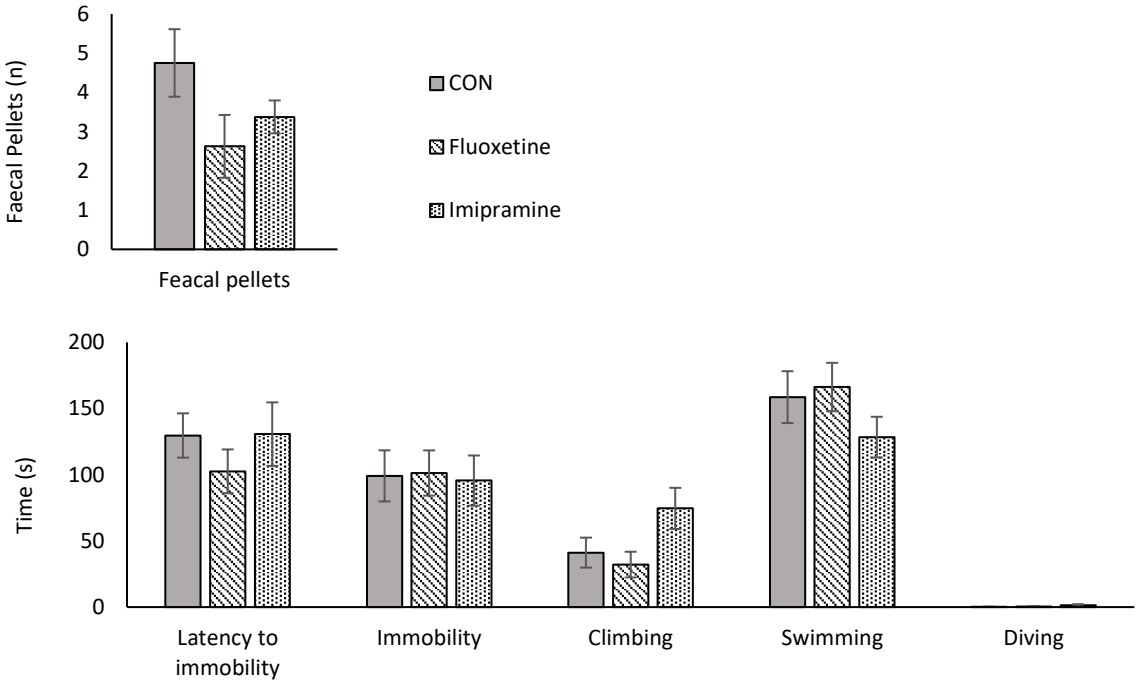

Figure 4.3. *Results of the Porsolt swim test validation study. Behaviour displayed by female Sprague-Dawley rats (n=8) in the Porsolt swim test, after injection at three prior time-points with antidepressant drug Imipramine or fluoxetine, or ethanol/saline vehicle. No difference was seen in behaviour between the three groups. Groups tested using ANOVA with p<.05. Results shown are mean and SEM. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20*

4.4.4 Discussion

The results of this PST validation study indicate that the female SD rats in our laboratory are not sensitive to the effect of anti-depressant drug fluoxetine in on the behaviours immobility, climbing and swimming, nor the anti-depressant drug imipramine on immobility. This is in

contrast to rats in other laboratories in which these doses and mode of administration are effective (e.g. Mora et al., 2005; Santiago et al., 2014; Slattery & Cryan, 2012). Imipramine may have increased climbing and decreased swimming, an expected result, but was underpowered for these variables to be significant.

Fluoxetine is a selective serotonin reuptake inhibitor and affects serotonergic systems, and typically increases swimming and decreases immobility (Slattery & Cryan, 2012). Imipramine is a tricyclic anti-depressant and which several neurotransmitters including serotonin, but has the strongest effect on the catecholaminergic systems, including norepinephrine and dopamine (Slattery & Cryan, 2012).

This study failed to validate the PST with immobility which is the key behavioural measure in the PST. Neither acute nor chronic stress induced depressive-like symptom of immobility in the PST in the female SD rats in our laboratory. The results suggest that in the female SD rats tested, the serotonergic systems are resistant to the effects of acute stress on immobility; and that increasing neurotransmission using anti-depressant drugs does not affect behaviour. The (non-significant) increase in climbing and decrease in swimming with imipramine indicates that the catecholaminergic system may have been affected by acute stress in these rats, and restored with imipramine, but the group size would need to be larger for this to be confirmed. It is plausible that that a serotonergic system which is robust to chronic stress as well is the reason that no depressive-like behaviours were observed in the chronic stress study reported in Chapter 3.

4.5 Study 3. Comparison of Male and Female Sprague-Dawley rats.

The results of Study 1 suggested that the sucrose concentration used in the SPT was appropriate, and therefore the results of increased sucrose preference are more likely to be due to the rat phenotype or the environmental conditions than the test itself. Study 2 indicated that the rats did not adequately respond to anti-depressant drugs under acute stress, suggesting the rat phenotype had a greater effect than the environmental conditions.

Published literature suggests that female SD rats may have different behavioural characteristics to male SD rats at baseline and following chronic stress in both the PST (Bielajew et al., 2003; Colom-Lapetina et al., 2017; Hong et al., 2012; Van den Hove et al., 2014), SPT (Konkle et al., 2003; Lu et al., 2015; Rincón-Cortés & Grace, 2017) and the light-dark box

(Lovelock & Deak, 2019). Male and female rats also show different baseline levels of neurotransmitters and their metabolites (Duchesne et al., 2009). Rat phenotypes and even genotypes also vary between different laboratories. (Colom-Lapetina et al., 2017; Hong et al., 2012; Van den Hove et al., 2014).

Perhaps the female SD rats used in the study reported in Chapter 3 have a unique neurotransmitter profile that promotes atypical behaviour responses or increased resilience under acute or chronic stress, compared with males. Response to acute and chronic stress has been shown to differ by sex in rodents (Lovelock & Deak, 2019; Luine et al., 2017). It is possible that the male Sprague-Dawley rats in our laboratory could show a more typical response to chronic stress, allowing the thesis hypothesis questions to be tested. The reason for the atypical responses in the female rats could then be investigated alongside responses from males.

4.5.1.1 Study aim

This study was undertaken to assess any differences in baseline behaviours (locomotive, anxiety-like and depressive-like behaviours) between male and female SD rats.

4.5.2 Method

4.5.2.1 Animals

For Study 3, eight male and eight female rats at 19-20 weeks of age were used. The weights of the males were 575 ± 35 g (mean ± SD), and the females were 312 ± 21 (mean ± SD). Power analysis was undertaken using G*Power software. Eight subjects were required per group for a 95% power , an alpha of 5%, and an effect size of 2.4 (based on results from Strekalova et al. (2004)). Procedures were approved by the Grasslands Animal Ethics Committee in accordance with the Animal Welfare Act 1999 (Application number 14504, modification number 2462).

The behavioural tests undertaken were the light-dark box, OFT, and PST using methods outlined in Chapter 3, Section 3.3.1. The light-dark box test was undertaken on day 1, the OFT on day 2, and then after two days break, the PST was completed on days 5 and 6. The OFT was scored using the automated software ANY-maze. Rats in the light-dark box and PST were (Strekalova et al., 2004)recorded using ANY-maze software, and behaviours were scored manually later, in the ANY-maze software, by a treatment-blinded researcher.

4.5.2.2 Behavioural tests

The SPT and light-dark box test were completed as described in Chapter 3.1. In addition, the PST was completed with a 15 minute pre-test on days 1 and 2, as per the published protocol (Slattery & Cryan, 2012). The OFT was conducted in opaque plastic boxes (50cm x 50cm x 50cm) with black paper on the base, replaced between rats. Testing began when the rats were placed in the centre of the field. Tests ran for 5 minutes and were filmed. Behavioural measures were automatically analysed using ANY-maze software.

4.5.2.3 Statistical analysis

Statistical analysis was conducted using the statistical programme GenStat v.20.1.2.24528. Results were compared using a two-tailed t-test. Differences with a p-value less than 0.05 were regarded as statistically significant. Data are presented as mean $\pm$ standard error.

4.5.3 Results

Problems with the software freezing meant that one round of testing (one male and one female) was not recorded in both the light-dark box test and the OFT, so there were seven rats per group for analysis which reduced power to 65%.

4.5.3.1 Light-Dark Box

Results are shown in Figure 4.4a. Time spent in the light box ($t(1, 12) = 2.25$, $p = .044$) and time rearing ($t(1, 12) = 2.61$, $p = .036$) was higher in the females compared with the males. There was no difference between groups in the head poke or "stretch-attend" behaviours.

4.5.3.2 Open Field Test

Results of the OFT are shown in Figure 4.4b-e. No difference was found between the male and female rats in the locomotion measures, total distance travelled, or average speed. The average time spent in the centre did not differ between groups, but time freezing did ($t(1, 7.87) = 4.77$, $p = .001$).

4.5.3.3 Porsolt Swim Test

There were no significant differences between any of the measures in the PST (see Figure 4.4f-g). As both locomotion and body weight can affect the PST (Bogdanova et al., 2013), results were adjusted for body weight and locomotion in the OFT. Neither weight nor mobility (distance travelled) significantly affected the result, based on analysis of covariance to adjust

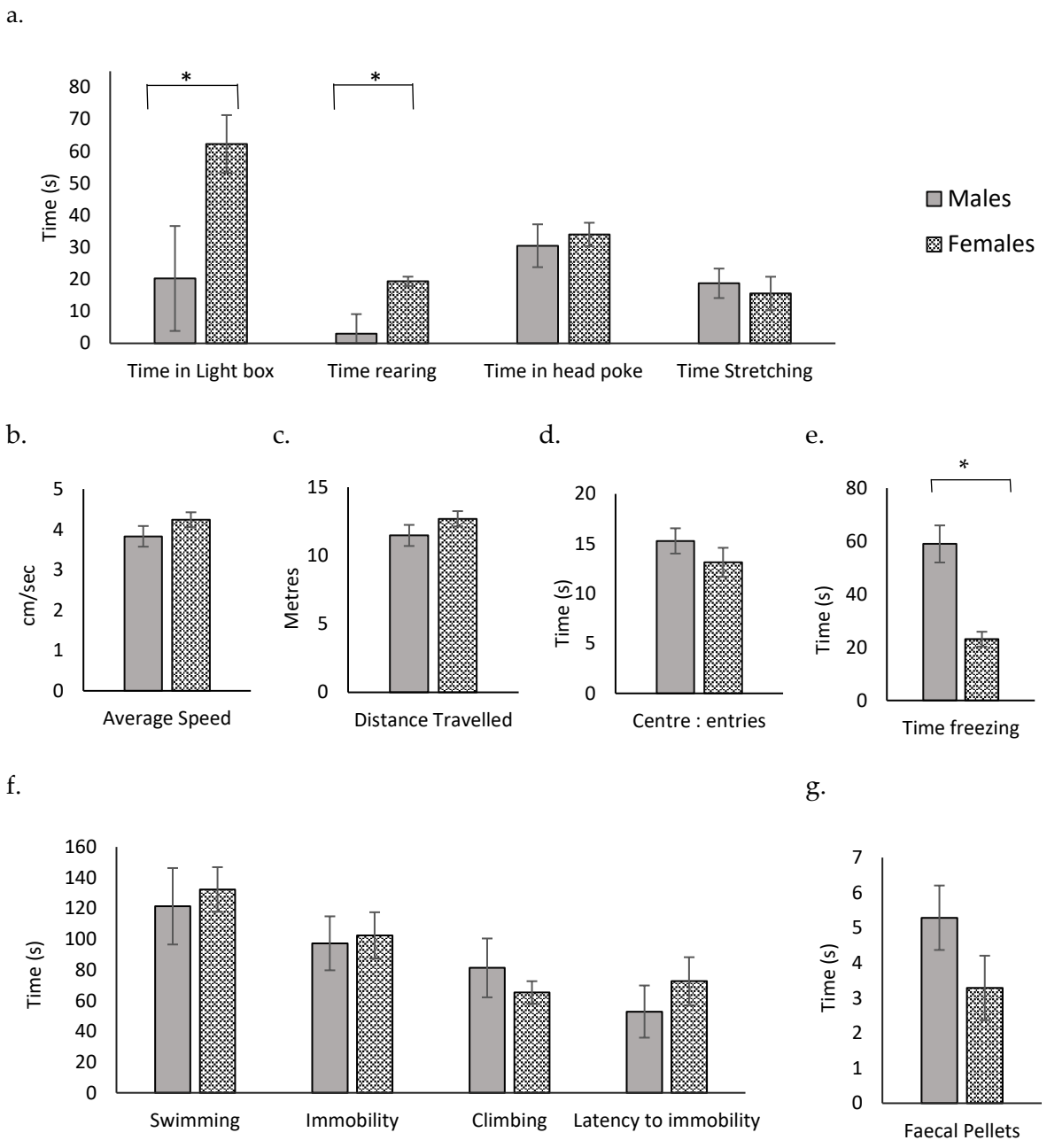

***Figure 4.4**. Results of baseline behavioural testing between male and female SD rats in a) the light-dark box, b).-e) the open field test and f)-g) the Porsolt swim test. n=7 per group. * = P< .05 difference between groups. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

for weight (F = .15 on 1 and 11 df, p = .708) and for mobility, F = .22 on 1 and 11 df, p = .648). The number of faecal pellets was also non-significantly increased in the males in the PST. The study was likely underpowered to assess any differences in faecal pellets, however, the trend may indicate differences in gut transit time under stress. More data would be needed to determine if this is the case.

4.5.4 Discussion

Measurement of baseline behaviours in the light-dark box and OFT showed that female rats displayed lower behavioural inhibition (anxiety-like behaviour) than male rats. However, no difference in behavioural despair (depressive-like behaviour) or coping styles in the PST were found.

Whether the differences in baseline behaviours between male and female rats would translate to differences under chronic stress is uncertain. Other studies have found differences between male and female rats in stress studies. However, results have been inconsistent among studies. Whether males or females are more resilient to chronic stress and the specific behaviours which change are also variable. After three weeks of chronic mild stress, immobility in the PST decreased, and acute stress-induced HPA activation increased in male but not female SD rats (Van den Hove et al., 2014). Following six weeks of chronic mild stress, Bielajew et al. (2003) found the duration of active behaviours in the PST increased in male SD rats, whereas the frequency, but not the duration of active behaviours, increased in females. Social isolation in SD rats caused an increase in sucrose intake in the female rats but a decrease (both non-significant differences) in the males. The female rats also showed an increase in climbing in the PST, whereas the males did not (Hong et al., 2012). Faraday (2002) found that male and female SD rats differed in their response to stress compared with the males in behaviour in the OFT with both groups reducing in locomotion on day 1 of chronic stress, but the males recovering by day 6. The duration of stress is likely to be important. In related studies, Haleem et al. (1988) and Kennett et al. (1986) found that male rats but not female rats showed reduced locomotion after acute stress (single two hour restraint), but after sub-chronic stress (five days of acute restraint stress) the males showed no difference to baseline, but the females showed reduced locomotion and increased defecation. This result was associated with increased corticosterone in the females (Haleem et al., 1988). It is possible that chronic stress will affect male SD rats in a more typical way compared with the female SD rats as reported in Chapter 3.

4.6 Conclusion

The results of the three studies reported in this chapter suggest that the rat phenotype may be the reason for the atypical behavioural responses of female SD rats in the study reported in

Chapter 3. The results of sucrose preference testing at different concentrations in non-stressed rats showed typical results, indicating that it was likely the response of the rats to stress rather than the test itself that caused the atypical results. A lack of clear response to anti-depressant drugs in the PST in females and similar baseline results in males and females could mean that depressive-like behaviours are not expressed in this rat phenotype. Lower anxiety-like behaviours and increased locomotion in the female SD rats compared with the male SD rats under acute stress (behavioural tests light-dark box and open field test) suggests that it is worth testing the response of male rats to UCMS as they may respond differently, possibly in a more typical way.

4.7 References

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Chapter 5.

Stress-Induced Changes in Caecal Digesta Neurotransmitters not associated with Serum Neurotransmitters or Anxiety-like Behaviour.

This chapter (following modification to a journal manuscript format) is intended to be submitted to the journal Frontiers in Endocrinology (Impact Factor 5.555) in the special issue "New Insights in Stress Resilience – Clues From the Gut". This special issue includes research which examines neurobiology of stress-resilience, including microbial metabolites.

5.1 Abstract

This study used four weeks of unpredictable chronic mild stress (UCMS) in male Sprague-Dawley rats, with an unexposed control group, to assess if stress-induced changes in caecal digesta neurotransmitters and/or lipopolysaccharide (LPS)-induced systemic inflammation due to increased gut permeability was associated with stress-resilience. Faecal corticosterone and anxiety-like and depressive-like behaviours in the light-dark box test, Porsolt swim test, sucrose preference test and open field test were measured before and after the stress intervention. Caecum and serum biogenic amines (including neurotransmitters dopamine, norepinephrine, epinephrine, serotonin and gamma-aminobutyric acid (GABA)), serum cytokines, and gut permeability marker lipopolysaccharide binding protein (LBP) were measured after the intervention period. In the UCMS group, faecal corticosterone and anxiety-like behaviours increased, and locomotion and exploratory behaviours decreased from baseline. The same results were also observed in the control group, with a larger change than the UCMS group indicating they also experienced stress, and at a higher level than the UCMS group. No correlation was found between behaviour and caecum biogenic amines or serum LBP or cytokines, indicating that these were not associated with stress-induced anxiety-like behaviour. There was also no correlation between serum and caecum biogenic amine concentrations indicating that the caecum concentrations may not be an important contributor to serum concentrations. No stress-induced change in depressive-like behaviours, LBP or caecum neurotransmitters occurred, so a relationship between these cannot be ruled out.

5.2 Introduction

Emerging research shows the gut microbiome to be an important influence on not just physical health, but also mental health. Depression and anxiety episodes are very commonly preceded by stress, and the gut microbiome has been shown to affect emotional behaviour in rodents and humans, and it is also affected by stress (Bear et al., 2021). It is plausible that stress-induced changes in the gut microbiome and associated factors could act as a partial mediator or moderator for stress-induced changes in mood. In fact, pre-existing composition of the faecal microbiota has been shown to be associated with stress-sensitivity or stress-resilience in male Sprague-Dawley (SD) rats (Tanelian et al., 2022). The effects of a stress-altered microbiome are not well established but many microbiome-gut-brain-axis studies which have found an ameliorative effect on mood of probiotics or foods, have used a stress model of depression/anxiety.

Several mechanisms have been proposed by which stress-induced changes in the gut microbiota could affect emotional behaviour. Increased gut permeability and systemic inflammation have been shown to be associated with increased severity of depression symptoms (Maes et al., 2012). In addition, one of the mechanisms by which systemic and brain inflammation have been linked to depression is through modulation of the kynurenine pathway. Inflammation-induced activation of the indoleamine 2,3-dioxygenase (IDO) enzyme causes the conversion of tryptophan to kynurenine to be increased, while the conversion of serotonin decreases. Increased levels in the brain of excitatory metabolites in the kynurenine pathway and low serotonin concentrations have been associated with depression (O'Connor et al., 2008).

The gut microbiota also produce some of the same neuromodulating substances found in the nervous system of animals, including acetylcholine, dopamine, serotonin, and norepinephrine (Holzer & Farzi, 2014; Lyte, 2011; Roshchina, 2010; Ross et al., 2010). Host serotonin released into the gut from enterochromaffin cells is also partly regulated by microbiota or microbial metabolites e.g., SCFA (Fukumoto et al., 2003). GABA, acetylcholine and norepinephrine are all immunomodulatory (Bjurstöm et al., 2008; de Jonge, 2013). Serotonin mainly affects gut motility but can also activate the vagus nerve and enter the circulation (Fukumoto et al., 2003). It has also been proposed that gut microbial metabolites could be mediators of the

microbiome-gut-brain-axis (MGBA) either through immunomodulation in the gut or by increasing the levels of systemic and brain neurotransmitters (Lyte & Cryan, 2014).

Gut permeability and inflammation can increased under psychological stress due to physiological processes (Ait-Belgnaoui et al., 2012; Demaude et al., 2006; Kiliaan et al., 1998; Meddings & Swain, 2000; Saunders et al., 1994; Saunders et al., 2002; Soderholm et al., 2002), but are also directly by alterations in the gut microbiota composition (Cani et al., 2008; Cani et al., 2009; Wang et al., 2018). Stress-induced changes in the gut microbiota could also change the concentration of microbially-produced neurotransmitters. It is therefore plausible that individual differences in stress-induced changes in these biological markers could contribute to how susceptible an individual is to developing stress-induced behavioural changes.

In the study using female rats reported in Chapter 3, the rats did not develop typical depressive symptoms following six weeks of unpredictable chronic mild stress (UCMS) and showed at trend of lower anxiety-like behaviour compared with the control group. There was a change in caecal microbiota composition in the UCMS exposed rats but no increase in serum lipopolysaccharide-binding protein (LBP), a systematic marker of gut permeability. The studies reported in Chapter 4 also observed that female SD rats in our laboratory do not show a typical response to anti-depressant drugs with acute stress and show lower levels of anxiety-like behaviours in behavioural tests than male SD rats. Male SD rats may show a more typical response to chronic stress than female SD rats. Therefore, the study reported in this chapter uses UCMS rodent model of depression, with male SD rats to investigate possible biological mechanisms associated with stress-resilience.

Gut permeability marker lipopolysaccharide (LBP), and serum cytokines, as well as serum biogenic amines in the tryptophan pathway including kynurenine, tryptophan, kynurenine, 5-HTP, and serotonin were measured to determine if the development of depressive-like behaviours in the stress-sensitive UCMS-exposed rats may be associated with inflammation induced modulation of the kynurenine pathway caused by increased gut permeability.

Additionally, in the study reported in Chapter 3, correlations were found between several caecal microbiota taxa and anxiety-like behaviours in both groups, but there was no correlation of either with the gut permeability marker lipopolysaccharide (LBP). While several caecal microbiota taxa correlated with plasma cytokines, the cytokines were not associated

with behaviour. This indicates that if the caecal microbiota have a causative association with behaviour, it is not mediated through gut permeability or stress-induced changes in systemic inflammation. It is plausible that changes in caecal microbial metabolites could influence behaviour. Therefore, the current study investigates a range of caecal and serum biogenic amines including neurotransmitters dopamine, norepinephrine, epinephrine, serotonin and gamma-aminobutyric acid (GABA), and their metabolites and precursors. This is to investigate if the caecal concentrations of biogenic amines correlate with serum concentrations of biogenic amines, and if either are associated with behaviour.

5.2.1 Study Aims

The study aimed to use behavioural responses of male SD rats to the chronic stressor UCMS to identify stress-sensitive and stress-resilient rats, and to assess potential mechanisms of stress-resilience. Serum inflammatory markers (cytokines), serum LBP, and levels of metabolites in the tryptophan pathway (tryptophan, serotonin, kynurenine, 5-HTP, and 5-hydroxyindoleacetic acid) were measured. In addition, several neurotransmitters in the tryptophan, tyrosine and GABA pathways were measured in caecal digesta and serum. Faecal bacteria composition before and after the stress was originally intended to be measured but unfortunately the faecal samples were lost, and the gut microbiota analysis could not be undertaken.

Six hypotheses were explored. These were:

1. The stress-sensitive rats will have increased levels of systemic pro-inflammatory cytokines and LBP compared with the control group and stress-resilient male rats.
2. Depressive-like and anxiety-like behaviours will be negatively correlated with systemic serotonin and/or positively correlated with kynurenine and/or kynurenine metabolites in all rats.
3. Decreased serotonin pathway metabolites and increased kynurenine pathway metabolites in the caecal digesta and serum will be associated with an increase in serum LBP and pro-inflammatory cytokines.
4. The stress-sensitive rats will have altered serum and/or caecal digesta metabolites compared with the control group and stress-resilient male rats.

5. Caecum metabolites will be positively correlated with serum metabolites and behaviour differences.
6. Caecum and/or serum metabolites GABA, acetylcholine and/or noradrenaline will be correlated with inflammatory markers.

5.3 Method

5.3.1 Design

The study was a two-by-two factorial quasi-experimental design with an experimental group (n=20) exposed to four weeks of chronic stress (UCMS group) and a control group (n=10) not exposed to stress (control group). The aim was to divide the stress group into stress-sensitive and stress-resilient groups following four weeks of stress based on depressive behavioural measures. The procedure was altered from the UCMS study in female rats reported in Chapter 3. The length of the UCMS was reduced from six to four weeks, because significant changes in sucrose intake were found by that time. Behavioural tests were undertaken both before and after the UCMS to be able to compare post-stress results to baseline results as well as between groups. This is in contrast to only doing behavioural tests at the end of the UCMS procedure in the previous study. The lighting was not reversed in case undertaking the behavioural tests in the dark phase was the reason for the atypical results reported in Chapter 3. Most laboratories tend to undertake UCMS in the light phase because it is more convenient and practical.

Baseline sucrose preference was measured before the rats were assigned randomly to the control or UCMS group. Sucrose preference values were averaged to check that the groups were balanced. The UCMS group was subjected to a UCMS stress protocol, as described in Chapter 3, Section 2.2.1.3. Behavioural tests were undertaken before and after the UCMS procedure (Figure 5.1).

5.3.2 Animals

Male SD adult rats (n = 30, weighing 476 g + 30 g standard deviation (SD) at the beginning of the study) were obtained from the animal breeding facility at Plant and Food Research Institute, Palmerston North, New Zealand. The rats were maintained on a 12:12 h dark: light cycle (0700/1900 h), individually housed in shoebox cages, and provided *ad libitum* food and water. Procedures were approved by the Grasslands Animal Ethics Committee in accordance

with the Animal Welfare Act 1999 (approval number 14507). The number of rats was determined using the same power analysis conducted for the female UCMS study (outlined in Chapter 3, Section 3.3.2) due to the same intended primary outcome that half the UCMS group developing lowered sucrose preference following UCMS.

5.3.3 Procedure

The UCMS procedure and behavioural tests (sucrose preference test, light-dark box test), were conducted as per the protocols described in Chapter 3, Section 3.2.3. The PST was completed with a 15 minute pre-test on day 1 and a 5 minute test on day 2, as per the published protocol (Slattery & Cryan, 2012). The open-field test was performed as described in Chapter 4, Section 4.2.4.

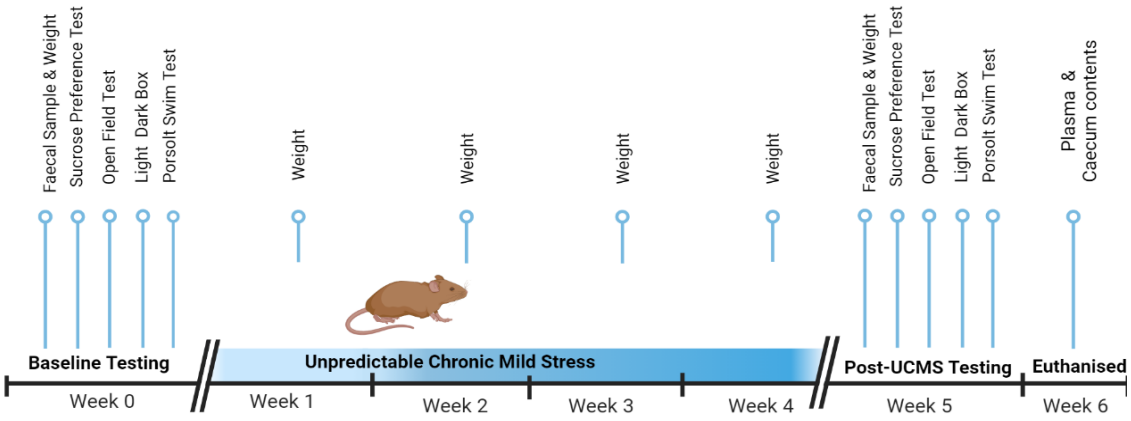

Figure 5.1. *Timeline of the UCMS study in male Sprague-Dawley rats.*

5.3.4 Stress Resilience

Stress-sensitivity was defined a sucrose preference of less than 60% in the UCMS exposed rats in the 24 hour SPT following exposure to the UCMS

5.3.5 Sample collection

Faecal samples were collected after placing the rats into clean cages. If they did not defecate within 5 minutes, their tails were gently lifted to induce defaecation. The first two pellets were collected immediately, placed in sterile tubes and frozen at -80 °C until analysis. Rats were euthanised using CO_2 gas 48 hours after the day after the last behavioural test (PST). Caecal contents were collected into sterile tubes, snap- frozen in dry ice and then stored at -80 ^{0}C until

analysis. Blood for serum was collected using cardiac puncture and deposited into plastic serum tubes (BD Vacutainer; Cat Ref 367837). The tubes were left upright at room temperature (18 °C) and then centrifuged for 15 minutes at 7180 x g at room temperature (18 °C) (Heraeus Multifuge X1R; Thermo Scientific). Serum was removed by pipette, aliquoted into Eppendorf tubes and stored at -80 °C until analysis.

5.3.6 Biogenic amine analysis using liquid chromatography-mass spectrometry

The biogenic amines measured were phenylethylamine and its metabolites tyrosine, 3,4-dihydroxyphenylalanine (l-DOPA), neurotransmitter dopamine (DA), 3-methoxy-p-tyramine (3MT), 3,4,-dihydroxyphenylacetic acid, homovanillic acid (HVA), neurotransmitter norepinephrine (NE), neurotransmitter epinephrine (E), DL-metanephrine (MN), normetanephrine (NM), 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylglycol (MHPG), and vanillylmandelic acid (VMA). We also analysed tryptophan, kynurenine and its metabolites, 5-hydroxytryptophan (5-HTP), neurotransmitter 5-hydroxytryptamine (serotonin), and 5-hydroxyindole-3-acetic acid (5-HIAA) and the glutamic acid metabolites, alpha-aminobutyric acid (AABA) and neurotransmitter gamma-aminobutyric acid (GABA). The samples (100 µL of digesta) were treated to remove proteins and derivatised in two stages to acetylate alcohol and amine functional groups and esterify acid groups, and 5 µL of the sample was then used for liquid chromatography-mass spectrometry (LCMS) analysis using an MS-probe and stable isotope coding method (detailed in Appendix B).

5.3.7 Faecal corticosterone

Faecal corticosterone concentration was measured using the same method as described in Chapter 3, Section 3.2.3.9.

5.3.8 Serum cytokines and lipopolysaccharide binding protein

Serum concentrations of cytokines interferon-gamma (IFN-γ), cytokine-induced neutrophil chemoattractant type-1 (CINC-1), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-18, IL-12p70, IL-1β, IL-17A, IL-33, IL-1α, and IL-6 were measured using the same methods as reported in Chapter 3, Section 3.2.3.10.

5.3.9 Statistical analysis

Rat weight, faecal corticosterone concentration and the behavioural test results were compared over time and between groups with one-way repeated measures ANOVA. Log transformation was used if data were not normally distributed. Post hoc testing with the least significant difference (LSD) was used to determine significant differences between groups or time points. Cytokines and LBP concentration data were examined using a Shapiro-Wilk test for normality of distribution. Variables with a normal distribution were compared between groups using a two-sample, two-sided t-test. Non-normal data were compared using the Mann-Whitney U (Wilcoxon rank-sum) non-parametric test. A 5 % probability level was considered significant. PERMANOVA was used to compare the behavioural scores from each group in the light-dark box as a set.

For partial least squares (PLS) regression, variables with 50 % or more values at a lower or upper threshold value were removed (no time spent in behaviour during behavioural tests; or below the detection limit for cytokines and LBP). In addition, variables with a skewness greater than 1.8 or kurtosis great than 3.8 were also removed.

Correlations were undertaken using Spearman's Rank Coefficient. Due to the large number of comparisons, a probability level of 1 % was used, and correction for a false discovery rate of 5 % was carried out using the Benjamini-Hochberg procedure. In addition, scatterplots were visually checked for outliers and a visual correlational relationship made for both groups. All statistics except PERMANOVA were completed using the statistical software package GENSTAT (version 19.1.0.21390, VSN International Ltd). PERMANOVA was conducted in R (version 4.1.2).

5.4 Results

5.4.1 Sucrose Preference Test

Time of day affected the results of SPT. In the 24-hour test, no difference was found in either group over time (Figure 5.2a). A trend of increased sucrose intake was seen in the control group, but no significant difference was found (Figure 5.2b). In the light phase, both sucrose preference and sucrose intake increased in the UCMS group over time, whereas they did not in the control group (Figure 5.2c-d). In the dark phase, results were similar to those obtained at 24 hours, reflecting that most of the fluid intake was during the dark (active) phase.

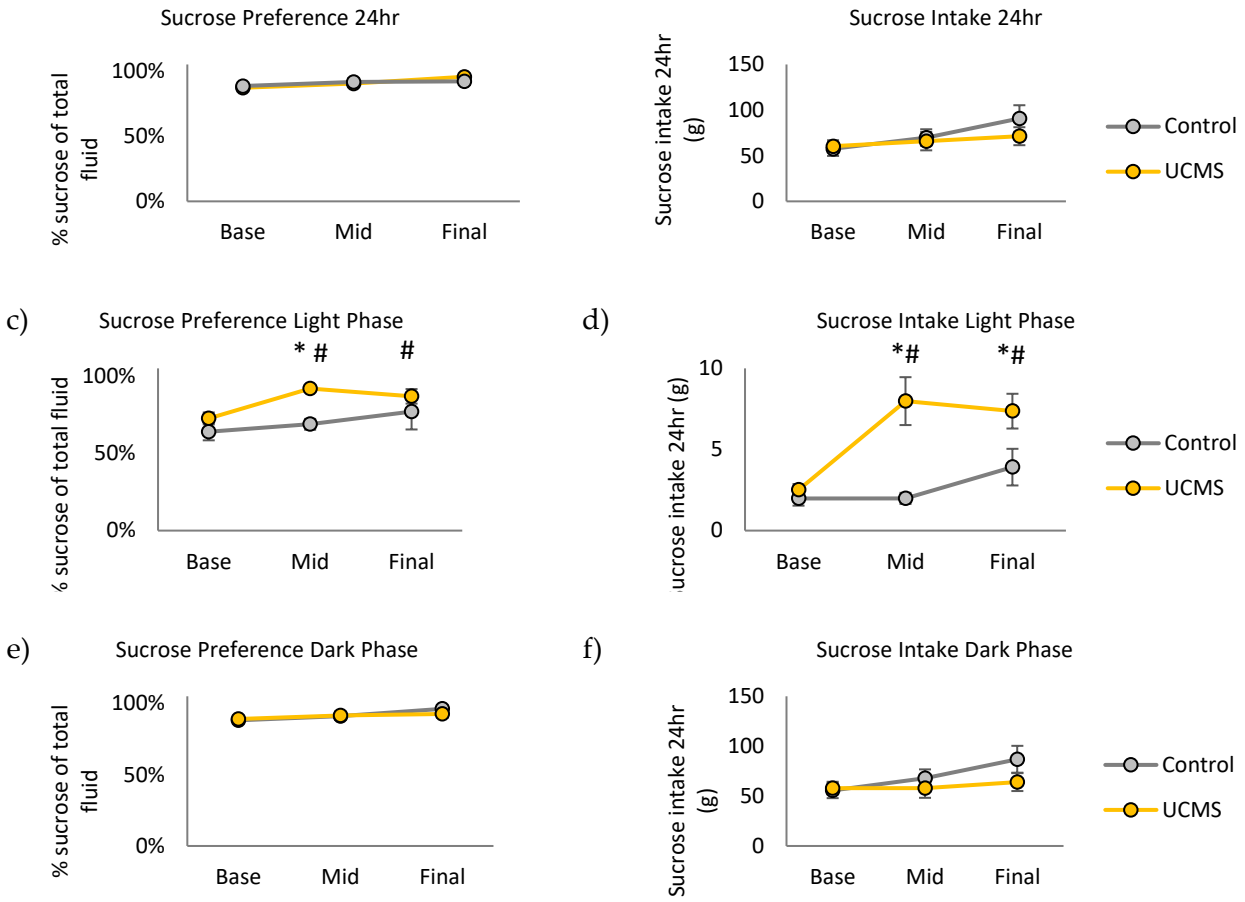

Figure 5.2. Behavioural parameters shown in the Sucrose Preference Test (SPT) of Sprague-Dawley rats following four weeks of unpredictable chronic mild stress (UCMS) procedure and control Sprague-Dawley rats. *A baseline test was undertaken prior to the UCMS (Base), at the midpoint of the UCMS (Mid) and after the UCMS was finished (Final). Sucrose preference and total sucrose solution intake were measured over a 24-hour period (a-b). Measurements were also taken after the first 12 hours following the light period (c-d) and from 12hours-24hours during the dark period (e-f). An increase in sucrose preference and intake from baseline was observed in the UCMS group in the light phase only. * Least significant differences (LSD) (5% level) show differences from the control group, # LSD (5%) shows differences from baseline. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

5.4.2 Porsolt Swim Test

No difference was found in any of the parameters measured in the PST between the control and UCMS groups. In addition, the post-stress scores did not differ from the baseline scores (Figure 5.3).

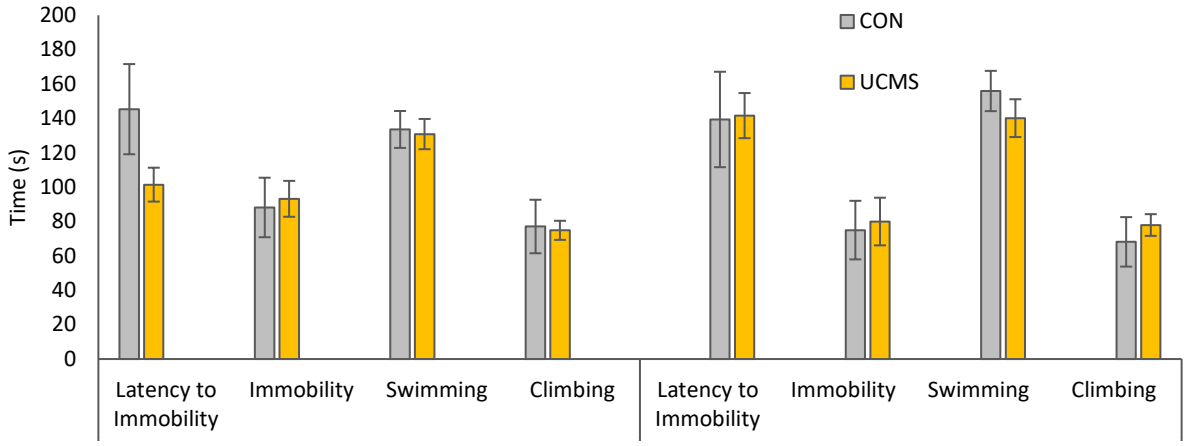

Figure 5.3. *Behaviour parameters in the Porsolt swim test. Male Sprague-Dawley rats unpredictable chronic mild stress (UCMS) exposed rats and a control group were tested were tested before and after the four weeks stressor protocol. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

5.4.3 Light-Dark Box

Several behaviours measured as part of the light-dark box test were affected by treatment. Time in the light box; transitions between zones; rearing time and rearing number, reduced in both groups after the experimental period compared to baseline (Figure 5.4 a-d). Latency to enter the light box increased in both groups (Figure 5.4 e). Time spent in stretch attend posture (SAP), and the number of times SAP occurred, was reduced in both groups from baseline, but decreased more in the control group (Figure 5.4 f-g). Finally, the overall time of head poke behaviour did not change, but the number of head pokes was reduced in the control group only. (Figure 5.4h-i).

Comparison of the behaviours as a group between UCMS and control using PERMANOVA showed no difference between the groups at baseline ($F = .05$ on 1 and 25 df, $p = .952$) or at five weeks ($F = 1.137$ on 1 and 21 df, $p = .326$).

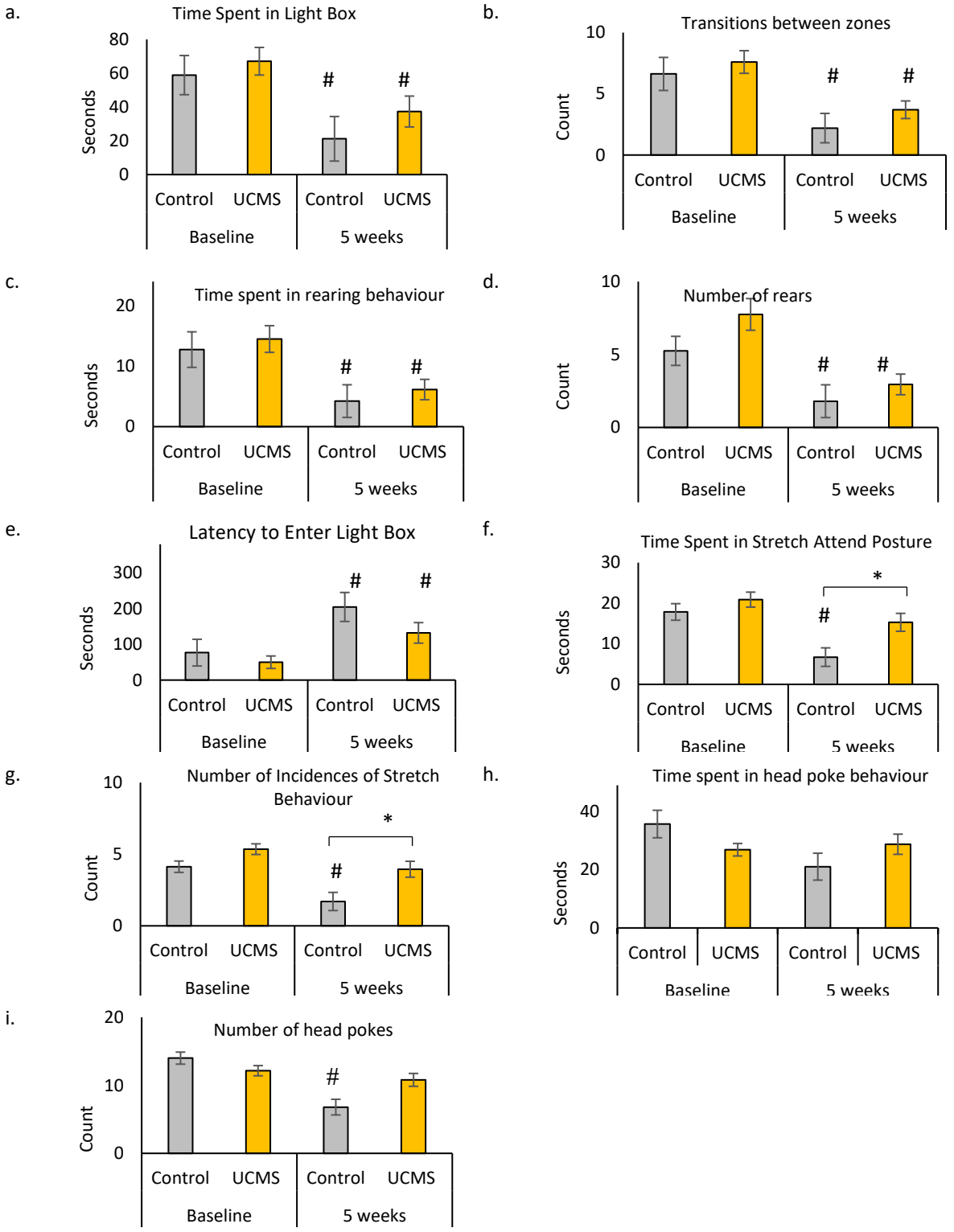

Figure 5.4. *Behaviour parameters measured in the light-dark box test. Male Sprague-Dawley rats showed changes in behaviour from baseline in both the control group and in the group exposed to four weeks of unpredictable chronic mild stress (UCMS). Behaviour parameters: (a), transitions between zones (b), time spent in rearing behaviour (c), number of rears (d), latency to enter light box (e), time spent in stretch attend posture (SAP) (f), number of SAP incidences (g), time spent in head poke behaviour (h), and number of head pokes (i). * Least significant differences (LSD) (5% level) show differences from the control group, # LSD (5%) shows differences from baseline. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

5.4.4 Open Field Test

Several open field test behavioural measures differed between the control and UCMS groups (Figure 5.5). Repeated measures ANOVA showed an effect of time on total distance travelled ($F(1,28) = 59.4476$, $p = <0.001$), average speed ($F(1,28) = 58.2962$, $p = <0.001$), and number of entries to the centre zone ($F(1,23) = 66.32$, $p = 0.000$)). An interaction between time and treatment also occurred for total distance ($F(1,28) = 12.99$, $p = 0.001$), average speed ($F(1,28) = 12.48$, $p = 0.001$) and entries to the centre ($F(1,23) = 10.98$, $p = 0.003$) but not for time in the centre. Post hoc analysis using an LSD of 5% showed the control group had a lower total distance, average speed, and entries to the centre than baseline and the UCMS group. Time spent in the centre zone was reduced over time in both groups ($F(1,28) = 9.56$, $p = 0.004$), but there was no difference between treatments.

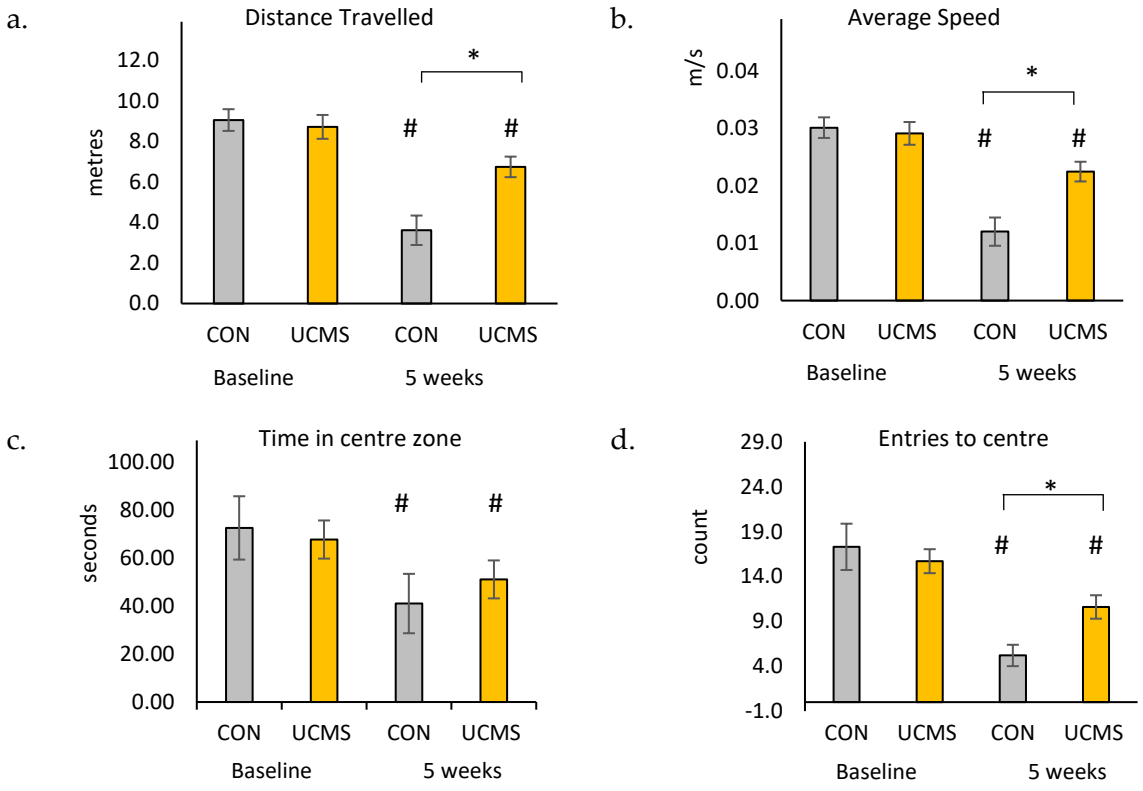

Figure 5.5. *Behaviours assessed with the Open field test. Male Sprague-Dawley rats exposed to four weeks of unpredictable chronic mild stress (UCMS) procedure as well as the control group decreases in behaviours from baseline. Behaviour parameters: distance travelled (a), average speed (b), time in centre zone (c) and entries to centre (d). * Least significant differences (LSD) (5% level) show differences from the control group, # LSD (5%) shows differences from baseline. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

5.4.5 Weight

As expected, the bodyweight of all rats increased over the course of the study. The bodyweights of the UCMS group and the control group were similar (Figure 5.6a).

5.4.6 Faecal corticosterone concentration

Comparison of the faecal corticosterone concentrations with repeated measures ANOVA showed an effect of UCMS ($F(1,28) = 4.90$, $p = .035$) and time ($F(1,28) = 42.06$, $p < .001$). There was also an interaction between UCMS and time ($F(1,28) = 9.94$, $p = .004$). Post hoc analysis using LSD showed no difference in faecal corticosterone concentrations between UCMS and control groups at baseline, and both groups were increased from baseline (Figure 5.6b). The post-UCMS levels of faecal corticosterone were higher in the control group.

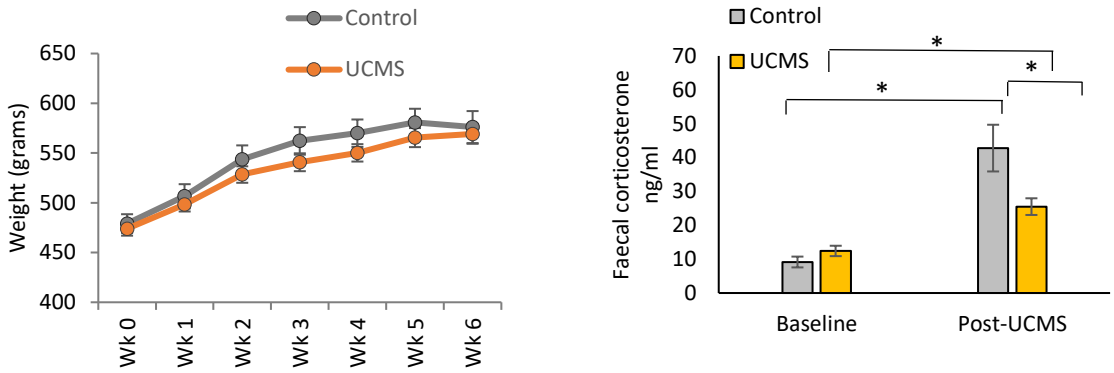

***Figure 5.6**. Body weight (a) and faecal corticosterone (b) in male Sprague-Dawley rats over the six-week study time period. Body weight increased in rats exposed to four weeks of unpredictable chronic mild stress as well as the control group, but there was no difference between the groups. Following four weeks of the UCMS procedure in male Sprague-Dawley rats, faecal corticosterone increased in both UCMS exposed rats and the control group but was increased more in the control group. Mean and SEM shown. * = p<.05. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20.*

5.4.7 Stress Resilience

No reduction in sucrose preference in the 24-hour SPT was observed in the rats (Figure 5.2), meaning that the rats could not be classified as stress-resilient and stress-sensitive. Sucrose intake and preference in the light phase, and behaviours in the light-dark box were therefore also assessed if they could identify rats who have been affected by stress and those who have not.

The percentage change from baseline was used so that the effect of the stress on individuals could be observed. However, the percentage change from baseline did not always match the magnitude of change in the actual scores. For example, a 200 % increase (large increase) was observed when a rat displayed a behaviour for one second in the baseline test and two seconds in the final test (inconsequential increase). Therefore, the actual scores were examined alongside the 'percentage of baseline' value.

Stress-sensitivity was defined as a stress-induced change in behaviour with a value greater than one standard deviation from the mean.

Both increased and decreased sucrose preference was observed in the light phase, but both the control or UCMS group had only 1-4 rats which showed a stress-induced change greater than one SD (Figure 5.7a-b). Sucrose intake showed a similar pattern (Figure 5.7c-d). These numbers were insufficient to provide power for statistical testing of a separate "stress-sensitive group".

Behaviour measures from the light-dark box test was assessed in the same way. Most of the behavioural variables had several rats with scores which categorised them as stress sensitive. However, there was not consistency between measures as to which rats were defined as stress sensitive. This meant that an overall stress-sensitive group could not be determined.

Because no grouping of stress-sensitive and stress-resilient rats was observed, a different statistical approach was taken to assess if the stress-altered behaviours were associated with stress-induced biological changes. Partial least squares and correlational analyses were undertaken to investigate if any potential mechanistic variables (LBP, cytokines, serum, and caecum digesta biogenic amines) were correlated with each other or any behavioural variables.

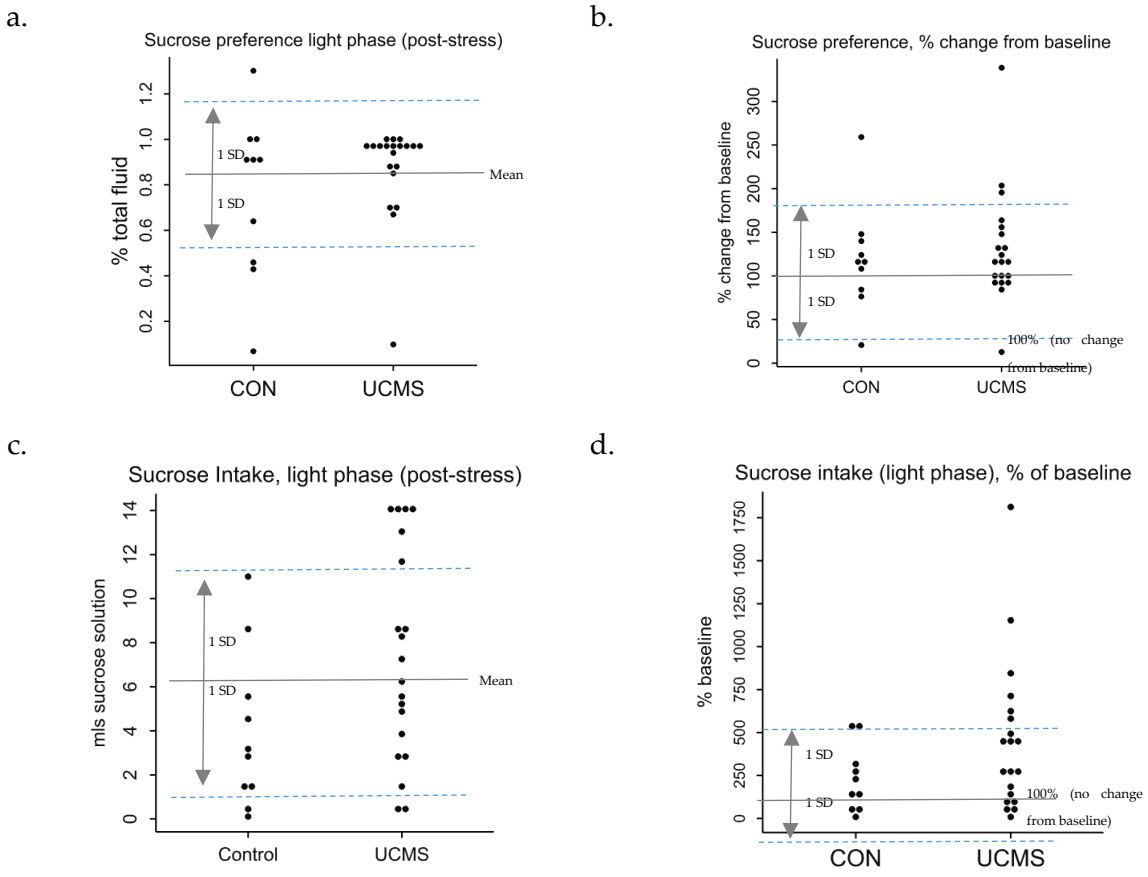

***Figure 5.7**. Individual measurements in the sucrose preference test during the light phase. Stress-resilience was not able to be determined in Sprague-Dawley rats following four weeks of unpredictable chronic mild stress procedure using the sucrose preference test.*

5.4.8 Serum lipopolysaccharide-binding protein

There was no difference in LBP between groups (U (10,20) = 63.0, p = .109, Figure 5.8a) as shown by Wilcoxon rank sum test. Spearman's rank correlation and PLS analyses showed that LPB concentration was not associated with behaviour, cytokine concentration, neurotransmitter concentration, or corticosterone concentration.

5.4.9 Serum cytokines

An increase in serum cytokine concentrations was observed, and PERMANOVA analysis showed the cytokine profile differed between UCMS and control groups (F = 8.38 on 1 and 28 df, p = .002; Figure 5.8b). The difference was not due to a difference in spread, as beta dispersion was not significantly different (F = 1.83 on 1 and 27 df, p = .192).

Several cytokines showed a difference in serum concentration: IL-10 (U (10,19) = 41.0, p = .012), GM-CSF (u (10,19) = 43, p = .016), IL-18 (u (10,19) = 22.0, p < .001), IL-17A (U (10,19) = 26.0, p < .001), IL-33 (u (10,19) = 33.0, p = .003), and IL-6 ((u (10,19) = 28.0, p = .001), (Figure 5.9). The concentrations of IFN-γ, KC, MCP1, TNF-α, IL12p70, IL-1β, IL-1α, IL-18 in serum were similar between groups (p > 0.05) (Figure 5.9).

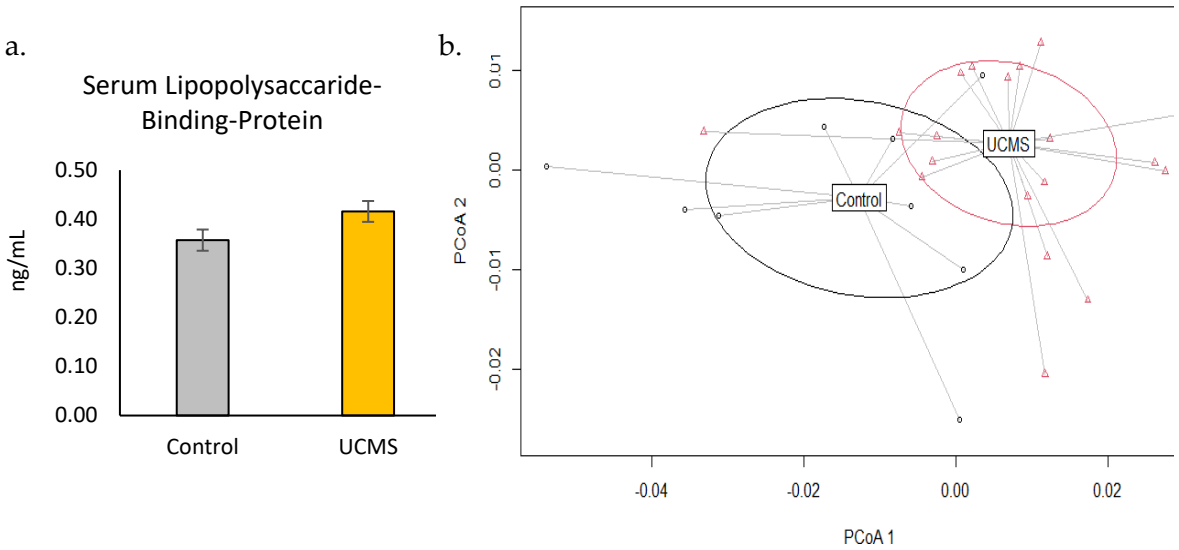

***Figure 5.8**: Serum concentration of lipopolysaccharide-binding protein (LBP), data presented as mean +/- SEM(a), and PCA plot of serum cytokine dispersion (b) of Sprague-Dawley rats following four weeks of unpredictable chronic mild stress (UCMS) procedure and control Sprague-Dawley rats. Control group n = 10, UCMS group n=20.*

5.4.10 Caecal digesta and serum biogenic amines

PERMANOVA analysis and comparison with t-tests showed no difference in caecal digesta biogenic amines between the control and UCMS groups (F = 1.267 on 1 and 21 df, p = .295). PERMANOVA analysis also showed no difference between the concentration of biogenic amines in serum between the control and UCMS groups (F = 1.20 on1 and 21 df, p = .293).

However, there were changes in concentration among individual biogenic amines in both caecum and serum (Table 5.1). In the UCMS group compared to the control group, concentrations were reduced of phenylethyl amine (p = .004), dopamine (p < .001), norepinephrine (p = .004), norepinephrine metabolite 3,4-dihydroxyphenylglycol (DHPG)(p = .013), GABA (p = .001), alpha-aminobutyric acid (AABA (p < .001) the ratio of kynurenine:

tryptophan ($p = .035$). The concentration of 3-methoxy-4-hydroxyphenylglycol (MHPG) ($p = .003$), vanillylmandelic acid, ($p < .001$) and 5-HTP ($p= .005$) was higher in the UCMS group than in the control group.

In the caecum, concentrations of phenylethylamine ($p= .017$), tyrosine ($p = .017$), HVA ($p = .035$) and VMA ($p = .039$) was lower in the UCMS group compared to the control.

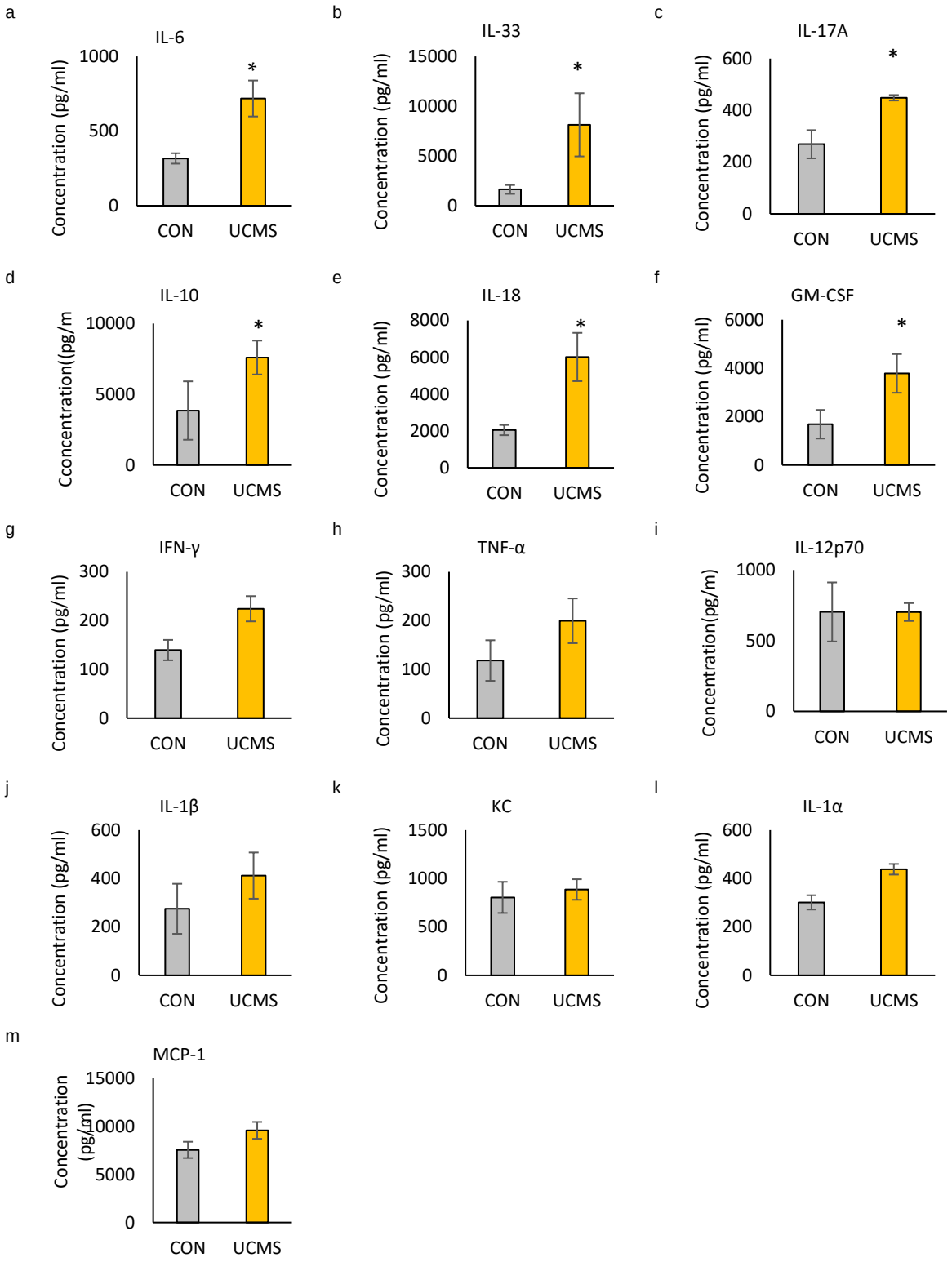

***Figure 5.9**. Serum cytokine concentrations of Sprague-Dawley rats following four weeks of unpredictable chronic mild stress (UCMS) procedure and control Sprague-Dawley rats (CON) .Cytokines include interferon-gamma (IFN-γ), cytokine-induced neutrophil chemoattractant type-1 (CINC-1), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-18, IL-12p70, IL-1β, IL-17A, IL-33, IL-1α, and IL-6. Data are presented as mean +/- SEM, control group n = 10, UCMS group n=20. Significance was assessed at a p-value of 0.05 or less and indicated by *.*

***Table 5.1**. Serum and caecum neurotransmitter concentrations of Sprague-Dawley rats following four weeks of unpredictable chronic mild stress (UCMS) procedure and control Sprague-Dawley rats. Data are presented as the mean of each experimental group ± S.E.M. (n = 10 Control group, n = 20, UCMS group). Bold numbers = p<0.05 between UCMS and control group.*

Pathway	Neurotransmitter	Caecum						Serum					
		Control ng/ml			UCMS ng/ml			Control ng/ml			UCMS ng/ml		
Phenylethyl- amine													
	Phenylethyl amine	**63.91**	**±**	**13.30**	**35.20**	**±**	**5.31**	**0.58**	**±**	**0.05**	**0.42**	**±**	**0.02**
Tyrosine	Tyrosine (TYR)	**11094.20**	**±**	**1449.88**	**7969.56**	**±**	**1175.95**	12413.22	±	781.79	1248.77	±	415.69
	3,4-dihydroxyphenylalanine (DOPA)	172.17	±	18.27	146.27	±	12.07	4.57	±	1.18	3.26	±	0.41
	Dopamine (DA)	101.92	±	14.25	90.72	±	10.26	**0.56**	**±**	**0.04**	**0.40**	**±**	**0.02**
	3-methoxytyramine (3MT)	5.28	±	1.39	4.97	±	1.21	0.21	±	0.01	0.18	±	0.01
	3,4-dihydroxyphenylacetic acid (DOPAC)	1351.55	±	570.06	945.04	±	405.26	3.10	±	0.39	2.91	±	0.25
	Homovanillic acid (HVA)	**30.81**	**±**	**5.63**	**23.57**	**±**	**10.27**	1.84	±	0.24	1.85	±	0.12
	Norepinephrine (NE)	17.14	±	1.98	17.66	±	1.49	**41.43**	**±**	**3.59**	**29.96**	**±**	**1.68**
	3,4-dihydroxyphenylglycol (DHPG)	74.13	±	4.60	78.20	±	3.37	**2.12**	**±**	**0.10**	**1.80**	**±**	**0.08**
	3-methoxy-4-hydroxyphenylglycol (MHPG)	15.13	±	3.13	12.36	±	3.15	**2.88**	**±**	**0.13**	**3.47**	**±**	**0.11**
	Normetanephrine (NM)	9.22	±	1.93	7.52	±	1.06	2.91	±	0.21	2.63	±	0.24
	Epinephrine (E)	3.09	±	0.33	3.16	±	0.34	36.39	±	4.97	28.95	±	2.87
	Metanephrine (MN)	1.49	±	0.28	1.26	±	0.16	0.87	±	0.11	0.90	±	0.08
	Vanillylmandelic acid (VMA)	**37.39**	**±**	**4.22**	**26.79**	**±**	**2.07**	**0.53**	**±**	**0.07**	**1.12**	**±**	**0.13**
Tryptophan	Tryptophan (TRP)	4384.37	±	1519.77	2767.53	±	516.56	12206.87	±	570.84	14065.97	±	681.88
	Kynurenine (KYN)	1219.50	±	193.42	1004.12	±	172.38	3518.87	±	354.76	2923.52	±	101.38
	5-hydroxytryptophan (5-HTP)	30.91	±	7.94	19.63	±	2.44	**10.93**	**±**	**0.62**	**13.84**	**±**	**0.58**
	Serotonin	103.78	±	12.50	111.58	±	9.62	1267.55	±	104.39	1427.32	±	109.22
	5-hydroxyindoleacetic acid (5-HIAA)	116.27	±	33.80	120.92	±	18.08	11.71	±	1.00	11.26	±	0.53
	Ratio 5-HT/KYN	0.13	±	0.03	0.17	±	0.03	0.39	±	0.04	0.50	±	0.04
	Ratio KYN/TRP	0.39	±	0.11	0.36	±	0.04	**0.29**	**±**	**0.03**	**0.22**	**±**	**0.01**
	Ratio 5-HT/TRP	0.06	±	0.02	0.07	±	0.01	0.11	±	0.01	0.11	±	0.01
GABA	Alpha-aminobutyric acid	99.35	±	14.46	101.45	±	7.47	**5.29**	**±**	**0.63**	**2.33**	**±**	**0.13**
	Gamma-aminobutyric acid	2116.66	±	130.51	2286.42	±	107.63	**160.65**	**±**	**9.72**	**119.0**	**±**	**4.54**

5.4.11 Multivariate analysis

PLS with Osten's test showed a significant, negative association between serum cytokines and serum biogenic amines, with the serum biogenic amines as the independent variable (f = 2.2 (df = 23,621), p <. 001). A correlation loading plot shows the correlations of serum cytokines and serum biogenic amines with the identified PLS dimension (Figure 5.10). Individual correlation analysis showed significant correlations between several serum biogenic amines and cytokines. Scatterplots of significant correlations are shown in Figure 5.11. AABA showed moderate (r>0.4) to strong negative correlations (r > .7) with IL-17a, IL-1a and IL-10. In addition, a moderate negative correlation (r = -.544) was found between IL-1a and GABA, and IL-1a with 3-methoxytyramine (r = -.473).

No significant dimensions were identified by PLS regression analysis with caecum biogenic amines as the independent variable. This result indicates there was no relationship between caecum biogenic amines and serum biogenic amines , cytokines, LBP, faecal corticosterone, or behaviour. LBP, faecal corticosterone, serum cytokines and serum biogenic amines were not associated with behaviour.

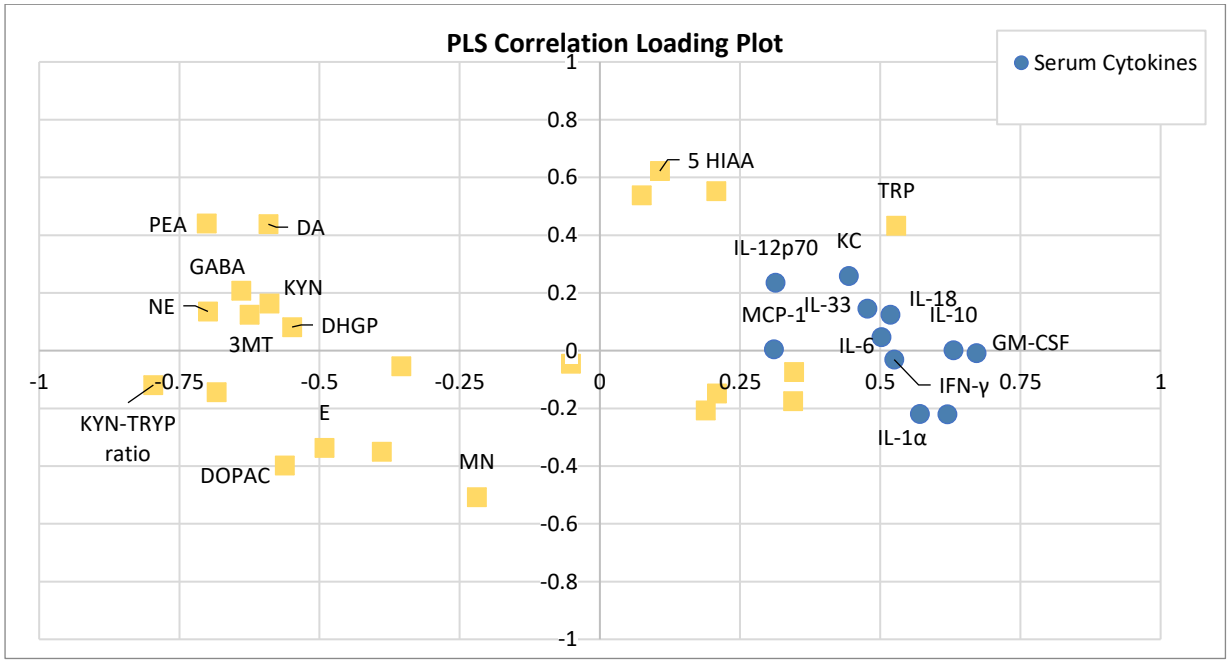

Figure 5.10. *Correlation loading plot of Partial Least Squares analysis of serum biogenic amines and serum cytokines of Sprague-Dawley rats following four weeks of unpredictable chronic mild stress procedure and control Sprague-Dawley rats. Only Dimension 1 was significant, meaning that variables that extend from the centre point in a similar direction along the x-axis are associated.*

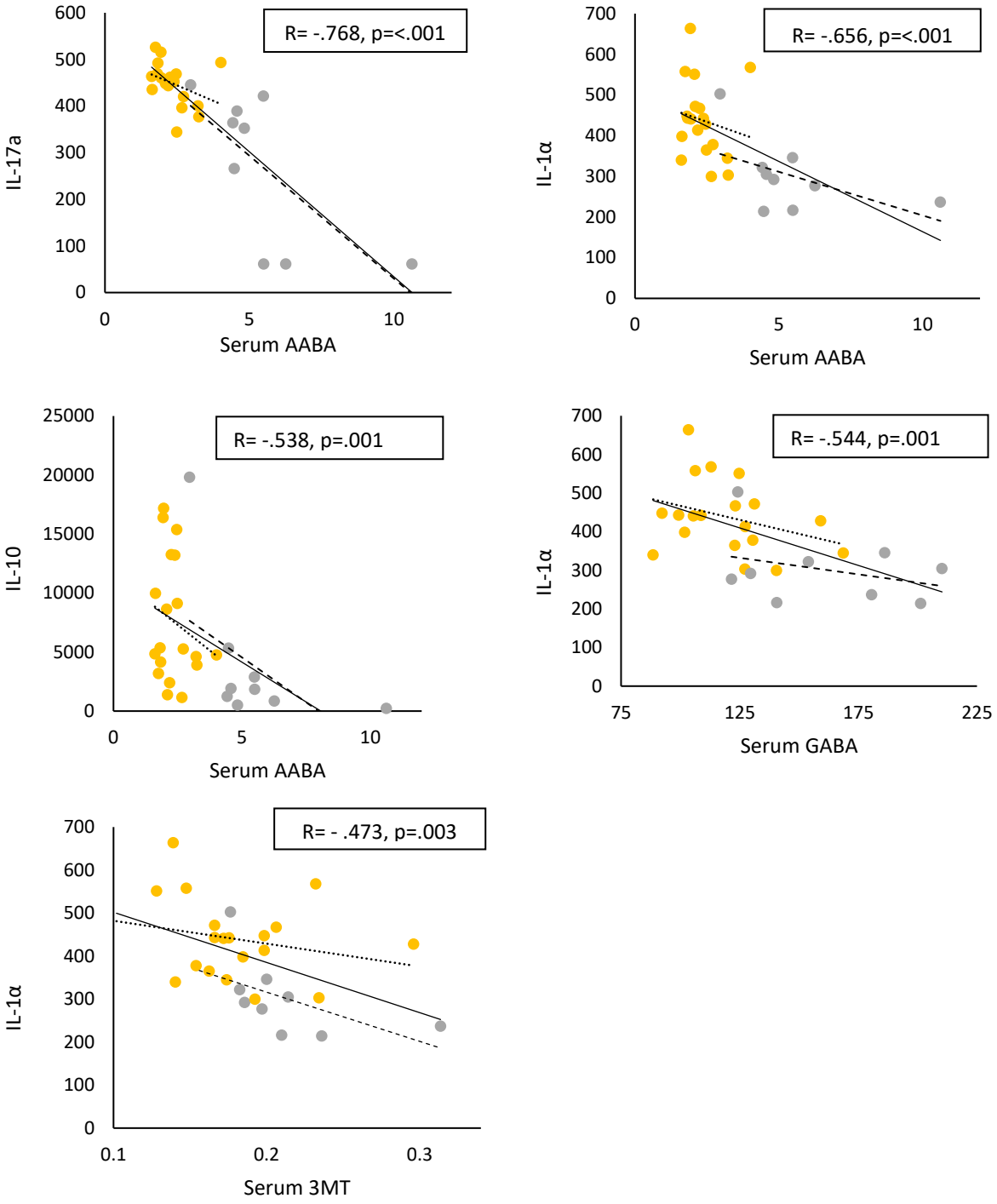

Figure 5.11. *Correlation plots between serum biogenic amines and cytokines in Sprague-Dawley rats following four weeks of unpredictable chronic mild stress (UCMS) procedure and control Sprague-Dawley rats. Abbreviations: IL-, Interleukin; ABBA, alpha-aminobutyric acid; GABA gamma-aminobutyric acid; 3MT, 3-methoxytyramine. Correction for an FDR rate of 5% using the Benjamini–Hochberg procedure indicated a critical p-value for the significance of 0.003.*

5.5 Discussion

The results of the current study suggest that the UCMS group experienced stress, which was demonstrated by an increase in faecal corticosterone, an increase in behavioural inhibition (reduced time in the light box and latency to enter the light box in the light dark box test, and decreased time in and entries into centre in the OFT), a decrease in locomotion in the OFT and a decrease in exploration in the light-dark box (rearing time, rearing number, transitions between zones) compared with baseline values. No typical depressive-like behaviours were observed, with no change in the PST and no decrease in sucrose preference in the 24hr SPT. An increase in sucrose preference and sucrose intake from baseline were found in the light phase of the SPT. The UCMS rats could not be differentiated into stress-sensitive and stress-resilient groups as per the first hypothesis.

5.5.1 Atypical Behavioural Results

Interestingly, the control group also showed an increase in faecal corticosterone from baseline and displayed similar behavioural changes to the UCMS group in the light-dark box test and OFT over time. Additionally, the change in faecal corticosterone and behaviour from baseline was greater in the control group, suggesting that the control group may have experienced a higher level of stress than the UCMS group.

As discussed in Chapter 3 section 3.4.1.1, the reason for stress in the control group is unclear but possible reasons include differences in the laboratory environment or handling (Balcombe et al., 2004; Morgan & Tromborg, 2007). It may have been isolation stress due to single housing, with the UCMS less affected by isolation stress due to UCMS induced adaptation. The current study is not directly comparable with the study undertaken in female SD rats reported in Chapter 3 because of changes in methodology in the current study, including the light cycle not being reversed, behavioural tests done in the light phase instead of the dark phase, and a different length of the UCMS procedure. Regardless, the results are generally consistent with the UCMS study reported in Chapter 3 and support the conclusion that the control rats were stressed in both studies.

In contrast to previous studies using the UCMS method, no depression-like behaviour was observed in either the 24-hour SPT or PST in the control or UCMS group. However, there was an increase in sucrose intake and sucrose preference in the UCMS group but only during the

light phase. An increase in sucrose intake over 24 hours was also observed in female SD rats in Chapter 3. Rats are typically less active in the light phase and sleep for much of it. It was suggested that a possible reason for the increased sucrose intake in the UCMS rats in Chapter 3 could be due to increased time awake during the light phase because of a disrupted circadian rhythm. The UCMS procedure used in both studies included altering the light schedule (lights on during the dark phase or alternating light and dark every two hours during the dark phase) which is likely to have caused some circadian disruption. This hypothesis is supported by the results from the current study, in which the increase was only seen in the light phase, and was only seen in the UCMS group, despite the control group having likely experienced a higher level of stress.

5.5.2 Effect of stress on physiological variables

No difference was found between the UCMS and control groups in gut permeability marker serum LBP, indicating that it was not affected differently by different stress levels. Reduced serum cytokine concentrations in the control group compared to the UCMS group were consistent with increased stress in the control group, due to anti-inflammatory effects of increased hypothalamic-pituitary-adrenal (HPA)-axis hormones (Sorrells & Sapolsky, 2007). However, none of the cytokines correlated with faecal corticosterone, epinephrine, or norepinephrine. Likely, the timing of the sample collection was sub-optimal for comparing these variables. Faecal corticosterone was taken prior to starting the behavioural tests, while the serum was collected at the time of euthanasia (two weeks later). HPA-axis hormones were likely to have increased acutely during euthanasia using CO_2 (Boivin et al., 2017), and it is unlikely that the short timeframe (minutes) would be enough for the anti-inflammatory effect of HPA-axis hormones to be effective. The cytokines would be more representative of the baseline levels in the days before euthanasia.

Differences in serum biogenic amines seem consistent with the control group being more stressed. The lower concentrations of serum phenylethylamine and HPA-axis neurotransmitter norepinephrine and its metabolites 3,4-dihydroxyphenylglycol and 3-methoxy-4-hydroxyphenylglycol in the UMCS group conflicts with previous research in male SD rats exposed to chronic noise stress (Di et al., 2011) or people exposed to acute stress

(Paulos & Tessel, 1982) but are consistent with a higher level of stress being experienced by the control group.

There was no difference between the groups in serum serotonin, which is in contrast with previous stress studies which have shown reduced levels of serum serotonin following chronic stress in rats (Di et al., 2011; Li et al., 2021; X. Xie et al., 2017) mice (Wang et al., 2019) and people (Paulos & Tessel, 1982), compared to unstressed controls. There was however reduced concentrations of serotonin precursor 5-HTP in the control group, as well as a higher ratio of kynurenine to serotonin, which suggests a shift in the tryptophan pathway towards kynurenine and away from serotonin production. It may be that shift was not large enough to reduce serotonin to a level which caused depressive-behaviours, and/or there was not enough of a difference between groups to show the effect of stress, since the comparison is between stressed rats and less stressed rats, rather than a stressed and unstressed group.

Lower concentrations of serum dopamine, GABA and AABA concentrations were observed in the UCMS group compared with the control group. The relationship between serum GABA, stress and mood is unclear. A difference was expected, as a bidirectional interaction between the neuroendocrine stress axis, and GABA synapse has been observed (Inoue et al., 2013; Inoue & Bains, 2014). In male BALB/c mice GABA was decreased following chronic stress along with the development of depressive-like symptoms (Wang et al., 2019). In contrast, plasma GABA levels were increased in humans with depression and were accurate as a predictive biomarker of depression (Pan et al., 2018). Similarly mixed results are seen with serum dopamine which has been shown to be both increased and decreased in rodents following chronic stress (Li et al., 2021; Wang et al., 2019; Xiaoxian Xie et al., 2017). In humans with depression, plasma GABA and dopamine was increased (Pan et al., 2018), and serum GABA was found to be not linked with anxiety (Zwanzger et al., 2003). Because there is no clear direction in which GABA and dopamine are altered following stress, the concentrations observed in the current study are plausibly consistent with stress in the control group.

A wide range of concentrations of caecal digesta biogenic amines was seen but a comparison of epinephrine, norepinephrine and dopamine concentrations showed they were within the range of concentrations reported in the caecum digesta of male BALB/c mice (Asano et al., 2012). There were lower concentrations of caecal digesta phenylethylamine, tyrosine, HVA

and VMA, in the UCMS group compared with the control group. Trace amines in the gut including phenylethylamine have been shown to act as anti-microbials, increased gut contractile activity, and stimulate secretion of monoamine neurotransmitters (reviewed in Bugda Gwilt et al., 2020). They have also been linked with neuropsychiatric and gut inflammatory disorders, in association with their receptors trace amine-associated receptors (TAARs) (Christian & Berry, 2018; Gainetdinov et al., 2018). Stress induced changes in trace amines in the gut could be linked with stress-induced flare-ups of these disorders, but more research is needed to determine what, if any effect the concentrations in the gut have.

Whether the changes in serum inflammatory markers, and serum and caecal biogenic amines were related to stress-induced behaviour in this study was assessed with correlation analysis.

5.5.3 Gut permeability, inflammation and tryptophan metabolism

One of the aims of the study was to assess if stress-induced behavioural changes in male rats were associated with increased gut permeability, increased systemic inflammation, and altered tryptophan metabolism. The hypotheses that stress-sensitive rats will have increased levels of systemic pro-inflammatory cytokines and LBP compared with the control group and stress-resilient male rats was not able to be directly tested because the UCMS exposed rats could not be differentiated into stress-sensitive and stress-resilient groups. Instead, the relationship between stress-induced behaviour and serum cytokines, LBP, caecal and serum biogenic amines was tested with correlation analysis.

No correlations were observed between behaviour outcomes, LBP concentrations, cytokine concentrations, serum serotonin, serum kynurenine or other serum tryptophan metabolites. This result suggests that the stress-induced changes in anxiety-like behaviour were not associated with gut-permeability associated inflammation. Increased serum concentration of serotonin precursor 5-HTP, and a reduced kynurenine: tryptophan ratio in serum in the UCMS group are suggestive of increased kynurenine pathway activity in the control group. This was likely not due to immune activation of the kynurenine pathway enzyme IDO, as serum cytokines were lower in the control group. Likely, the increased 5-HTP and the reduced kynurenine concentrations in serum were linked with corticosterone levels. Other studies have shown that corticosterone increases kynurenine pathway activity (Dalvi-Garcia et al., 2021; Qiu et al., 2021).

5.5.4 Caecal and Serum Biogenic amines

This study also aimed to determine whether changes in biogenic amine concentrations in the caecal digesta were associated with behavioural changes and whether they correlated with concentrations of serum biogenic amines or serum inflammatory markers.

No correlation was observed between any caecal digesta biogenic amine concentration and behavioural outcomes, serum biogenic amines concentrations, or serum cytokines. These results therefore indicate that caecum digesta biogenic amine concentrations were not a mediator for the stress-induced behavioural changes or serum neurotransmitter concentrations. It is still possible that neuro- and immunomodulatory effects could occur within the gut which could act as an MGBA mechanism.

The negative correlations between serum GABA, AABA , and 3MT with cytokines is consistent with the extensive crosstalk between the immune system and the nervous system (Tian et al., 2012). In addition, serotonin and GABA are both immunomodulatory molecules known to decrease pro-inflammatory processes (Bhandage et al., 2018; Herr et al., 2017). However, as neither cytokines nor serum neurotransmitter concentrations were associated with behaviour outcomes, it is unlikely either was the mechanism for changes in stress-induced behaviour changes in this study. Similarly, there was no change in depression-like behaviour despite changes in serum cytokines and biogenic amines, indicating no relationship.

5.5.5 Strengths and Limitations

The current study had several strengths. Well-established published methods were used, with standardised protocols. This makes the results comparable to other studies with the same methods. Having baseline measurements for faecal corticosterone as well as behaviour meant that the change in behaviour in the UCMS group was able to be assessed without the reliance on having an unstressed control group.

Assessing metabolites in caecal digesta rather than faecal samples meant that the concentrations were more accurate for the levels that occur in the gut and that the host is exposed to. Faecal samples have already had some nutrients and metabolites absorbed in the large intestine and may have altered levels due to further microbial metabolism in the large intestine. However, having the caecal digesta and serum samples only from the end timepoint

after the stress meant that a true stress-induced change in the variables measured from these samples was not able to be assessed, particularly as the control group was not unstressed.

The choice of the SD rat strain was intended to be a strength, and to make heterogeneous responses in the UCMS group more likely. However, the SD rats were likely too robust for the UCMS stressor protocol chosen, which used relatively mild stressors compared with the range of stressors commonly used (Antoniuk et al., 2019). It is possible that the SD rats may have needed a more stressful intervention.

5.6 Conclusion

The study showed that chronic stress in Sprague-Dawley male rats did not induce anhedonia or behavioural despair, both depressive-like behaviours, but reduced locomotion and exploration and increased anxiety-like behaviours. The four-week UCMS procedure combined with isolation stress single housing was less stressful than isolation stress alone in Sprague-Dawley male rats. The UCMS exposed rats were not able to be divided into stress-resilient and stress-sensitive groups.

The study aimed to assess if a stress-induced increase in gut permeability could be associated with stress-induced behaviour changes via inflammation and alterations to tryptophan metabolism. These variables were not found to be associated with anxiety-like behaviour. The results of this study do not however discount a positive association between increased gut permeability and serum immune markers with depressive-like behaviour. Much like the study in female rats reported in Chapter 3, it is possible that a lack of increase in gut permeability was the reason that the rats did not develop depressive-like symptoms.

The study also aimed to assess if stress-induced changes in caecal biogenic amines would be associated with the concentrations of serum biogenic amines, and/or stress-induced behaviour, and neither of these associations were found. The study is not able to exclude gut biogenic amines as a mechanism under different conditions, however the results are significant and indicate that changes in biogenic amines in the gut are not a sole mechanism for stress-induced anxiety changes.

The results of this study contribute unique knowledge to the growing body of MGBA research and move to determine mechanisms driving the relationships observed between the gut

microbiome and the brain and behaviour. It is probable that MGBA mechanisms for anxiety-like behaviour differ from those of depression-like behaviour and this deserves further follow-up. Further research into the implications of stress-induced changes in gastrointestinal biogenic amines would also be useful.

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Chapter 6.

Discussion, Conclusion and Areas of Further Research

6.1 Introduction

The high burden of mood disorders such as depression and anxiety on the healthcare system means there is a need for a better understanding of their pathophysiologic mechanisms, as well as identify more treatment options and preventative measures. Recent research shows links between the gut microbiome and diet with mood. Experiencing stress is a strong risk factor for developing an episode of depression or anxiety, and stress can alter the gut microbiota. It is plausible that stress-induced changes in the gut microbiome and associated microbiome-gut-brain-axis mechanisms could be a causative factor in whether an individual develops anxiety or depression following stress. The aim of this thesis was to investigate whether stress-induced changes in the gut microbiome, microbial metabolites, gut permeability, and/or systemic inflammation are associated with stress-sensitivity.

6.2 Summary of the research

The first study used chronic stress in female Sprague-Dawley (SD) rats with the aim of inducing anxiety-like and depressive-like symptoms in half of the stress exposed rats (stress-sensitive rats), for comparison with rats which did not develop these symptoms (stress-resilient rats) and with an unstressed control group. The caecal microbiota composition, caecal organic acids, a gut permeability marker (Lipopolysaccharide binding protein (LBP)), and systemic inflammatory markers (cytokines) were measured. The hypotheses were:

Hypothesis 1: Gut microbiota, SCFAs, plasma LBP, and cytokines differ between stress-resilient and stress-sensitive rats, but not differ between the stress-resilient rats and the control group, and/or

Hypothesis 2: Anxiety-like and depressive-like behaviours would correlate with any or all of the gut microbiota, SCFAs, plasma LBP, and cytokines.

The UCMS model of depression and anxiety did not induce the expected anxiety-like and depressive-like behaviours in the rats. The only significant behavioural change was an increase in sucrose solution intake during the sucrose preference test, which is not a behaviour which has an established meaning. In addition, the control group had higher faecal corticosterone than the UCMS group, and a non-significant pattern of higher anxiety-like behaviour in light-dark box test. These findings suggest that the control rats may have been more stressed than the UCMS rats. Stress resilience was unable to be determined, so associations between all variables were assessed using correlation analysis. Both positive and negative associations between several bacteria were found with anxiety-like behaviour and cytokines. No association was found between behaviour and cytokines or LBP.

The association between the relative abundance of some microbial taxa and anxiety behaviour was an interesting result, but the uncertainty of whether the UCMS group was stressed or not, and the lack of depressive-like behaviours in the rats made it difficult to draw a concrete conclusion. Understanding what caused these atypical results could help to develop the methodology for another study focussing on stress-resilience. Therefore, three studies were then completed to investigate possible reasons for the unusual results in study one.

A possible reason for the increased sucrose solution intake was first investigated. Because the sucrose solution concentration could affect results in the sucrose preference test, the amount of sucrose solution consumed by rats at different concentrations was tested to assess if the rats showed a similar pattern of intake to previous published findings. The findings showed that the pattern of consumption was in an inverse u-shape, similar to other studies, and that the concentration used was likely to be effective.

Because no difference was observed in the PST following chronic stress, a validation test of the PST using two antidepressant drugs was undertaken. Results showed that neither of the two administered drugs (fluoxetine and imipramine) reduced immobility or altered swimming or climbing time compared with a group given only a vehicle solution. This finding indicated that the female SD rats used are not sensitive to changes in serotonin.

Results at this point suggested that the female SD were not responding to either acute or chronic stress in a typical way. Options considered were to change to a different stress model of depression, a different rat strain, mice, or male rats. Because male and female rats were

readily available, it was decided to see if sex could be the reason for the atypical results in the female SD rats.

Male and female rats show different behaviours in the same tests and can also vary in different laboratories and so a comparison of male versus female baseline behaviours was undertaken. Differences in anxiety-like behaviours (Light-dark box test) and locomotion (OFT) but not depressive-like behaviours (PST) were found. This finding gave cause to investigate UCMS in male rats to determine if they responded in a more typical way than the initial chronic stress study in female rats.

A chronic stress study was then undertaken in the male SD rats, with the aim again of identifying stress-sensitive and stress-resilient rats. Adjustments to the study design were made. The length of the UCMS procedure was reduced to four weeks and behavioural testing was undertaken before and after the chronic stress protocol so that changes over time could be seen independently in each group.

Six hypotheses were explored. These were:

Hypothesis 1: The stress-sensitive rats will have increased levels of systemic pro-inflammatory cytokines and LBP compared with the control group and stress-resilient male rats.

Hypothesis 2: Depressive-like and anxiety-like behaviours will be negatively correlated with systemic serotonin and/or positively correlated with kynurenine and/or kynurenine metabolites in all rats.

Hypothesis 3: Decreased serotonin pathway metabolites and increased kynurenine pathway metabolites in the caecal digesta and serum will be associated with an increase in serum LBP and pro-inflammatory cytokines.

Hypothesis 4: The stress-sensitive rats will have altered serum and/or caecal digesta metabolites compared with the control group and stress-resilient male rats.

Hypothesis 5: Caecum metabolites will be positively correlated with serum metabolites and behaviour differences.

Hypothesis 6: Caecum and/or serum metabolites GABA, acetylcholine and/or noradrenaline will be correlated with inflammatory markers.

Faecal corticosterone results showed that both control and UCMS groups had experienced stress over the four-week period. The control group showed an increased level of faecal corticosterone suggesting the control group rats were more stressed than the UCMS rats. Higher levels of serum catecholamines in the control group was consistent with this possibility. Behavioural tests showed both groups had reduced locomotion and increased anxiety-like behaviours, with the biggest difference occurring in the control group. The UCMS group also showed increased serum cytokines, and several differences between groups in biogenic amines in the serum and caecum digesta were observed. Again, the rats were not able to be differentiated into stress-sensitive and stress-resilient and did not show differences over time in tests of depression-like behaviour: the 24hr sucrose preference test or PST. Increased sucrose preference and intake was observed in the UCMS group compared with the baseline and the control group during the light phase. This finding suggests that the behaviour may be related to disrupted circadian rhythm and increased wakefulness during the light phase.

Assessment of physiological variables showed that there was no association between behaviour and LBP, faecal corticosterone, serum cytokines, caecal digesta neurotransmitters or serum neurotransmitters. Serum neurotransmitters were found to negatively correlate with serum cytokines, both individually and as a group. No correlations were found between caecal digesta neurotransmitters and serum neurotransmitters.

6.3 Discussion of the main findings and further research ideas

The association found between the relative abundance of several caecal microbes and anxiety-like behaviour is an exciting find and adds to the growing body of research in the MGBA area. It is especially significant that the same families (*Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales Family_*XIII) have been found to be associated with anxiety-like behaviour in two other animal studies (van der Eijk et al., 2019; Verbeek et al., 2021). Neither of these studies nor those reported in this thesis can demonstrate causality, and it could be that the gut microbiota composition is strongly influenced by anxiety. One plausible reason is that physiological changes due to anxiety (e.g., increased hypothalamic-pituitary-adrenal axis and

sympathetic nervous system activation) could be creating a gut environment which is conducive to the growth of these specific bacteria.

It is interesting that the association was apparent in both groups and therefore not dependent on the stress intervention. This finding suggests a pre-existing difference, although it is possible both groups were stressed and had a similar stress-induced increase in the relative abundance of these bacteria. It is unfortunate that the faecal samples from the second chronic stress study were lost. The faecal samples taken before and after the intervention would have allowed comparison from baseline so that stress-induced changes in each group could be determined. It could also show whether pre-existing microbiota composition were associated with stress-induced changes in anxiety.

Comparing the relative abundance of specific microbes between treatment groups and studies is plagued with issues. Some specific microbes may be significant in the MGBA, however different microbes can occupy similar environmental niches and carry out similar functions. This means that bacteria showing an association with behaviour in one study may not be observed in another study because a different microbe is occupying the environmental niche. Looking at microbial gene expression and metabolic pathways using KEGG analysis and/or metagenomics analysis is more likely to allow researchers to determine patterns of change which are consistent between studies and therefore are key mediators/mechanisms.

The finding that caecal digesta neurotransmitter metabolites were not associated with serum metabolites is an important piece of the MGBA puzzle. Microbial-derived metabolites have been proposed as a key mechanism for the effects of the gut microbiota composition on emotional behaviour (Needham et al., 2020). Gut metabolites could still have an important effect though via changes in gut inflammation and neural activation.

The observed association between microbiota and anxiety-like behaviour, but no observed associations with possible mediators such as organic acids, neurotransmitters in caecal digesta, systemic inflammation or gut permeability is also a key finding. This finding could be due to reverse causality, with anxiety being the moderator of the microbiota; both the microbiota and anxiety-like behaviour being moderated by another variable; or could be due to a different mechanism mediating an effect of anxiety on microbiota composition.

Further research should include:

- A repeat of the UCMS study with both male and female SD rats with baseline samples of all variables to compare with post-UCMS samples.
- The association of the gut microbiota with anxiety-like behaviours could be tested for causation by undertaking faecal transplants from rats with high baseline anxiety-like behaviours to those with low baseline anxiety-like behaviours and vice versa.
- On a similar note, investigating effects on behaviour due to manipulation of the gut microbiome could be done using antibiotics, or comparing a high and low fibre diet.
- Measuring markers of gut inflammation alongside gut microbial metabolites and behaviour.

As discussed in Chapters 3 and 5, the lack of association between the gut microbiota composition and depressive behaviours could mean that there is no association, but counterintuitively, the lack of depressive-like behaviour occurring alongside a lack of change in stress-induced gut permeability to lipopolysaccharide is also a finding that supports the hypothesis that there is a link between gut permeability and depression.

- Further research could test this by artificially increasing gut permeability with non-steroidal anti-inflammatory drugs. It would be useful to do this with and without stress and to test the effect on behaviour and systemic inflammation.

It was unfortunate that the chronic stress used did not induce depressive-like behaviours in the rats, and that the rats were not able to be split into stress-sensitive and stress-resilient groups. Other studies have found that stress increases anxiety but not depression in SD rats (Lovelock & Deak, 2019; Riaz et al., 2015). The similarity in results between the current male and female UCMS studies suggests that under these conditions gender had little effect. It may be the effect of rat strain - SD rats are more resilient to stress and possibly they need a less mild version of chronic stress. Studies which have found depressive-like symptoms induced a more intense stressor such as restraint stress (Zhang et al., 2016), cold temperature swimming every day, tail clamp, heat stress, and food and water deprivation (Wang et al., 2016). The higher resilience to stress is likely due to genetic differences as SD rats have been shown to have a reduced corticosterone response to chronic stress compared to Long Evans rats (Bielajew et al., 2002), and decreased binding affinity of serotonin agonist [^{3}H]-DPAT to

the 5-HT_{1A} receptors in the hippocampus following chronic stress, compared with an increase in the more stress-sensitive WKY rats (Paré & Tejani-Butt, 1996).

There are differences in behavioural responses to any intervention, including stress between different laboratories with the same strain of rodent. While this variation is likely to be largely due to differences in the rodents' life experience (transport, cage size, researchers, noise), it is also entirely possible that differences in the gut microbiota could be partly responsible.

Comparing stress-resilient with stress-sensitive individuals is a useful way to investigate the key mechanisms behind stress resilience. If a model were to be used again to test this hypothesis there are several research approaches that could be taken.

- UCMS could be tried with a different set of chronic stressors, a different (more stress-sensitive) rat strain, or mice instead of rats.
- Alternatively, a different stressor could be used. Early life (post-natal) stress has been shown to affect the immune system, the gut microbiota and behaviour later in life (O'Mahony et al., 2009; Pusceddu et al., 2015; Shu et al., 2015). Interestingly, adolescent and early life stress can also be protective of stress-induced behavioural changes in adulthood (Rana et al., 2016; Shu et al., 2015), and comparing several groups with different combinations of no stress, early life stress and adulthood stress could create differing groups by which to compare stress-resilience and stress-sensitivity.
- Improving measurements of stress would help to determine the stress levels of the rodents. This could include measuring longitudinal faecal corticosterone levels, comparing plasma corticosterone with faecal corticosterone, and measuring adrenal weight.
- Comparison of stress levels and behaviour between socially isolated and group housed rats would also be useful.

There are limitations to using animal models as a proxy. Rodent behaviour used as a proxy for human anxiety and depression has face validity, which is a superficial similarity between the behaviours. Predictive validity, where interventions such as antidepressant drugs will change the behaviour is often tested, but the issue with this is that antidepressant drugs do not always work in people, and when they do it tends to be with chronic administration, whereas acute administration can change behaviours in rodents. Construct validity is based

on the behaviours being tested having the same underlying biological causes, but this becomes circular when the tests are used to determine and describe those same causes. Based on the difficulties with animal research, investigating the differences in the gut microbiota, gut permeability and systemic inflammation in humans would also be a good approach. The strength of using humans is that stress is easier to measure, with perceived stress able to be measured as well as physiological markers. However, inducing chronic stress would not be ethical so stressed and unstressed people would need to be recruited and the study would be more correlative than in a rodent study.

6.4 Concluding remarks

This thesis aimed to investigate associations between the gut microbiome and behaviour, and potential mechanisms, in the context of stress-sensitivity and stress-resilience in SD rats. The rats showed atypical results following the interventions, but the results still provided useful and unique information which contributes to the body of MGBA research. There is still a lot of research required to unravel and understand the interactions between the gut microbiome, diet, physiology, and psychology. While the research presented in this thesis did not find any relationship with stress-resilience and stress-sensitivity, this hypothesis is worth researching further, particularly in the context of depression and anxiety disorders.

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Appendix A. Table of correlation values between behaviour and plasma cytokines, caecal organic acids, and caecal microbiota

Table A.1. R values of correlations between behaviour and plasma cytokines, caecal organic acids, and caecal microbiota, before correction for false discovery rate.

		Coat state	Light Dark Box Test									Porsolt Swim Test				Sucrose Preference Test		
		Score week 5	Head poke time	head poke number	Latency to enter the light box	Time spent rearing	Number of rears	Time in stretch-attend posture	Number of stretch-attend postures	Time in the light box	Number of transitions	Climbing	Faecal Pellets	Immobility	Swimming	Sucrose Intake	Sucrose Preference (Baseline)	Sucrose Preference (Week 6)
	Cort_wk6																	
	Cytokine GM-CSF			0.393														
	Cytokine IFNγ			0.435														
	Cytokine IL-10			0.392														
	Cytokine IL-12p70													-0.471				
	Cytokine IL-17A													-0.414				
	Cytokine IL-18			0.384														
	Cyt_IL1alpha																	
	Cyt_IL6																	
	Cytokine IL-33													-0.404				
	Cytokine CINC-1																0.392	
	Cytokine MCP-1																0.388	
	Cytokine TNF-α													-0.503				
	LBP																	
	SCFA_Acetate											0.366			-0.317		0.421	
	SCFA_Butyrate																	-0.318
	SCFA_Formate																	
	SCFA_Heptanoate					0.343												
	SCFA Hexanoate											0.459	-0.354					
	SCFA_Isobutyrate	0.337													0.314	-0.326	-0.315	
	SCFA Isovalerate				-0.4	0.306	0.337			0.383	0.359					-0.389	-0.355	
	SCFA Lactate														-0.344			
	SCFA Propionate			-0.401								0.384				-0.589		-0.465
	SCFA Succinate							-0.337	-0.32								-0.406	
	SCFA Valerate				-0.324		0.316			0.32	0.328					-0.419		-0.311
Taxa_1	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Bifidobacterium;__	0.318				0.311				0.352	0.337							
Taxa_2	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Rothia;__			-0.397	-0.323												-0.344	
Taxa_3	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Atopobiaceae;D_5__Coriobacteriaceae UCG-002;D_6__uncultur															-0.327	0.306	
Taxa_4	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriales Incertae Sedis;D_5__uncultured;D_6__gut met				0.439	-0.339	-0.329			-0.422	-0.492			0.319				
Taxa_5	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Adlercreutzia;D_6__uncultured bacteriu				0.359					-0.372	-0.354				-0.302			-0.534
Taxa_6	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__DNF00809;D_6__uncultured bacterium				0.394					-0.407	-0.365							
Taxa_7	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Enterorhabdus;D_6__uncultured bacteri		-0.386															
Taxa_8	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Enterorhabdus;__											-0.438						
Taxa_9	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;__;__		-0.357	-0.349								-0.304						
Taxa_10	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__uncultured;D_5__uncultured bacterium;D_6__uncultured bacte													0.333				
Taxa_11	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidaceae;D_5__Bacteroides;__																-0.45	
Taxa_12	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;D_5__mouse gut metagenome;D_6__mouse gut meta												-0.33					
Taxa_13	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;D_5__uncultured bacterium;D_6__uncultured bacteri																	
Taxa_14	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;D_5__uncultured organism;D_6__uncultured organis							-0.343	-0.313					-0.381	0.412			
Taxa_15	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;__;__					-0.356	-0.326											
Taxa_16	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Alloprevotella;D_6__uncultured bacterium												-0.509					
Taxa_17	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Alloprevotella;__																	
Taxa_18	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 9;__					-0.459	-0.422											-0.465
Taxa_19	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae Ga6A1 group;D_6__uncultured b													0.566				
Taxa_20	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae NK3B31 group;D_6__uncultured			-0.362		-0.365	-0.358			-0.377	-0.371						-0.343	
Taxa_21	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae UCG-001;D_6__uncultured bacte									-0.302				-0.358				-0.396
Taxa_22	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Alistipes;D_6__uncultured bacterium	-0.422													0.373	0.391		
Taxa_23	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;D_6__uncultured ba															0.32		
Taxa_24	D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;D_4__uncultured bacterium;D_5__uncultured bacterium;D_6__	0.427																
Taxa_25	D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;D_4__uncultured rumen bacterium;D_5__uncultured rumen bac	-0.376		-0.328														
Taxa_26	D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;__;__;__														-0.303			
Taxa_27	D_0__Bacteria;D_1__Epsilonbacteraeota;D_2__Campylobacteria;D_3__Campylobacterales;D_4__Helicobacteraceae;D_5__Helicobacter;__	-0.332		0.335												0.474		0.327
Taxa_28	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Staphylococcaceae;D_5__Staphylococcus;__														-0.516			
Taxa_29	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus;__		-0.373				0.345											
Taxa_30	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Lactococcus;__		-0.321															-0.362
Taxa_31	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;D_6__Streptococcus mutans				0.317			-0.341	-0.321	-0.317	-0.319							
Taxa_32	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;D_6__Streptococcus sp. GDLAMI-SD2								-0.346	-0.357		0.311						
Taxa_33	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;__																	
Taxa_34	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__Christensenellaceae R-7 group;D_6__uncultured	0.324											-0.343				-0.437	
Taxa_35	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__Christensenellaceae R-7 group;__										-0.303		-0.304				-0.426	
Taxa_36	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__uncultured;D_6__uncultured bacterium														0.33			
Taxa_37	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Candidatus Arthromitus;__																-0.37	
Taxa_38	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Clostridium sensu stricto 1;__																	
Taxa_39	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiales vadinBB60 group;D_5__uncultured bacterium;D_6__uncultured				0.384	-0.348	-0.321			-0.358	-0.372			0.314	-0.408			
Taxa_40	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiales vadinBB60 group;__;__																	
Taxa_41	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Defluviitaleaceae;D_5__Defluviitaleaceae UCG-011;__					-0.301	-0.302	0.326										
Taxa_42	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Eubacteriaceae;D_5__Anaerofustis;D_6__uncultured bacterium											-0.364		0.308				
Taxa_43	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII AD3011 group;D_6__uncultured bacterium								-0.338									
Taxa_44	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII UCG-001;D_6__uncultured bacterium																-0.351	
Taxa_45	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__[Eubacterium] brachy group;D_6__uncultured bacterium				-0.411	0.406	0.41		0.366	0.385	0.363				0.322			
Taxa_46	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__[Eubacterium] nodatum group;D_6__uncultured organism							-0.404	-0.399									
Taxa_47	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;__;__		0.364		0.591	-0.544	-0.55		-0.342	-0.633	-0.647							-0.401
Taxa_48	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__A2;D_6__uncultured bacterium			-0.327	0.53	-0.505	-0.505			-0.519	-0.481				-0.309			-0.33
Taxa_49	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__A2;__									-0.318		0.402			-0.343			-0.577
Taxa_50	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__ASF356;D_6__uncultured bacterium											0.31	-0.35	-0.401	0.444			
Taxa_51	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Acetatifactor;D_6__uncultured bacterium																0.521	
Taxa_52	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Acetatifactor;__																	
Taxa_53	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Acetitomaculum;D_6__Firmicutes bacterium CAG_19					0.381	0.405											
Taxa_54	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia;D_6__Lachnospiraceae bacterium DW59					0.309	0.328		0.362									
Taxa_55	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia;__			-0.397										-0.374	0.403			
Taxa_56	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Butyrivibrio;D_6__uncultured bacterium																	
Taxa_57	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Coprococcus 2;__																	
Taxa_58	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Dorea;__											-0.302		0.327				
Taxa_59	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__GCA-900066575;D_6__uncultured bacterium																	
Taxa_60	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnoclostridium;__		0.407															
Taxa_61	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospira;__		0.305															
Taxa_62	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae FCS020 group;D_6__uncultured pro							-0.333	-0.334								0.302	
Taxa_63	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae FCS020 group;__							0.333								0.327		
Taxa_64	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NC2004 group;D_6__uncultured Clo											-0.361			0.366			
Taxa_65	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__Clostridiales												0.334					
Taxa_66	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__Lachnospirace																	
Taxa_67	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__Trichinella ps																	-0.338
Taxa_68	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__uncultured Cl			0.361					-0.31									
Taxa_69	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__uncultured ba				0.349	-0.301		-0.62	-0.59									
Taxa_70	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;__																	
Taxa_71	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4B4 group;D_6__uncultured bact																	
Taxa_72	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-001;D_6__uncultured Clostridi																	
Taxa_73	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-001;D_6__uncultured bacteriu							-0.318						-0.336	0.46			
Taxa_74	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-001;__	0.318										-0.36						
Taxa_75	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-006;D_6__uncultured bacteriu																	
Taxa_76	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Marvinbryantia;__							-0.316	-0.312									
Taxa_77	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Roseburia;D_6__uncultured bacterium					-0.332	-0.324											
Taxa_78	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Roseburia;__												-0.465					
Taxa_79	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella 3;D_6__uncultured bacterium					0.318	0.362										-0.377	
Taxa_80	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella;D_6__uncultured bacterium							-0.326										
Taxa_81	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Bacteroides] pectinophilus group;D_6__uncultured			0.328														
Taxa_82	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ruminantium group;D_6__uncultured												0.346					-0.31
Taxa_83	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ventriosum group;D_6__uncultured C																	-0.477
Taxa_84	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] xylanophilum group;D_6__uncultured																	
Taxa_85	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] xylanophilum group;__		-0.422															

Appendix A

		Coat state	Light Dark Box Test									Porsolt Swim Test				Sucrose Preference Test		
		Score week 5	Head poke time	head poke number	Latency to enter the light box	Time spent rearing	Number of rears	Time in stretch-attend posture	Number of stretch-attend postures	Time in the light box	Number of transitions	Climbing	Faecal Pellets	Immobility	Swimming	Sucrose Intake	Sucrose Preference (Baseline)	Sucrose Preference (Week 6)
Taxa_86	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__Clostridium sp. Culture-27																	
Taxa_87	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__Clostridium sp. Culture-54																	
Taxa_88	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__uncultured bacterium							-0.377	-0.334									
Taxa_89	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;__							0.353	0.322			0.365			-0.432			
Taxa_90	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;__;__	-0.308										0.345		-0.308				
Taxa_91	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__Peptococcus;D_6__uncultured bacterium												-0.434					
Taxa_92	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured;D_6__uncultured bacterium				0.469					-0.524	-0.519		-0.484			-0.309		
Taxa_93	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured;D_6__uncultured organism	0.33				-0.391	-0.382				-0.364						-0.509	
Taxa_94	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured;__							0.327	0.321									
Taxa_95	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Romboutsia;__			-0.398														
Taxa_96	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerofilum;D_6__uncultured bacterium													0.475	-0.472	0.303		
Taxa_97	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerotruncus;D_6__uncultured bacterium																	-0.341
Taxa_98	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Angelakisella;__		0.332															
Taxa_99	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Butyricicoccus;D_6__uncultured bacterium				0.317					-0.388	-0.312		-0.367					
Taxa_100	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Butyricicoccus;__	0.307												0.332	-0.303			
Taxa_101	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Candidatus Soleaferrea;__														-0.488			
Taxa_102	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Fournierella;__			-0.34														
Taxa_103	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__GCA-900066225;D_6__uncultured bacterium				0.324			-0.384	-0.305	-0.339				-0.403	0.362			
Taxa_104	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__GCA-900066225;__							0.364	0.355									
Taxa_105	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Harryflintia;D_6__uncultured bacterium					-0.414	-0.438											
Taxa_106	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Intestinimonas;D_6__uncultured bacterium																	
Taxa_107	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Intestinimonas;__	-0.42																
Taxa_108	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter;D_6__Oscillibacter sp. 1-3			0.423	-0.347			0.306	0.313	0.376	0.397						0.389	0.345
Taxa_109	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter;D_6__uncultured bacterium	0.324			0.425			-0.364	-0.401	-0.373	-0.409					-0.308		
Taxa_110	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter;__																	
Taxa_111	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Papillibacter;D_6__uncultured bacterium				0.446	-0.321				-0.353	-0.337		-0.331				0.338	
Taxa_112	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Pygmaiobacter;D_6__uncultured bacterium	0.341		0.347														
Taxa_113	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__Ruminiclostridium sp. KB															0.346		
Taxa_114	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__uncultured Clostridiales			-0.339														
Taxa_115	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__uncultured bacterium					-0.302		-0.33	-0.355	-0.344								
Taxa_116	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;__																	
Taxa_117	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 6;D_6__uncultured bacterium																0.372	
Taxa_118	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 9;D_6__uncultured bacterium			0.357														
Taxa_119	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 9;__				0.337	-0.309	-0.336			-0.432	-0.358							
Taxa_120	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium;D_6__uncultured bacterium															0.331		
Taxa_121	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;D_6__Lachnospir							-0.346	-0.324									
Taxa_122	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;D_6__uncultured																	
Taxa_123	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;__					-0.351	-0.325		-0.351									
Taxa_124	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-003;D_6__uncultured bacter																	
Taxa_125	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-004;D_6__uncultured bacter		0.322			-0.319	-0.33										0.434	
Taxa_126	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-005;D_6__uncultured Clostr				0.319	-0.38	-0.322			-0.34	-0.356							
Taxa_127	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-005;__												-0.358					
Taxa_128	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-007;D_6__uncultured bacter					-0.337	-0.337							0.439	-0.374			
Taxa_129	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-009;D_6__uncultured bacter				0.484	-0.423	-0.358			-0.488	-0.421							
Taxa_130	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-009;D_6__uncultured organ	-0.547																
Taxa_131	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-009;__	0.342														-0.336		-0.499
Taxa_132	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-010;__		-0.401									-0.388	0.31	0.474				
Taxa_133	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013;D_6__uncultured bacter											0.313						
Taxa_134	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013;D_6__uncultured rumen																	
Taxa_135	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013;__															-0.404	-0.435	-0.487
Taxa_136	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;D_6__uncultured bacter				0.338	-0.36	-0.309	-0.343	-0.324	-0.467	-0.394							
Taxa_137	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;D_6__uncultured rumen							0.388						0.488	-0.342	0.355		0.484
Taxa_138	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;D_6__unidentified																	
Taxa_139	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;__											-0.433					-0.43	
Taxa_140	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;D_6__Ruminococcus flavefaciens													-0.438		-0.33		
Taxa_141	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;D_6__uncultured bacterium															0.398	0.315	
Taxa_142	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;__									-0.33	-0.371	0.367	-0.666					
Taxa_143	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 2;D_6__uncultured bacterium					0.33	0.37			0.311	0.338							
Taxa_144	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 2;__												0.339		0.383			
Taxa_145	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__UBA1819;D_6__uncultured bacterium	0.455			0.376	-0.332	-0.349	-0.442	-0.549		-0.332					-0.419		-0.38
Taxa_146	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__UBA1819;__		-0.335															
Taxa_147	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;D_6__Clost		-0.378															
Taxa_148	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;D_6__gut r																	
Taxa_149	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;D_6__uncu															-0.372		
Taxa_150	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;__	-0.312										0.34						
Taxa_151	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__uncultured;__					-0.409	-0.398					-0.332						
Taxa_152	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;__;__																	
Taxa_153	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;__;__;__					0.331												
Taxa_154	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Turicibacter;D_6__uncultured bacteriu															-0.346	0.327	
Taxa_155	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__uncultured;D_6__uncultured bacteriu		-0.319	-0.335														
Taxa_156	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;__;__	-0.312	-0.353															
Taxa_157	D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Veillonella;__																	
Taxa_158	D_0__Bacteria;D_1__Patescibacteria;D_2__Saccharimonadia;D_3__Saccharimonadales;D_4__Saccharimonadaceae;D_5__Candidatus Saccharimonas;D_		-0.385															
Taxa_159	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Bilophila;D_6__uncultured	-0.555														0.437		0.387
Taxa_160	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio;D_6__uncult	-0.338	-0.438			0.418	0.368		0.317							0.357		
Taxa_161	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio;__							-0.607	-0.555									
Taxa_162	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__uncultured;D_6__unculture			0.474													-0.322	
Taxa_163	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__uncultured;D_6__unculture	-0.349										-0.375				0.532		0.403
Taxa_164	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__uncultured;D_6__unculture	-0.383	-0.369													0.391		0.504
Taxa_165	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae;D_5__Parasutterella;__															-0.378		-0.38
Taxa_166	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella;__				-0.407													
Taxa_167	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales;D_4__Pasteurellaceae;D_5__Rodentibacter;__		-0.352															
Taxa_168	D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Anaeroplasmatales;D_4__Anaeroplasmataceae;D_5__Anaeroplasma;D_6__uncultured bacter				0.378	-0.314	-0.339			-0.327	-0.367		-0.349					

Table A.2. R values of correlations between plasma lipopolysaccharide, plasma cytokines, caecal microbiota and caecal organic acids, before correction for false discovery rate.

		Cytokine GM-CSF	Cytokine IFNγ	Cytokine IL-10	Cytokine IL-12p70	Cytokine IL-17A	Cytokine IL-18	Cyt_IL1alpha	Cyt_IL6	Cytokine IL-33	Cytokine CINC-1	Cytokine MCP-1	Cytokine TNF-α	Lipopolysaccharide Binding Protein
	SCFA_Acetate													
	SCFA_Butyrate	0.399	0.385	0.5	0.44	0.459	0.508	0.453	0.484	0.528	0.405			
	SCFA_Formate	*	*	*	*	*	*	*	*	*	*	*	*	*
	SCFA_Heptanoate													
	SCFA Hexanoate													
	SCFA_Isobutyrate	-0.307		-0.303							-0.416	-0.377		
	SCFA Isovalerate										-0.407	-0.368	-0.335	
	SCFA Lactate													
	SCFA Propionate	-0.376	-0.441	-0.325					-0.345		-0.51	-0.31		
	SCFA Succinate	-0.469	-0.432	-0.38			-0.414	-0.358	-0.436	-0.437	-0.301			
	SCFA Valerate		-0.381						-0.309		-0.372	-0.317		
Taxa_1	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Bifidobacterium;__													
Taxa_2	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Rothia;__	-0.448	-0.425	-0.34			-0.378	-0.331	-0.385	-0.445		-0.431		
Taxa_3	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Atopobiaceae;D_5__Coriobacteriaceae UCG-002;D_6__uncultured bacterium							0.307						
Taxa_4	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriales Incertae Sedis;D_5__uncultured;D_6__gut metagenome	-0.404	-0.373	-0.41	-0.427	-0.441	-0.442	-0.475	-0.399	-0.446	-0.445	-0.311		
Taxa_5	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Adlercreutzia;D_6__uncultured bacterium													
Taxa_6	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__DNF00809;D_6__uncultured bacterium													
Taxa_7	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Enterorhabdus;D_6__uncultured bacterium													
Taxa_8	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Enterorhabdus;__													
Taxa_9	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;__;__													
Taxa_10	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__uncultured;D_5__uncultured bacterium;D_6__uncultured bacterium				-0.418	-0.308		-0.314			-0.327			
Taxa_11	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidaceae;D_5__Bacteroides;__	-0.314										-0.334	-0.448	
Taxa_12	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;D_5__mouse gut metagenome;D_6__mouse gut metagenome													
Taxa_13	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;D_5__uncultured bacterium;D_6__uncultured bacterium													
Taxa_14	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;D_5__uncultured organism;D_6__uncultured organism													
Taxa_15	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Muribaculaceae;__;__													0.31
Taxa_16	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Alloprevotella;D_6__uncultured bacterium													0.339
Taxa_17	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Alloprevotella;__												0.311	
Taxa_18	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 9;__													
Taxa_19	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae Ga6A1 group;D_6__uncultured bacterium	-0.454	-0.361	-0.374	-0.316	-0.322	-0.404	-0.444	-0.364	-0.419	-0.438	-0.39	-0.45	
Taxa_20	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae NK3B31 group;D_6__uncultured bacterium													
Taxa_21	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae UCG-001;D_6__uncultured bacterium													
Taxa_22	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Alistipes;D_6__uncultured bacterium								0.301					
Taxa_23	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;D_6__uncultured bacterium													
Taxa_24	D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;D_4__uncultured bacterium;D_5__uncultured bacterium;D_6__uncultured bacterium	-0.317												
Taxa_25	D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;D_4__uncultured rumen bacterium;D_5__uncultured rumen bacterium;D_6__uncultured rumen bac													
Taxa_26	D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;__;__;__													
Taxa_27	D_0__Bacteria;D_1__Epsilonbacteraeota;D_2__Campylobacteria;D_3__Campylobacterales;D_4__Helicobacteraceae;D_5__Helicobacter;__										0.31			
Taxa_28	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Staphylococcaceae;D_5__Staphylococcus;__													
Taxa_29	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus;__	-0.482	-0.439	-0.492	-0.311		-0.499	-0.486	-0.463	-0.473	-0.39	-0.478		
Taxa_30	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Lactococcus;__	0.33		0.415	0.408	0.376	0.316	0.404	0.324		0.435			
Taxa_31	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;D_6__Streptococcus mutans													
Taxa_32	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;D_6__Streptococcus sp. GDLAMI-SD2											0.312		
Taxa_33	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;__	-0.509	-0.501	-0.502	-0.334	-0.309	-0.448	-0.502	-0.478	-0.424	-0.419	-0.494		
Taxa_34	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__Christensenellaceae R-7 group;D_6__uncultured prokaryote													0.395
Taxa_35	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__Christensenellaceae R-7 group;__	-0.431	-0.423	-0.378	-0.301		-0.384	-0.328	-0.497	-0.421	-0.404			
Taxa_36	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__uncultured;D_6__uncultured bacterium													
Taxa_37	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Candidatus Arthromitus;__													
Taxa_38	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Clostridium sensu stricto 1;__													
Taxa_39	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiales vadinBB60 group;D_5__uncultured bacterium;D_6__uncultured bacterium													
Taxa_40	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiales vadinBB60 group;__;__													
Taxa_41	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Defluviitaleaceae;D_5__Defluviitaleaceae UCG-011;__													
Taxa_42	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Eubacteriaceae;D_5__Anaerofustis;D_6__uncultured bacterium													
Taxa_43	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII AD3011 group;D_6__uncultured bacterium		-0.348	-0.359	-0.353	-0.395	-0.364	-0.359	-0.363	-0.351				
Taxa_44	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII UCG-001;D_6__uncultured bacterium			-0.318			-0.346							
Taxa_45	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__[Eubacterium] brachy group;D_6__uncultured bacterium													
Taxa_46	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__[Eubacterium] nodatum group;D_6__uncultured organism			0.317	0.413	0.379	0.338	0.417			0.332	0.558		
Taxa_47	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;__;__					-0.31					-0.357			
Taxa_48	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__A2;D_6__uncultured bacterium													
Taxa_49	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__A2;__													
Taxa_50	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__ASF356;D_6__uncultured bacterium													
Taxa_51	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Acetatifactor;D_6__uncultured bacterium	0.398	0.433	0.375	0.382		0.354	0.366	0.457	0.412	0.492	0.434		
Taxa_52	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Acetatifactor;__													
Taxa_53	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Acetitomaculum;D_6__Firmicutes bacterium CAG_194_44_15													
Taxa_54	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia;D_6__Lachnospiraceae bacterium DW59													
Taxa_55	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia;__													
Taxa_56	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Butyrivibrio;D_6__uncultured bacterium													
Taxa_57	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Coprococcus 2;__													0.339
Taxa_58	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Dorea;__													
Taxa_59	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__GCA-900066575;D_6__uncultured bacterium													
Taxa_60	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnoclostridium;__													
Taxa_61	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospira;__			0.317		0.373		0.325						
Taxa_62	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae FCS020 group;D_6__uncultured prokaryote													
Taxa_63	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae FCS020 group;__		0.332	0.327										
Taxa_64	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NC2004 group;D_6__uncultured Clostridiaceae bacterium	0.326	0.388	0.318			0.346	0.303	0.408	0.33	0.396			
Taxa_65	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__Clostridiales bacterium CIEAF 020													
Taxa_66	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__Lachnospiraceae bacterium COE1												0.457	-0.36
Taxa_67	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__Trichinella pseudospiralis													
Taxa_68	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__uncultured Clostridiales bacterium													
Taxa_69	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__uncultured bacterium													
Taxa_70	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;__													0.413
Taxa_71	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4B4 group;D_6__uncultured bacterium													
Taxa_72	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-001;D_6__uncultured Clostridiales bacterium													0.461
Taxa_73	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-001;D_6__uncultured bacterium													
Taxa_74	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-001;__													
Taxa_75	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-006;D_6__uncultured bacterium			-0.313				-0.302			-0.36	-0.342		
Taxa_76	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Marvinbryantia;__													
Taxa_77	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Roseburia;D_6__uncultured bacterium													
Taxa_78	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Roseburia;__													
Taxa_79	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella 3;D_6__uncultured bacterium													
Taxa_80	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella;D_6__uncultured bacterium													
Taxa_81	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Bacteroides] pectinophilus group;D_6__uncultured bacterium	-0.302		-0.323							-0.309			
Taxa_82	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ruminantium group;D_6__uncultured bacterium													
Taxa_83	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ventriosum group;D_6__uncultured Clostridiales bacterium													0.337
Taxa_84	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] xylanophilum group;D_6__uncultured bacterium													
Taxa_85	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] xylanophilum group;__									-0.342				
Taxa_86	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__Clostridium sp. Culture-27													
Taxa_87	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__Clostridium sp. Culture-54				-0.379	-0.349			-0.303					
Taxa_88	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__uncultured bacterium													
Taxa_89	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;__													
Taxa_90	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;__;__	0.407	0.317	0.351	0.427	0.439	0.332	0.39	0.377	0.325	0.342	0.389		
Taxa_91	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__Peptococcus;D_6__uncultured bacterium													0.327
Taxa_92	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured;D_6__uncultured bacterium													
Taxa_93	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured;D_6__uncultured organism		-0.321								-0.419			
Taxa_94	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured;__													
Taxa_95	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Romboutsia;__		-0.307											
Taxa_96	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerofilum;D_6__uncultured bacterium													
Taxa_97	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerotruncus;D_6__uncultured bacterium												0.309	
Taxa_98	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Angelakisella;__													
Taxa_99	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Butyricicoccus;D_6__uncultured bacterium													
Taxa_100	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Butyricicoccus;__		-0.31		-0.396	-0.352	-0.324	-0.318	-0.336	-0.348	-0.36			0.484

Appendix A

		Cytokine GM-CSF	Cytokine IFNγ	Cytokine IL-10	Cytokine IL-12p70	Cytokine IL-17A	Cytokine IL-18	Cyt_IL1alpha	Cyt_IL6	Cytokine IL-33	Cytokine CINC-1	Cytokine MCP-1	Cytokine TNF-α	Lipopolysaccharide Binding Protein
Taxa_101	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Candidatus Soleaferrea;__													
Taxa_102	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Fournierella;__													
Taxa_103	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__GCA-900066225;D_6__uncultured bacterium												0.314	-0.523
Taxa_104	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__GCA-900066225;__													
Taxa_105	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Harryflintia;D_6__uncultured bacterium									0.304				
Taxa_106	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Intestinimonas;D_6__uncultured bacterium	0.316									0.324		0.318	0.357
Taxa_107	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Intestinimonas;__													
Taxa_108	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter;D_6__Oscillibacter sp. 1-3													
Taxa_109	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter;D_6__uncultured bacterium													
Taxa_110	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter;__													
Taxa_111	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Papillibacter;D_6__uncultured bacterium													
Taxa_112	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Pygmaiobacter;D_6__uncultured bacterium													
Taxa_113	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__Ruminiclostridium sp. KB18												0.375	-0.324
Taxa_114	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__uncultured Clostridiales bacterium					-0.351		-0.305						
Taxa_115	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__uncultured bacterium													
Taxa_116	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;__												0.31	
Taxa_117	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 6;D_6__uncultured bacterium	0.623	0.592	0.571	0.372	0.312	0.482	0.481	0.56	0.447	0.523	0.566		
Taxa_118	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 9;D_6__uncultured bacterium											0.322		0.514
Taxa_119	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 9;__													
Taxa_120	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium;D_6__uncultured bacterium	0.51	0.506	0.477		0.388	0.513	0.426	0.542	0.468	0.463	0.379		
Taxa_121	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;D_6__Lachnospiraceae bacterium 19gly4													0.319
Taxa_122	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;D_6__uncultured bacterium	0.522	0.606	0.57	0.572	0.419	0.495	0.52	0.576	0.504	0.614	0.585	0.354	
Taxa_123	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;__	-0.44	-0.399	-0.491	-0.465	-0.403	-0.441	-0.414	-0.408	-0.394	-0.465	-0.325		
Taxa_124	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-003;D_6__uncultured bacterium													0.394
Taxa_125	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-004;D_6__uncultured bacterium	0.557	0.549	0.519	0.372	0.372	0.53	0.449	0.612	0.53	0.395	0.432		
Taxa_126	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-005;D_6__uncultured Clostridiales bacterium													
Taxa_127	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-005;__	-0.353	-0.341	-0.331					-0.314	-0.34	-0.39			
Taxa_128	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-007;D_6__uncultured bacterium												-0.309	
Taxa_129	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-009;D_6__uncultured bacterium								0.337			0.311		
Taxa_130	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-009;D_6__uncultured organism													
Taxa_131	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-009;__												0.319	0.335
Taxa_132	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-010;__												-0.42	
Taxa_133	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013;D_6__uncultured bacterium		0.306	0.343										
Taxa_134	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013;D_6__uncultured rumen bacterium													
Taxa_135	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013;__													
Taxa_136	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;D_6__uncultured bacterium													0.306
Taxa_137	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;D_6__uncultured rumen bacterium				-0.344									
Taxa_138	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;D_6__unidentified	-0.309	-0.335	-0.34	-0.365		-0.313	-0.355	-0.363	-0.333	-0.522		-0.475	
Taxa_139	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;__													0.503
Taxa_140	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;D_6__Ruminococcus flavefaciens				0.345									-0.449
Taxa_141	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;D_6__uncultured bacterium	0.417	0.435	0.421			0.333	0.33	0.367		0.499	0.321		
Taxa_142	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;__													
Taxa_143	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 2;D_6__uncultured bacterium													
Taxa_144	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 2;__													
Taxa_145	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__UBA1819;D_6__uncultured bacterium	-0.429	-0.356	-0.412	-0.415	-0.516	-0.407	-0.394	-0.333		-0.474	-0.345	-0.319	
Taxa_146	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__UBA1819;__													
Taxa_147	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;D_6__Clostridiales bacterium 42_27													
Taxa_148	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;D_6__gut metagenome	-0.313	-0.384	-0.342	-0.331	-0.341	-0.341	-0.308	-0.436	-0.408	-0.4			
Taxa_149	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;D_6__uncultured bacterium													
Taxa_150	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group;__													
Taxa_151	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__uncultured;__	0.395	0.474	0.438	0.493	0.444	0.39	0.49	0.467	0.404	0.425	0.443		
Taxa_152	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;__;__													
Taxa_153	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;__;__;__													
Taxa_154	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Turicibacter;D_6__uncultured bacterium													
Taxa_155	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__uncultured;D_6__uncultured bacterium		-0.341		-0.396	-0.351			-0.392	-0.341				
Taxa_156	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;__;__													
Taxa_157	D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Veillonella;__													
Taxa_158	D_0__Bacteria;D_1__Patescibacteria;D_2__Saccharimonadia;D_3__Saccharimonadales;D_4__Saccharimonadaceae;D_5__Candidatus Saccharimonas;D_6__uncultured bacterium												-0.312	
Taxa_159	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Bilophila;D_6__uncultured bacterium													
Taxa_160	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio;D_6__uncultured bacterium	0.316	0.332						0.309	0.36			0.333	
Taxa_161	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio;__													
Taxa_162	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__uncultured;D_6__uncultured Desulfovibrionaceae bacterium	0.339	0.338	0.306	0.478	0.435	0.419	0.394	0.377	0.461	0.324	0.31	0.326	
Taxa_163	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__uncultured;D_6__uncultured Desulfovibrionales bacterium					-0.303							-0.356	
Taxa_164	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__uncultured;D_6__uncultured bacterium	-0.464	-0.385	-0.493	-0.387	-0.358	-0.474	-0.561	-0.426	-0.477	-0.419	-0.446		
Taxa_165	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae;D_5__Parasutterella;__													
Taxa_166	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella;__													
Taxa_167	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales;D_4__Pasteurellaceae;D_5__Rodentibacter;__									-0.304			-0.339	0.392
Taxa_168	D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Anaeroplasmatales;D_4__Anaeroplasmataceae;D_5__Anaeroplasma;D_6__uncultured bacterium													

Appendix B. Method Description for Biogenic Amine Analysis using Liquid Chromatography-Mass Spectrometry

Method Description

Standards and internal standards used included 3,4-dihydroxyphenyl acetic acid (DOPAC), 3,4 dihydroxyphenylalanine (L-DOPA), homovanillic acid (HVA), dopamine hydrochloride (DA), DL-normetanephrine HCL (NM), norepinephrine bitartrate (NE), epinephrine bitartrate (E), DL-5-hydroxytryptophan (5HTP), 5-hydroxyindole-3-acetic acid (5HIAA), DL-tyrosine (TYR), DL-metanephrine HCL (MN), DL-4-hydroxy-3-methoxymandelic acid (VMA), and gamma-aminobutyric acid (GABA), purchased from Sigma–Aldrich (St Louis, MO). Phenylethylamine (PEA) was purchased from Acros Organics (Thermo Fisher Scientific, New Jersey, US), and 3-methoxy-p-tyramine HCL (3MT), rac 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylglycol (MHPG), d_3-homovanillic acid (d_3-HVA), rac normetanephrine-d_3-HCL (d_3-NM), DL-norepinephrine-d6 HCL (d6-NE), rac epinephrine-d_3 (d_3-E), 5-hydroxyindole-3-acetic acid-d_5 (d_5-5HIAA), rac 3,4-dihydroxyphenylethylene glycol-d_5 (d5-DHPG), rac metanephrine-d_3 HCL salt (d_3-MN), , 4-hydroxy-3-methoxymandelic acid-d_3 (d_3-VMA) and gamma-aminobutyric acid-d_6 (d_6-GABA), L-tyrosine-d_4 (d_4-TYR) were purchased from Toronto Research Chemicals (TRC, Toronto, Canada); d4-Dopamine hydrochloride (d_4-DA) and 3,4 dihydroxyphenylalanine-d_3 (L-DOPA-d_3) were purchased from CDN Isotopes (Quebec, Canada). ReagentPlus grade acetic anhydride (AA) (≥99%), acetic anhydride-d_6 (AA-d6), 99 atom % D and ReagentPlus grade 2,2,2-trifluoroethanol (TFE) (≥99%) were also purchased from Sigma–Aldrich. 2,2,2-trifluoroethanol-d_2 (D, 98%) was purchased from Cambridge Isotope Laboratories, (Andover, USA) and sodium bicarbonate and sodium carbonate (anhydrous) Analar grade from BDH (King of Prussia, PA, USA).

This method uses $^1H/^2H_6$-acetic anhydride and $^1H/^2H_2$ -2,2,2-trifluoroethanol to quantitatively convert the BAs to their corresponding acetate or ester to increase their analysis sensitivity. Isotope label coding is enabled using d_6-acetic anhydride and d_2-2,2,2-trifluoroethanol to create an internal standard (IS X-DP) for each BA. Stock solutions of the 21 BA's and 17 deuterated BAs were individually prepared in 50% aqueous acetonitrile plus 1% formic acid at a concentration of 1 mg/mL, with the exception of tyrosine, and d4-tyrosine (0.35 mg/mL).

Preparation of IS dX: A mixed standard solution comprising 100 ng/mL of each of the 17 deuterated BAs was prepared by diluting the appropriate volume of each stock solution with 50% aqueous acetonitrile plus 1% formic acid.

Preparation of STD-P: A mixed standard solution comprising 40 μg/mL of each of the 21 BAs and 17 deuterated BAs (dX) was made by diluting the appropriate volume of each stock solution with 50% aqueous acetonitrile plus 1% formic acid followed by derivatisation with acetic anhydride and trifluoroethanol (TFE) (Figure S2a). The derivatisation workflow is as described for the samples below but with the omission of the Phree plate clean-up step.

Preparation of IS X-DP: A mixed standard solution comprising 40 μg/mL of each of the 21 BAs and 17 deuterated BAs (dX) was made by diluting the appropriate volume of each stock solution with 50% aqueous acetonitrile plus 1% formic acid followed by derivatisation with d6-acetic anhydride and d2-trifluoroethanol (Figure S2b). The derivatisation workflow is as described for the samples below but with the omission of the Phree plate clean-up step.

Preparation of calibration standards: Calibration standards were prepared from STD-P to give concentrations of 0.02 to 1000 ng/mL. A fixed volume of IS X-DP (100 μL) was added to each calibration standard.

The caecum digesta samples (100 μL) were each added to an individual well of a Phree™ phospholipid removal 96-Well Plate (Phenomenex, Torrance, CA 90501 USA) followed by the addition of labelled internal standard (IS dX) (20 μL) and 25% acetic anhydride in acetonitrile (400 μL). The mixture was vortexed (1000 rpm) for 5 min and the Phree 96-Well Plate was placed on top of a 96 Multi-Tier Micro Plate System (TOPAS) containing 2 mL conical bottom glass vials (J.G Finneran Associates, Inc., Vineland, NJ 08360 USA) for collection of the filtrate. A positive pressure Manifold (Waters, Milfor, MS, USA) set at 2 to 5 psi was applied to the plate to elute the supernatant. An additional 100 μL water in 400 μL 25% acetic anhydride in acetonitrile (v/v) was added to the Phree plate, followed by positive pressure elution. Bicarbonate-carbonate buffer (200 μL; 0.7644 g sodium bicarbonate and 0.0954 g of sodium carbonate (anhydrous) in 100 mL water) was added to the combined collected filtrate and the mixture was evaporated to dryness using a CentraVap® Refrigerated Centrifugal Concentrator, (10°C), (Labconco, Kansas City, USA). Acetic anhydride (25 μL) and TFE (75 μL) were then added to each sample well and the TOPAS system was sealed with a Molded PTFE/Silicone Mat, 96 Plugs (JG Finneran), and heated at 80°C with agitation using a ThermoMixer® C (Eppendorf, Hamburg, Germany) at 300 rpm for 6 h, then 50°C for a further 18 h before removing the seal and evaporating to dryness using the CentraVap® Refrigerated

Centrifugal Concentrator, (10°C). Samples were re-derivatised with acetic anhydride (50 μL) and heated at 100°C with agitation using a ThermoMixer® C at 500 rpm for 2.5 h. Then to each sample was added IS X-DP (100 μL), acetonitrile (100 μL) and aqueous ammonium formate (250 μL; 0.06% ammonium formate in water pH adjusted to 4.8 with formic acid). Samples were vortex mixed prior to filtration through a 0.7-μm GF 96 well plate filter, and the filtrate collected in a 2-mL 96-deepwell plate (Phenomenex, Torrance, CA, USA). An aliquot (5 μL) was injected for LC-MS.

LC-MS experiments were carried out on a 5500 QTrap triple quadrupole/linear ion trap (QqLIT) mass spectrometer equipped with a Turbo V™ ion source and electrospray source (ESI) probe (AB Sciex, Concord, ON, Canada) coupled to an Ultimate 3000 UHPLC (Dionex, Sunnyvale, CA, USA). Chromatographic separation was performed on an Acquity UPLC® CSH™ C18 (2.1 x 150 mm, 1.7 μm) column (Waters, Dublin, Ireland), using 5% acetonitrile 95% (0.06% ammonium formate pH adjusted to 4.8 with formic acid) (solvent A) and acetonitrile (solvent B) as the mobile phase for gradient elution. The column flow rate was 0.4 mL min^{-1}; the column temperature was 65°C, and the autosampler was kept at 5°C. The initial mobile phase, 0% B, was held for 1 min, then ramped linearly to 10% B at 6 min, held for 1 min, then 20% B at 13 min, held for 2 min, then 25% B at 20 min, 45% B at 20.5 min, 50% B at 21 min, 57% B at 23 min, 90% B between 23.5 to 30 min before resetting to the original conditions.

MS data were acquired in the positive mode using a multiple reaction monitoring method using Analyst 1.6 software and was processed using MultiQuant 3.0.2 software (AB Sciex, Concord, ON, Canada). Quantitation uses the internal standard ratio method. The transitions monitored (Q1 and Q3) are listed in Supplementary Table S1. Other operating parameters were as following: ion spray voltage 2500 V; temperature 700°C; curtain gas 50 psi; ion source gas 1 40 psi; ion source gas 2 50 psi; collision gas set to medium.

Appendix C. Statement of Contribution to Published Work

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Tracey Bear
Name and title of main supervisor:	Professor Jane Coad
In which chapter is the manuscript/published work?	Chapter 2, Section 2.1
What percentage of the manuscript/published work was contributed by the student?	80%

Describe the contribution that the student has made to the manuscript/published work:

The manuscript was written, and edited for journal review comments by Tracey Bear. The co-authors reviewed the manuscript and made recommendations.

Please select one of the following three options:

- (●) **The manuscript/published work is published or in press**

  Please provide the full reference of the research output:

  Bear, T. L. K., Dalziel, J. E., Coad, J., Roy, N. C., Butts, C. A., & Gopal, P. K. (2020). The Role of the Gut Microbiota in Dietary Interventions for Depression and Anxiety. Advances in Nutrition, 11(4), 890-907. doi: 10.1093/advances/nmaa016

- ( ) **The manuscript is currently under review for publication**

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Student's signature:	Tracey Bear — Digitally signed by Tracey Bear Date: 2023.03.07 09:56:05 +13'00'	Main supervisor's signature:	Jane Coad — Digitally signed by Jane Coad Date: 2023.06.06 18:03:16 +12'00'

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Tracey Bear
Name and title of main supervisor:	Professor Jane Coad
In which chapter is the manuscript/published work?	Chapter 2, Section 2.2
What percentage of the manuscript/published work was contributed by the student?	80%

Describe the contribution that the student has made to the manuscript/published work:

The manuscript was written, and edited for journal review comments by Tracey Bear. The co-authors reviewed the manuscript and made recommendations.

Please select one of the following three options:

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  Bear, T., Dalziel, J., Coad, J., Roy, N., Butts, C., & Gopal, P. (2021). The Microbiome-Gut-Brain Axis and Resilience to Developing Anxiety or Depression under Stress. Microorganisms, 9(4). doi: 10.3390/microorganisms9040723

- ( ) **The manuscript is currently under review for publication**

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- ( ) **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Tracey Bear — Digitally signed by Tracey Bear Date: 2023.03.07 09:56:05 +13'00'	Main supervisor's signature:	Jane Coad — Digitally signed by Jane Coad Date: 2023.06.06 18:03:16 +12'00'

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