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Comparative Compositional Analysis of the Gut Microbiome in Animal Models of Addiction and Stress

Thesis presented by

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Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

All of the work conducted by author with the following exceptions.

Chapter 2 – Nick Jury, Holmes Lab NIH, conducted CIE animal studies.

Chapter 3 – Kshitij Jadhav, Boutrel Lab, Univ. Lausanne conducted alcohol phenotyping animal studies and dopamine mRNA expression PCR analysis. Grace Ahern, Teagasc performed 16S library preparation of caecal samples. Fiona Fouhy, Teagasc ran 16S sequences through Teagasc Moorepark pipeline to create OTU tables.

Chapter 4 – Abe Palmer group, UCSD, including Oksana Polesskaya, coordinated breeding of HS rats and shipped microbiome samples. Jerry Richards group and Paul Meyers group, Univ. Buffalo, including Jordan Tripi and Chris King, performed behavioural testing for animals. From Paul Cotter's lab: 1) Vickey Murrey performed DNA extraction of samples, 2) Fiona Crispie performed sequencing and management of samples 3) Raul Cabrera-Rubio ran 16S sequences through Teagasc Moorepark pipeline to create OTU tables and provided mentorship for further biostatistical analysis.

Chapter 5: Aurelijus Burokas, APC Microbiome Ireland assisted in animal study and performed FST testing/scoring and performed CORT assay. In Colin Hill's group, APC Microbiome Ireland 1) Marion Dalmasso performed faecal bacteriome DNA extraction and supervised virome DNA extraction, 2) Lorraine Draper performed caecal bacteriome DNA extraction and

supervised library preparation of bacteriome and virome. Raul Cabrera-Rubio performed full bioinformatic analysis of bacteriome and virome data.

Signed

Veronica L Peterson

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I would like to thank my mother for raising me with compassion, intelligence, creativity, and a desire to help others. Ultimately this thesis is dedicated to my father, a man who became a profound scientist without an advanced degree because his intelligence surpassed all norms. He has been my mentor through life, teaching me how to analyse problems in a new way and live a humble life (despite his exceptional genius). In the end, I finished this PhD for my father.

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First Author Publications:

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Co-Author Publications:

- Golubeva, A. V., Joyce, S. A., Moloney, G. M., Moloney, G. M., Burokas, A., Sherwin, E., Arboleya, S., Flynn, I., Khochanskiy, D., Moya-Perez, A., **Peterson, V. L**., Rae, K., Murphy, K., Makarova, O., Buravkov, S., Hyland, N. P., Stanton, C., Clarke, G., Gahan, C. GM., …, Cryan, J. F. (2017). Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine*, [Epub ahead of print]. sciencedirect.com/science/article/pii/S2352396417303742
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- Burokas, A., Arboleya, S., Moloney, R.D**., Peterson, V. L.**, Murphy, K., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2017). Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice. *Biological Psychiatry*, 82(7), 472-487. dx.doi.org/10.1016/j.biopsych.2016.12.031
- Doherty, F. D., O'Mahony, S. M., **Peterson, V. L.**, O'Sullivan, O., Wigmore, P., King, M. V., Cryan, J. F, & Fone, K. CF. (2017). Post-Weaning Social Isolation of Rats Leads to Long-term Disruption of the Gut Microbiota-Immune-Brain Axis. *Brain, Behavior, and Immunity*, Accepted 2017.10.28.
- Ryan, P., Patterson, E., Kent, R. M., Stack, H., O'Connor, P. M., Murphy, K., **Peterson, V. L.**, Mandal, R., Wishart, D. A., Dinan, T. G., Cryan, J. F, Seeley, R. J., Stanton, C., & Ross, R. P. (2017). Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice. *Biological Psychiatry*, 82(7), 472-487. nature.com/articles/s41598-017-14010-x
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Conference Posters:

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Abstract

Intro: The microbiome-gut-brain axis (MGBA) has been shown to be instrumental to brain and behaviour, including psychological disorders affecting stress, depression, and anxiety. Many of the aforementioned disorders are comorbid with substance abuse disorders, and on a larger scale with addiction. In this body of work, we seek to characterize addiction-related phenotypes in the microbiome and correlate with measures of addiction-related behaviours, along with changes in the virome following chronic social stress.

Aims: We first examined if vapor administration of ethanol is capable of altering the microbiome and to assess if substances of abuse, such as ethanol, can alter the microbiome outside of oral/gastrointestinal administration. To characterize addictive phenotypes behavioural measures of impulsivity, reward learning, and dopaminergic response to novelty were used.

Methods: All studies were carried out in rodents. Following behavioural testing, gut microbiota samples were analysed with 16S rRNA gene amplicon sequencing. In the chronic social stress study, the virome was also sequenced via shot-gun metagenomic sequencing of faecal filtrate. Correlations were performed between microbiome measures and biological measures of behaviour, cytokines, corticosterone, and dopamine mRNA expression.

Results: Chapter 2) Investigation of microbiome and vapor alcohol administration in mice showed significant alterations in microbiome; Chapter 3) A pre-clinical model of alcohol-addiction showed no significant differences in the microbiome by grouped phenotype. However, low abundance genus-level bacteria correlated to striatal dopamine mRNA expression. Chapter 4) A large heterogenous study of male and female rats revealed that females' microbiome composition is more associated to addictive measures. However, within both male and female operational taxonomic units (OTUs) were significantly correlated to impulsivity measures. Chapter 5) Chronic social stress in male mice significantly affected bacterial and viral

composition. Viral richness was increased alongside decreases in bacterial richness.

Discussion: Results from this body of work show that the microbiome may be impacted by addiction and psychosocial stress. Our findings suggest that there are subtle yet significant differences in microbiome composition between rats with different behavioural phenotypes. These findings indicate that associations to addictive-related behaviour occur in low-abundance (<5%) bacteria and are sex-specific. Furthermore, microbiome (bacteriome and virome) is impacted top-down, brain to gut via the hypothalamic-pituitary-adrenal axis, during chronic psychosocial stress. These initial investigations into stress and gut bacteriophage (aka phage) indicate that increases in phage richness are associated to decreases in bacterial richness and alpha diversity during stress. These novel findings seed the foundation for new research investigating MGBA in stress, substance abuse, and addiction. Further research is necessary to elucidate the mechanisms involved in this cross-communication of the MGBA. New insights into these challenging disorders of addiction and stress may provide unique therapies targeting the gut microbiome.

1 General Introduction

1.1 The Gut Microbiome

In the last decade a new field of research has blossomed, demonstrating how interconnected our bodies are to the microbes that reside in and on us. Research on gut microbiota underpins just how connected organs and biological processes are to each other. The microbes populating the gut are a focal point in microbiota research. In a microbiota sample there also exist a large pool of bacteria, viruses -- largely bacteriophage, viruses that target bacteria --, archaea, fungi, and multi-cellular organisms, such as helminths. The microbiota is the community of organisms that inhabit a certain site in their host, while the microbiome is the total genetic composition of all organisms within that community. Research into the microbiome has revealed connections between host health and composition of microbiome. This includes the immune system (Kau, Ahern et al. 2011, Honda and Littman 2016, Thaiss, Zmora et al. 2016), gastrointestinal function and disease (Benjamin, Hedin et al. 2012, Fraher, O'Toole et al. 2012, Mayer, Labus et al. 2015, Moloney, Johnson et al. 2016), metabolomic processes (Joyce, MacSharry et al. 2014, Lin, An et al. 2016, Barton, Penney et al. 2017), and brain and behaviour (Bercik, Verdu et al. 2010, Bravo, Forsythe et al. 2011, Dinan, Stanton et al. 2013, Kelly, Borre et al. 2016). Bacteria in the gastrointestinal system have been shown to alter immune signalling, such as T-regulatory cells (Round and Mazmanian 2010, Hsiao, McBride et al. 2013), and linked to inflammatory cytokine production (Bailey, Dowd et al. 2011, Kelly, Borre et al. 2016, Scott, Ida et al. 2017). Gastrointestinal function has particularly been associated with gut microbiome in inflammatory bowel disease (Scanlan, Shanahan et al. 2006, Gevers, Kugathasan et al. 2014). Gut microbiome has been shown to alter metabolomic profiles of the host, particularly in conditions such as exercise (Barton, Penney et al. 2017, Cronin, Barton et al. 2018) and obesity (Everard, Belzer et al. 2013, Joyce, MacSharry et al. 2014, Thaiss, Zeevi et al. 2014, Ryan, Patterson et al. 2017).

1.1.1 Bacteriome

The bacteriome has been the focal point of the host microbiome. Gut microbiota are involved in intestinal integrity, digestion, and utilization of nutrients (Kau, Ahern et al. 2011, Mutlu, Gillevet et al. 2012, Leclercq, Matamoros et al. 2014). Bacteria have been most characterized in terms of disease versus healthy conditions; however efforts have been made to understand the microbiome within the entirety of the human population (Lloyd-Price, Abu-Ali et al. 2016, Zhernakova, Kurilshikov et al. 2016). At the phylum level Bacteroidetes and Firmicutes are commonly the most highly abundant bacteria. Both in human and animal studies alterations in enteric microbiota impact critical areas of health. Major functions effected by the gut microbiota include the metabolic pathway (Barton, Shanahan et al. 2015), endocrine system (Clarke, Stilling et al. 2014), intestinal permeability (Miele, Valenza et al. 2009), immune system and inflammatory pathway (Littman and Pamer 2011, Honda and Littman 2012, El Aidy, Dinan et al. 2014).

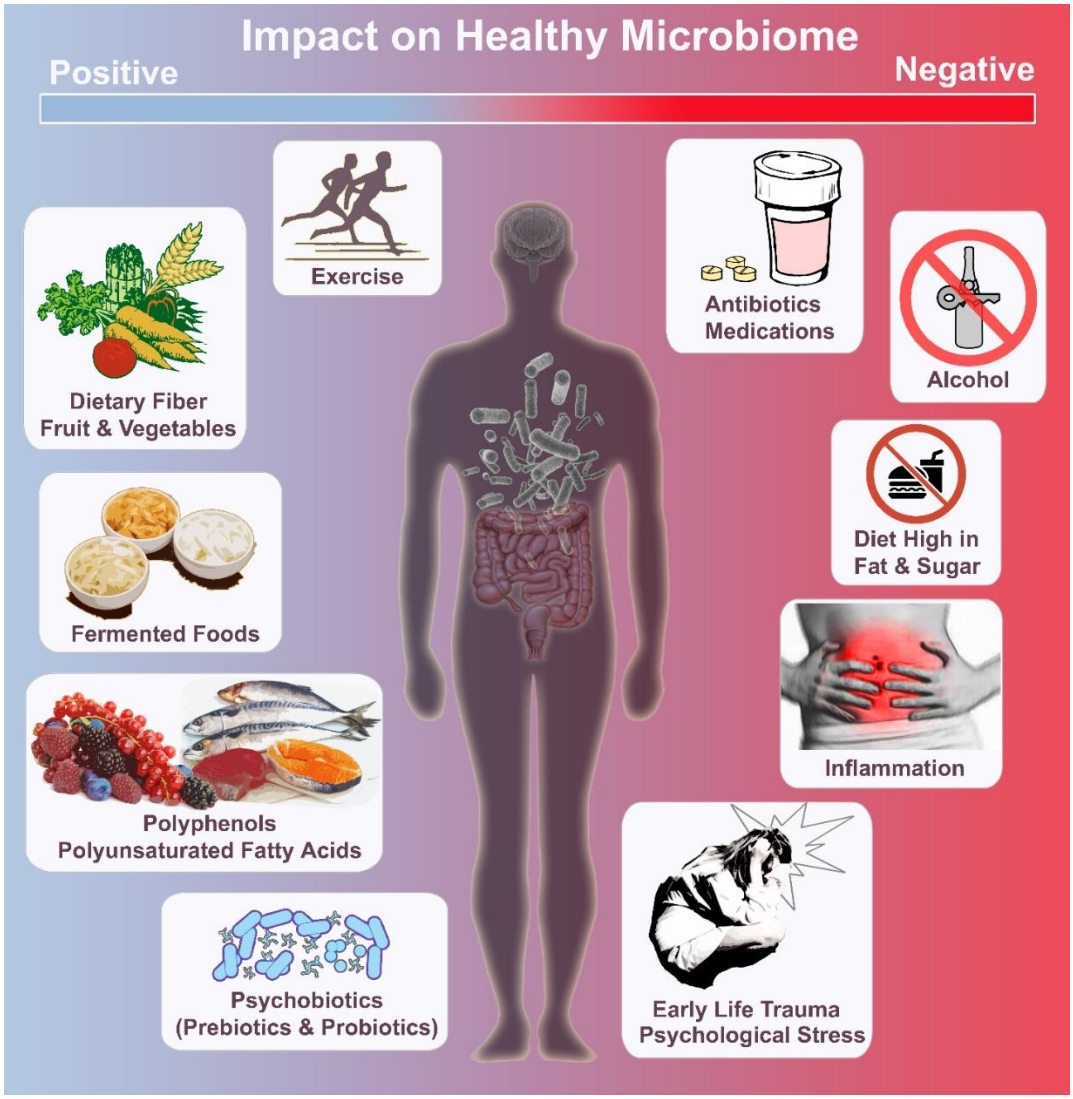

Figure 1.1.1: Factors influencing microbiome composition:

Images in white boxes indicate factors that have a positive (blue, left) or negative (red, right) impact on a healthy microbiome. Most factors known to have an impact on overall health (i.e., diet, exercise, mental health) also impact on the gut microbiome in the same manner. Figure designed by Veronica L. Peterson.

1.1.2 Virome - Bacteriophage and Other Viruses

1.1.2.1 Bacteriophage

Over the past 100 years, bacteriophage have been instrumental in fundamental scientific discoveries, such as the Hershey-Chase experiments which demonstrated that DNA, not protein, was the genetic material of life (Hershey 1952). Bacteriophages are a virus which only targets bacteria. Therefore, they are benign to other living organisms, and beneficial in some instances. Research into phage therapy began soon after the discovery of bacteriophage as scientists, such as George Eliava (Kutateladze 2015), recognized the therapeutic potential of a virus which specifically eliminates pathogenic bacteria. To date, phage therapy is used in food production (Lipson, Sethi et al. 2007, Catel-Ferreira, Tnani et al. 2015) and is used in former Soviet countries, such as Georgia, for antibiotic resistant infections (Biswas 2002, Cao, Wang et al. 2015, Broecker, Klumpp et al. 2016). Bacteriophage contribute to the stability and dynamics of bacterial populations and represent a targeted approach to influencing specific populations of bacteria in the gut (Chevallereau, Blasdel et al. 2016, Wahida, Ritter et al. 2016). Identification of specific bacteriophage and their target bacteria involved in disease may significantly advance therapeutic treatments for a broad range of disorders.

Research into the role of gastrointestinal microbiota in health focuses almost exclusively on bacteria (> 95% of publications), yet the number of bacteriophages vastly outnumber bacteria in the gut. Available research has shown alterations in bacteriophage associated to Crohn's disease (Broecker, Klumpp et al. 2016), Immunodeficiency Syndrome (Monaco, Gootenberg et al. 2016), and Clostridium difficile infection (Pérez-Brocal et al., 2015) with effects attributed to host immunity (Stashak, Baker et al. 1970), intestinal permeability (Tetz and Tetz 2016), and maintaining a bacteriome stability (Stashak, Baker et al. 1970, Dalmasso, Hill et al. 2014, De Paepe, Leclerc et al. 2014, Manrique, Bolduc et al. 2016). There are no published studies on the role of bacteriophage in the microbiota-gut-brain axis. Novel investigation

of bacteriophage and its involvement in brain and behaviour may lead to therapies for psychological disorders involving the microbiota-gut-brain axis.

1.1.2.2 Other Viruses

While bacteriophages are strictly prokaryotic viruses, only infecting bacteria, other viruses exist in the virome. Commensal eukaryotic viruses have not been extensively characterized in the mammalian microbiome. Research has found pathogenic giant viruses (*Mimivirus, Marseillevirus*) in clinical samples of human microbiome (Lagier, Drancourt et al. 2017). Other eukaryotic viruses that dwell in the microbiome are potentially pathogenic to the host. Of note, the genus *Flavivirus* which evades detection by inhibiting host cellular inhibition, such as interferon (Munoz-Jordan, Laurent-Rolle et al. 2005). Furthermore, eukaryotic viruses, such as *Flavivirus,* are sensitive to cellular stress and oxidation (Gullberg, Jordan Steel et al. 2015), similar to temperate bacteriophages (Dalmasso, Hill et al. 2014). Overall composition of the microbiome has been shown to influence viral infections. AIDS disease progression was linked to microbiome composition and changed independently from treatment (Monaco, Gootenberg et al. 2016), revealing that AIDS treatment did not correct alteration in microbiome. Finally, psychological stress can induce virulence of viruses through immunosuppression (Freeman, Sheridan et al. 2007).

1.1.2.3 Other Organisms

While the majority of microbiome research focuses on bacteria, other organisms exist within this community including fungi, archaea, protozoa, and intestinal worms, such as helminths. Like viruses, these organisms have been researched to a lesser extent in terms of the mammalian microbiome. However, these organisms may be as important as bacteria and viruses in terms of influencing host health.

1.1.2.3.1 Fungi - Mycobiome

Of the other microbes, fungi have been most researched and characterized in the microbiome (termed the mycobiome). Fungi in the human mycobiome consist of both commensal and pathogenic species, for example the genus *Candida* (Huffnagle and Noverr 2013, Huseyin, O'Toole et al. 2017). While fungal communities are much less abundant, relative to the entirety of the microbiome, many of these communities are implicated in health and diet (Hoffmann, Dollive et al. 2013, Huffnagle and Noverr 2013, Huseyin, O'Toole et al. 2017). Noteworthy is the genus *Saccharomyces,* which includes *Saccharomyces cerevisiae* and *Saccharomyces buyanus* that are used in the fermentation of creating alcoholic beverages. A variety of the *Saccharomyces cerevisiae* species has also been used as probiotic treatment for gastroenteritis (Huffnagle and Noverr 2013). As with the majority of organisms inhabiting the host, fungi are able to alter immune response and pathogenic fungi increase opportunistically in immune-compromised subjects (Huseyin, O'Toole et al. 2017).

1.1.2.3.2 Archaea

Archaea are prokaryotic microorganisms, like bacteria, which are also seen in the mammalian gastrointestinal tract. Methanogens are highly abundant in the gastrointestinal tract, for example genus *Methanobrevibacter* (Eckburg, Bik et al. 2005, Samuel and Gordon 2006). Furthermore, these methanogens for mutualistic relationships with bacteria to enhance fermentation of dietary compounds, thus increasing nutrients available for the host (Samuel and Gordon 2006, Turner, Ramakrishnan et al. 2013).

1.1.2.3.3 Protozoa

Protozoa are a eukaryotic single-celled organism occasionally identified in the gut microbiome (Turner, Ramakrishnan et al. 2013). Most protozoa known to inhabit mammals are pathogenic, such as malaria. However, in livestock commensal protozoa have been characterized and believed to metabolize plant carbohydrates (Turner, Ramakrishnan et al. 2013). Like

archaea, these commensal protozoa are believed to have a mutualistic relationship with certain bacteria.

1.1.2.3.4 Helminths

Other multicellular organisms are known to inhabit the mammalian gastrointestinal system, notably intestinal worms. Helminths inhabiting the intestines can be both beneficial and harmful to the host, however the majority are considered to be parasitic. In most developed countries, helminths are not seen in the human gastrointestinal tract. However, this decrease in intestinal worms has been associated with decreased alpha diversity of bacteria (Broadhurst, Ardeshir et al. 2012, Lee, Tang et al. 2014) and, thus, a hygiene hypothesis has been proposed for helminths to explain proliferation of certain autoimmune diseases (Weinstock and Elliott 2009, Williamson, McKenney et al. 2016). Various studies have shown therapeutic applications of helminths for treating inflammatory bowel disorders by regulating the immune system and reducing mucosal inflammation (Weinstock 2004, Broadhurst, Ardeshir et al. 2012, Osborne, Monticelli et al. 2014). Additionally, colonization of pregnant rats attenuates excessive cytokine response in the brain during pathogenic bacterial infection in offspring, which is mediated through microglia, thus preventing cognitive dysfunction (Williamson, McKenney et al. 2016).

1.2 The Microbiome-Gut-Brain Axis (MGBA)

The gut-brain axis is a complex cellular network that connects the central and enteric nervous system. This communication network in the gut is affectionately termed ‘the second brain’ because it can function autonomously from the central nervous system (CNS). Descending signals from the brain communicate to the gut via the autonomic nervous system and more directly through the dorsal motor nucleus of the vagus. Ascending signals from the gut are sent directly to the vagus, reaching the nucleus tractus solitarius in the dorsal medulla oblongata. This bidirectional network, the gut-brain axis, mediates many vital processes necessary for homeostasis

and response to environmental stimuli in the periphery and CNS (Mayer 2011, Mussa and Verberne 2013).

The gut microbiota involvement in gut-brain communication is termed the microbiota-gut-brain axis (MGBA) (Burokas, Moloney et al. 2015). Interestingly, brain and behaviour have been linked to gut microbiota composition. Through this axis it has been demonstrated that bacteria that reside in the gut are capable of influencing cognition and behaviour (Dinan and Cryan 2015). Communication between the gut microbiota and the brain include the inflammatory pathway (Leclercq, De Saeger et al. 2014), vagus nerve (Bravo, Forsythe et al. 2011), and hypothalamic-pituitary-adrenal (HPA) axis (Clarke, Stilling et al. 2014). A large array of psychiatric disorders have been linked to gut microbiota, including depression (Kelly, Borre et al. 2016, Zheng, Zeng et al. 2016), neurodevelopmental disorders such as autism (Hsiao, McBride et al. 2013, Golubeva, Joyce et al. 2017), and the stress response (Bailey, Dowd et al. 2011, Bharwani, Mian et al. 2016, Burokas, Arboleya et al. 2017). Animal research has shown that changes in gut microbiota can influence gene expression in key brain regions critical for mood and behaviour (Bravo, Forsythe et al. 2011, Stilling, Dinan et al. 2014). It has also been shown that the animal behavioural phenotype can be transferred from one animal to another through faecal microbiota transplantation (Collins et al., 2013; Kelly et al., 2016). However, these studies have focussed on the bacteria in the transferred material as being the main determinants influencing behaviour. Bi-directional communication between host and gut microbiome can influence one another, thus must be considered when investigating the microbiota-gut-brain axis (bottom up) or brain-gut-microbiome axis (top down).

1.2.1 Overview: Bacteria & MGB Axis

Numerous studies have shown that aspects of animal behaviour, including anxiety and depressive-like behaviours can be influenced by the bacteria that inhabit the gastrointestinal tract (Bailey, Dowd et al. 2011, Bravo, Forsythe et al. 2011, Collins, Kassam et al. 2013). Previous research reveals that

treatment with a particular strain of bacteria can induce anxiolytic properties by altering inhibitory GABAergic innervations (Bravo, Forsythe et al. 2011). Clinical trials have begun looking at the effects of probiotics on mood and cognition (Tillisch, Labus et al. 2013, Schmidt, Cowen et al. 2015). Notably, fMRI studies by Tillisch and colleagues found that women who consumed a fermented milk product had decreased activity in the insular cortex -- a region active during drug craving (Tillisch, Labus et al. 2013).

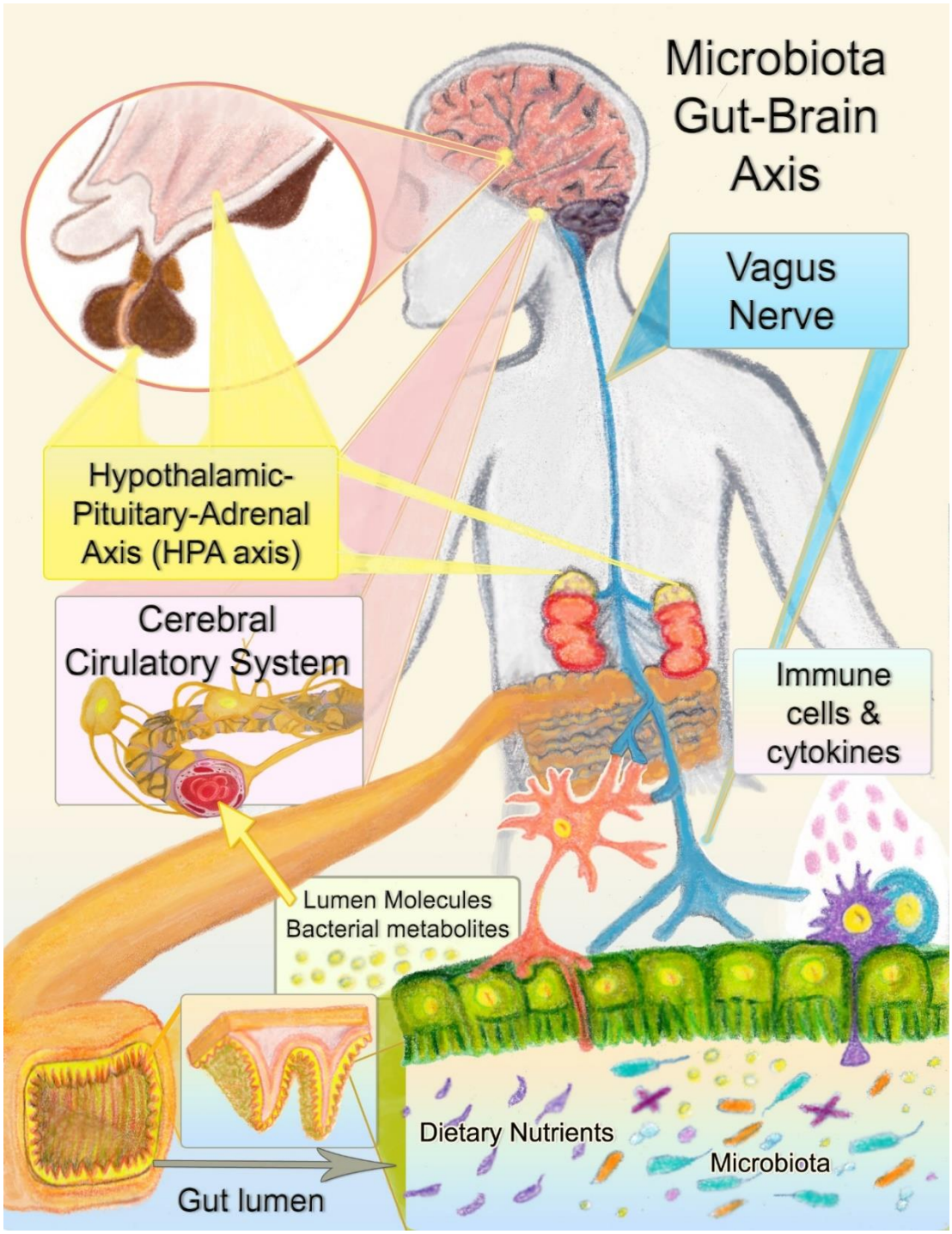

Figure 1.2.1: Anatomy of the Microbiome-Gut-Brain Axis (MGBA) and pathways of communication*: MGBA signalling from microbiota to intestinal epithelium, on to neurons of the vagus nerve, influencing HPA axis and Cerebral Circulatory System (Artwork hand-drawn by Veronica L. Peterson).*

1.2.2 Overview: Viruses & Microbiome-Gut-Brain Axis (MGBA)

To date, links between viral infections and psychological disease have only been observed and may be primarily caused by systemic inflammation or psychological distress associated with diagnosis of infection. Epidemiological analysis has found links between anxiety/depression and viral infections, including influenza, herpes simplex, hepatitis C, and AIDs/HIV (Coughlin 2012). The neurovirologist Dr. Robert Yolken has published several studies suggesting a link between viruses and incidence of schizophrenia, cognitive dysfunction, and bipolar disorder (Shirts, Prasad et al. 2008, Yolken, Jones-Brando et al. 2014, Tanaka, Matsuda et al. 2017). While many of Yolken's studies have investigated herpes simplex, he has also published work showing alterations in the oropharyngeal phageome in schizophrenic patients (Yolken, Severance et al. 2015).

1.2.3 Methods of Communication

For further growth of the gut microbiome field, it is now critical that new studies focus on methods/mechanisms of how gut microbiota communicate with the brain. Recent studies have begun to reveal methods of communication in the microbiome-gut-brain axis.

1.2.3.1 Immune system

One of the most characterized mechanisms of how the bacteriome influences host health is via the immune system (Littman and Pamer 2011). Certain bacteria inhabiting the host gut microbiome can promote either an anti-inflammatory or pro-inflammatory profile in the host (Li, Lin et al. 2016). Furthermore, gut microbiota can regulate host immunity by altering immune cell activity. The interface between host immune system and microbiome is a focus of interest, not only for inflammatory-related disorders, but also for psychiatric diseases associated to systemic inflammation – such as depression (Miller and Raison 2015).

Regulatory T cells (Treg) are a suppressor T cell within a subpopulation of T cells that modulate the immune system. These Treg cells are, generally, immunosuppressive and therefore implicated in autoimmune disease. Furthermore, the immunosuppressive activity of Treg cells has been shown to be beneficial in neurological diseases associated with inflammation, such as Alzheimer's (Kunis, Baruch et al. 2013). Chronic social stress has been associated to trends in reduced Treg cells and IL-10 alongside altered microbiome composition (Bharwani, Mian et al. 2016). Reductions in Treg cells and anti-inflammatory cytokines in peripheral blood have also been characterized in depression (Miller and Raison 2016). In the brain, Treg cells can reduce inflammation and promote neural integrity (Kipnis, Avidan et al. 2004, Miller and Raison 2016). Gut bacteria have been shown to alter Treg cells within the intestine (Round and Mazmanian 2010, Honda and Littman 2016). Indeed, colonization of a human isolated *Bacteriodes fragilis* NCTC9343 and Clostridium species (clusters IV, XIVa, XVIII) in germ-free mice increased differentiation of Treg cells, immunosuppressive activity, and increased anti-inflammatory cytokine production in the gut (Round and Mazmanian 2010, Atarashi, Tanoue et al. 2013). The mechanism of this action was shown to be mediated through polysaccharide A and short-chain fatty acids (SCFAs) altering conversion of CD4+ T cells to FOX3+ Treg cells which produce the anti-inflammatory cytokine IL-10 (Round and Mazmanian 2010, Atarashi, Tanoue et al. 2013, Smith, Howitt et al. 2013).

1.2.3.2 Microbial Toxins and Sickness Behaviour

Many bacteria in the gut microbiome have compounds toxic to the host, when released. The most common example is the endotoxin lipopolysaccharide (LPS), a large molecule lipoglycans in gram-negative bacteria. Binding of LPS to host immune cells triggers production of pro-inflammatory cytokines. Shiga toxins, although released by bacteria, originate from the genome of lambda prophage (Friedman 2001). These highly toxic compounds are associated to dysentery (*Shigella dysenteriae)* and certain *Escherichia coli* infections. Shiga toxin, once entered into the host cell, halts protein synthesis thus causing cascade of cell death and inflammation (Sandvig and van Deurs 2000, Melton-

Celsa 2014). Other bacterial toxins include those produced only within certain strains of bacterial species. Bacterial species *Bacteroides fragilis,* for example, contains some strains that secret a metalloprotease toxin (termed *B. fragilis* toxin – BFT). Mutation from a commensal to toxic *B. fragilis* appears to be dependent on many external, currently unidentified, factors. BFT cleaves tight junction proteins in the host epithelium, causing diarrhoea and haemorrhage (Sears 2001).

Bacterial toxins are capable of inducing an anxious, lethargic, and/or depressive state termed as sickness behaviour. Sickness behaviour is characterized as a pro-inflammatory cytokine profile affecting the central nervous system. Cortical inflammation brings on symptoms of depression and/or anxiety, lack of concentration, fatigue, reduced sociability, altered sleep and appetite. Bacterial toxins and immune response to pathogen-associated molecular patterns (PAMPs) have been shown to cause sickness behaviour in humans and other animals (Kelley, Bluthé et al. 2003).

1.2.3.3 Bacterial Metabolites & Host Metabolism

Metabolism is another important mechanism linking gut bacteria to host health. Metabolites produced by gut bacteria are capable of influencing host biological processes. Additionally, gut microbiota is linked to alterations in host metabolism. Research employing the metabolic effects of gut microbiome has been explored in diabetes (Plovier, Everard et al. 2017, Ryan, Patterson et al. 2017), obesity and weight loss (Joyce, MacSharry et al. 2014, Plovier, Everard et al. 2017, Ryan, Patterson et al. 2017), exercise (Barton, Penney et al. 2017, Cronin, Barton et al. 2018), and drug use (Davey, Cotter et al. 2013, Elamin, Masclee et al. 2013).

Importantly, short-chain fatty acids (SCFAs) are influential metabolites produced by bacteria. SCFAs, which include propionate, butyrate, and acetate, are produced by the fermentation of dietary fibre in the colon. SCFAs have been linked to reduced inflammation (Smith, Howitt et al. 2013, Erny, Hrabe de Angelis et al. 2015), stress resilience (Burokas, Arboleya et al. 2017), and mental/neurological health (Erny, Hrabe de Angelis et al. 2015,

Kelly, Kennedy et al. 2015, Stilling, van de Wouw et al. 2016). Hence, this promising data makes SCFA-producing probiotics and prebiotics (dietary fibre precursors) marketable therapeutic products to pursue. Another metabolite that must be considered in the microbiome is the production of gaseous hydrogen sulphite by sulfate-reducing bacteria (SRBs). A majority of these bacteria are classed into the genus *Desulfovibrio*. Hydrogen sulphite has been shown to impure working memory and navigation (Ritz, Burnett et al. 2016).

A large body of work has been done on the effect of diet on gut microbiome. Investigation of how gut microbes alter nutrient availability and host metabolism is one of the key topics in this field, particularly in relation to obesity and diabetes. Indeed, certain gut microbes are capable of influencing various metabolic pathways including neutralization of reactive oxygen species (Olson, Vuong et al. 2018), glycolysis and gluconeogenesis (Everard, Belzer et al. 2013, Ryan, Patterson et al. 2017).

1.2.3.4 Bacteriome & Intestinal and BBB Permeability

Tight-junction proteins, such as occludin and claudins, creates a protective barrier within the intestinal epithelium and endothelium of the blood-brain-barrier (BBB). Increases in intestinal permeability have been characterized alongside a pro-inflammatory state, altered microbiome composition, and psychological illness (Forsyth, Farhadi et al. 2009, Mutlu, Keshavarzian et al. 2009, Forsyth, Shannon et al. 2011, Leclercq, Cani et al. 2012, Moreira, Texeira et al. 2012, Summa, Voigt et al. 2013, Leclercq, Matamoros et al. 2014, Kelly, Kennedy et al. 2015, Leclercq, de Timary et al. 2017). Germ-free mice have increased BBB permeability, which decreased when colonized with a pathogen-free gut microbiota believed to harbour butyrate-producing bacteria (Braniste, Al-Asmakh et al. 2014). Recent research has shown that beneficial effects of a ketogenic diet on neurovascular function may be mediated through the microbiome (Ma, Wang et al. 2018). *Akkermansia muciniphila* inhabits the mucosal layer of the intestine and capable of mucin degradation, thus its proximal relationship has implications for intestinal permeability. Indeed, *Akkermansia* has a profound impact on both

permeability in the intestine and brain (Li, Lin et al. 2016, Ma, Wang et al. 2018, Olson, Vuong et al. 2018). This work exemplifies how gut microbiome is capable of altering host health, through intestinal and brain barrier function.

1.3 Psychological Stress

Psychological stress causes systemic changes in the brain and the periphery. Acute stress activates the sympathetic nervous system. Acute stress in humans activates the sympathoadrenal-medullary system, resulting in norepinephrine and glucocorticoid release, pupil dilation, and increases in heart rate, blood pressure and respiration. If the acute stressor is severe, neural signalling becomes sensitized and overactive resulting in a condition known as post-traumatic stress disorder (Hammamieh, Chakraborty et al. 2012). Chronic psychological stress activates the hypothalamic-pituitary-adrenal (HPA) axis and has profound implications to health, causing inflammation (Bailey, Dowd et al. 2011), increased intestinal permeability (Kelly, Kennedy et al. 2015), and impaired neurogenesis, memory and cognitive function (Pittenger and Duman 2008). Many physical and psychological symptoms are paralleled in sickness behaviour. Indeed, chronic psychological stress alters gut bacteria and is linked to pro-inflammatory cytokines and changes in immune cells (Bailey, Dowd et al. 2011, Bharwani, Mian et al. 2016, Burokas, Arboleya et al. 2017).

1.3.1 Animal Models of Stress

The diversity of experimental models of stress are as numerous as the bacteria in the microbiome. They include physical stress, chemical stress, psychological stress, immune stress, and environmental stress. Each has applications in a variety of research fields, however all stress regardless of paradigm activates the hypothalamic-pituitary-adrenal (HPA) axis, alterations in vagal signalling, and in the immune system.

1.3.1.1 Chronic Stress

Chronic stress is the repeated exposure of a noxious stimulus over an extended period. In rodent models the use of repeated exposure to water/moisture, bright light, loud sounds, altered night/day cycles, and restraint or confinement in a small space are used. The chronic unpredictable stress model employs a battery of these stressful stimuli randomly and repeatedly. A random schedule is often implemented as it prevents the rodent from habituating to one type of stressful stimulus.

1.3.1.2 Chronic Social Stress

Chronic social stress is one of the most widely used animal models to investigate the psychological stress, biological impact of psychological stress, and progression of chronic psychological stress to depression. Response to social stress is inherent in animals, therefore social stressors are more resistant to habituation. Protocols for chronic social stress vary, however all involve repeated exposure of a smaller experimental rodent to a larger, more aggressive rodent. The repeated social defeats of the experimental rodent by the aggressor result in a highly stressed phenotype showing depressive behaviour, elevated corticosterone and pro-inflammatory cytokines, alongside other deleterious alterations in brain, gut, and immune system (Reber, Obermeier et al. 2006, Finger, Dinan et al. 2011, Finger, Dinan et al. 2012, Tramullas, Dinan et al. 2012, Bharwani, Mian et al. 2016, Huang, Zhao et al. 2016, Lehmann, Cooper et al. 2016, McKim, Patterson et al. 2016, Watanabe, Arase et al. 2016, McCann, Rosenhauer et al. 2017).

1.3.1.3 Behavioural Tests of Anxiety and Depression

Anxious and depressive phenotypes are common in animal models of stress (described above). Furthermore, addiction is often co-morbid with anxiety and depression, and hence both are often measured in addiction models. In this thesis a number of behavioural tests of anxiety and depression are used.

1.3.1.3.1 Behavioural Tests of Anxiety

Tests of anxiety behaviour include exploratory approach-avoidance behaviour in an open field arena or elevated plus maze (EPM) (Cryan and Holmes 2005). Anxiety induced by novelty can also be measured in the marble burying test and novelty supressed feeding (Cryan and Sweeney 2011). These behavioural tests are based on thigmotaxis, the response of the animal to a certain stimulus. These behaviours are affected by the dopaminergic system, one of the major pathways associated to addiction (Simon, Dupuis et al. 1994).

1.3.1.3.2 Behavioural Tests of Depression

Depression is measured in paradigms resembling learned-helplessness in humans, two versions include the forced swim test (FST) and tail-suspension test (TST), both measuring time immobile following an allotted struggle time. These tests of learned helplessness are purposed to be mediated through the serotonergic neurochemical pathway, as administration of serotonergic antidepressants reduces immobility time (Cryan and Holmes 2005). Depression is often accompanied by anhedonia (Slattery, Markou et al. 2007); thus, behavioural tests of depression also assess interest in rewarding stimuli, such as time male rodents spend smelling the pheromones of a female in oestrus, amount of sucrose water consumed, and interest in socializing with a new conspecific.

1.4 The Addicted Microbiome: Role of Gut Microbiota in Addiction

1.4.1 Drug Addiction and Substance Abuse Disorders

Drug addiction is a chronic relapsing disorder in which compulsive drug-seeking and drug-taking behaviours persist despite serious negative consequences (Koob and Volkow 2010). The addiction cycle is broken up into three stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (Koob 2015). Many factors increase the risk of

addiction including heredity (Epps and Wright 2012), childhood trauma (Enoch, Hodgkinson et al. 2010), and psychological comorbidities (Samaha and Potvin 2014).

1.4.1.1.1 Cortical Physiology of Addiction

The major brain neurochemical pathway involved in addiction is the mesolimbic dopaminergic pathway, also known as the brain reward pathway (Pierce and Kumaresan 2006). Regions in this circuitry include the ventral tegmental area (VTA), nucleus accumbens (NAc) as well as other key regions such as the hippocampus, prefrontal cortex (PFC), and amygdala which are altered by chronic drugs of abuse (Koob and Volkow 2010). Chronic drug abuse is associated with alterations in gene expression and dendritic morphology in regions that reinforce drug-related cues and drug-seeking behaviour (Farris, Harris et al. 2015, Peterson, McCool et al. 2015, Radke, Jury et al. 2015). Additionally, drugs of abuse are known to alter behaviours and neuroplasticity of the PFC (Kroener, Mulholland et al. 2012) which contributes to the persistence of addictive behaviours (Koob 2013).

1.4.1.1.2 Behavioural Phenotype of Addiction

The addictive phenotype can be characterized by impulsive behaviours and compulsive reward seeking (Koob 2013). These aberrant behaviours have been linked to altered function in the prefrontal cortex (PFC) and dopaminergic pathways (Dalley, Everitt et al. 2011). Loss of executive function by damaging the PFC is associated with overeating, gambling, and substance abuse (Crews and Boettiger 2009). In animal models of addiction, research has revealed that increased impulsivity, incentive-salience, and attentional deficits can confer an addictive phenotype, known as goal/sign-tracking (Tomie, Grimes et al. 2008, Lovic, Saunders et al. 2011, Nasser, Chen et al. 2015, King, Palmer et al. 2016).

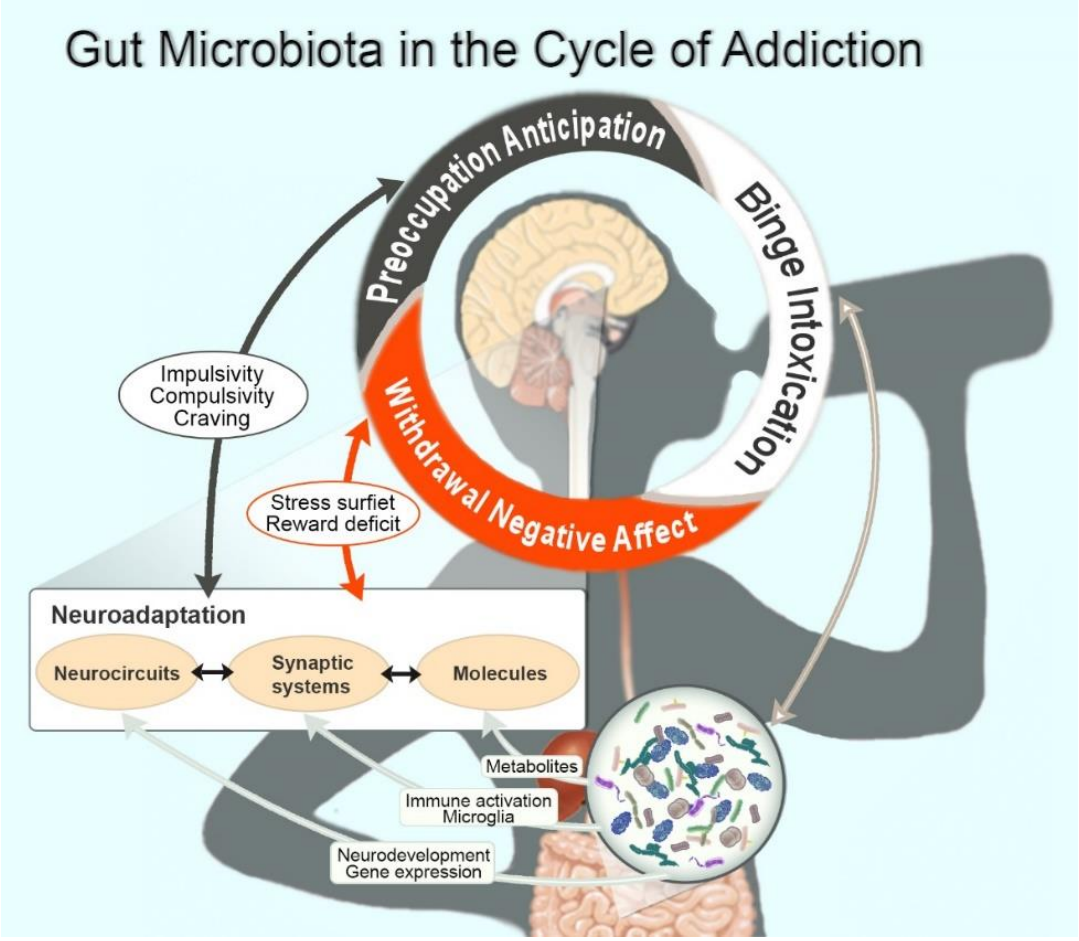

***Figure 1.4.1: Gut Microbiota in the cycle of addiction**: purposed mechanisms of how gut microbiota affect the addiction cycle; adapted from a figure by Wise & Koob (Wise and Koob 2014).*

1.4.2 Psychological Comorbidities

The co-occurrence of mental health disorders in populations suffering from substance abuse cannot be overlooked. In the United States 8.9 million adults have been diagnosed with co-occurring substance use and mental health disorders (Priester, Browne et al. 2016). Moreover, co-occurring psychological disorders cannot easily be disentangled from addiction. Among these, stress, anxiety, and depression are commonly comorbid in patients with substance abuse disorders (Samaha and Potvin 2014) and are influenced by gut microbiota (Dinan and Cryan 2013, Naseribafrouei, Hestad et al. 2014, Jiang, Ling et al. 2015, Kelly, Borre et al. 2016, Morell Miranda, Bertolini et al. 2018).

Addiction has been purposed to be a stress surfeit disorder where over-activation of the HPA axis drives the addiction cycle (Koob 2015). Exposure to stress is known to enhance craving, drug seeking, drug-related anxiety, and is believed to be one of the leading factors in relapse (Goeders 2003, Koob, Buck et al. 2014). Administration of corticosterone increases sensitivity to low doses of cocaine, mediated through the HPA axis, and by severing this connection with adrenalectomy, administration of cocaine ceases (Goeders 2003). Acute drug abuse activates the HPA axis thereby increasing sensitivity to stress (Koob, Buck et al. 2014), which can then cause dysbiosis of the gut microbiota (Bailey, Dowd et al. 2011, Bharwani, Mian et al. 2015). Glucocorticoids and catecholamines alter intestinal barrier function, bacterial adherence to mucosa, and populations of microbes within the gut (Lyte, Vulchanova et al. 2011). In animal studies, an anxious phenotype can be transferred into a new host via faecal microbiota transplantation (Collins, Kassam et al. 2013). It begs the question, could compulsive drug administration behaviour be increased by an anxious microbiota when exposed to stress?

Certain microbes have been shown to have psychoactive properties in the host (Dinan, Stanton et al. 2013). In our lab, *Lactobacillus rhamnosus* was shown to have anxiolytic properties mediated through the vagus nerve which alters GABA receptors expression in cortical regions (Bravo, Forsythe et al. 2011).

Given the shared use of the HPA axis in stress, drug abuse, and microbiota-gut-brain communication it is likely that gut microbiota could influence addiction through this pathway.

Eating disorders share behavioural, neurochemical and genetic similarities to drug addiction (Mancino, Burokas et al. 2015). Rodent behavioural models assessing the goal/sign-tracking phenotype show that the more addictive sign-tracking phenotype assign higher motivational value to food rewards (Pitchers, Flagel et al. 2015, Yager and Robinson 2015, Flagel and Robinson 2017). Data suggests a link between eating behaviours and the microbiome (Schellekens, Dinan et al. 2013, Alcock, Maley et al. 2014). Human studies have found eating disorders comorbid with alcohol abuse (Avena, Carrillo et al. 2004), increased incidence of eating disorders with heightened antibiotic usage (Raevuori, Lukkariniemi et al. 2016), and alterations in microbiome in eating disorders linked to increased depression and anxiety severity (Kleiman, Watson et al. 2015). The microbiota affect ghrelin, a protein critical in hunger and satiety as well as natural and drug-related rewards (Vengeliene 2013). In rodent studies, antagonism of the ghrelin receptor reduced alcohol intake (Suchankova, Steensland et al. 2013) and decreased operant responding for sucrose (Skibicka, Hansson et al. 2012).

1.4.3 Genetics in Addiction

Genetics is also an important aspect of addiction (Palmer and de Wit 2012). Implications for the gut microbiota on the genetic level include epigenetic modulation and host genetics shaping the gut microbiota. It has been shown that bacterial production of butyrate can alter DNA histone methylation in the host (Stilling, Dinan et al. 2014). Interestingly, a study has demonstrated that host miRNAs can promote the growth of bacteria commonly found in mammals (Liu, da Cunha et al. 2016). In an animal model of addiction, antibiotic-treated subjects had increased locomotor response and place preference to cocaine which correlated to alterations in synaptic protein transcripts in the brain (Kiraly, Walker et al. 2016). Alterations in gut

microbiota may induce neuroplasticity associated to addiction. Furthermore, gut microbiota are able to alter gene expression in cortical regions such as BDNF and GABA (Bravo, Forsythe et al. 2011, Dinan, Stanton et al. 2013).

1.4.4 Neurodevelopment and Addiction

The human brain has several critical periods of neurodevelopment, during the embryonic stage, childhood and at puberty (Borre, O'Keeffe et al. 2014, Neufeld, Luczynski et al. 2016). Research during the embryonic stageshowed altered reward behaviour in offspring through maternal immune activation by administering LPS to pregnant rodents (Straley, Van Oeffelen et al. 2016). The connection between childhood trauma and the incidence of drug abuse is well documented (Enoch, Hodgkinson et al. 2010). In animal studies, early life stress alters dendritic morphology of the NAc and PFC (Muhammad, Carroll et al. 2012) as well as causing marked changes in gut microbiota (Golubeva, Crampton et al. 2015) that lead to increased anxious behaviours in adulthood (Clarke, Grenham et al. 2013, Hsiao, McBride et al. 2013, Desbonnet, Clarke et al. 2015, Stilling, Ryan et al. 2015). A link between childhood trauma and neurodevelopment mediated through the gut microbiota may be involved in addiction. Disruption of the microbiota during puberty may also have profound effects on mental health, including risk of substance abuse (McVey Neufeld, Luczynski et al. 2016). In particular, myelination in the PFC persists to 20 years of age (Bartzokis 2005) during which time humans continue to develop executive control over behaviours. Intriguing research from our lab shows that an absence of gut microbiota in germ-free mice disrupts normal myelination in the PFC which can be corrected with transplantation of a conventional mouse microbiota at postnatal day 21 (Hoban, Stilling et al. 2016). It has been proposed that abnormal myelination of the PFC may result in a lack of inhibitory control, thus producing addictive behaviours (Bartzokis 2005). In addition to perpetuating the cycle of addiction by exacerbating stress and inflammation, the gut microbiota may be a factor in risk of addiction by altering neurodevelopment in cortical regions critically involved in addiction.

1.4.5 Inflammation and Addiction

Severe inflammation caused by pathogenic microbes have been reported in intravenous heroin use (Collignon 1983, Finn 2003) and alcohol abuse (Happel and Nelson 2005). Studies have implicated inflammation in several psychiatric diseases (Grenham, Clarke et al. 2011, Felger and Lotrich 2013) including alcohol dependence (Kelley and Dantzer 2011, Gorky and Schwaber 2016) and depression (Miller and Raison 2015). Drugs of abuse activate microglia and increase innate immune genes in the brain, which can enhance negative affect and cause persistence of drug-seeking behaviours (Miguel-Hidalgo 2009, Crews, Zou et al. 2011). Commensal microbes in the gut are capable of regulating the immune system and this immune modulation is known to impact the CNS (El Aidy, Dinan et al. 2014). Recent research has shown that gut microbiota can regulate microglia (Erny, Hrabe de Angelis et al. 2015, Lee, Vuong et al. 2018). Gut microbiota may also influence aspects of addiction through brain-immune system interactions (Cryan and Dinan 2015, Plein and Rittner 2018).

1.4.6 Substance Abuse and the Gut Microbiota

1.4.6.1 Alcohol Abuse Disorder and the Gut Microbiota

Alcohol use disorder is a substance abuse disorder in which an individual is physically or psychologically dependent upon drinking alcohol, irrespective of the consequences. Research shows that chronic alcohol abuse alters the composition of the gut microbiota, causing overgrowth of harmful bacteria (Nilsson 2007, Mutlu, Keshavarzian et al. 2009, Yan, Fouts et al. 2011, Mutlu, Gillevet et al. 2012, Bull-Otterson, Feng et al. 2013, Lowe, Gyongyosi et al. 2017, Kosnicki, Penprase et al. 2018). Alcoholic liver disease is the most prevalent illness associated to chronic alcohol abuse (O'Shea, Dasarathy et al. 2010). Consumption of alcohol irritates the gastrointestinal tract, which injures the lining of the intestines (Bull-Otterson, Feng et al. 2013, Elamin, Masclee et al. 2013, Leclercq, Matamoros et al. 2014). Poor intestinal barrier function allows bacterial endotoxins to pass from the intestines into the blood circulation (Bode 2003, Leclercq, Matamoros et al. 2014, Lowe, Gyongyosi et

al. 2017). Circulating toxins reach the liver causing inflammation, injury, and impaired hepatic function. A study in rodents found that increased intestinal permeability is one of the leading causes of alcohol-induced liver damage and this effect is mediated through gut bacteria (Bull-Otterson, Feng et al. 2013, Canesso 2014). Furthermore, human studies have found that altered microbiota composition correlated to increased intestinal permeability, resulting in increased inflammation and negative affect during alcohol withdrawal (Leclercq, Matamoros et al. 2014). This research by Leclercq suggests that alterations in gut microbiota can result in enhanced craving, which is mediated through the gut-brain-liver axis. Similar alterations in bacterial taxa occur in human studies of alcohol abuse, chronic inflammation, and psychological disorders (Table 1).

1.4.6.2 Other Substances of Abuse and the Gut Microbiota

Available data on the effects of other substances of abuse on the gut microbiota indicate similar effects as alcohol. Clinical studies investigating tobacco smokers have associated smoking and cessation to changes in the gut microbiome and inflammation (Benjamin, Hedin et al. 2012, Biedermann, Zeitz et al. 2013, Biedermann, Brulisauer et al. 2014, Allais, Kerckhof et al. 2016). Changes in gut microbiota following smoking cessation share similarities to the bacterial composition in an obese phenotype; this finding offers an explanation as to why individuals who stop smoking commonly gain weight (Biedermann, Zeitz et al. 2013). Chronic exposure to tobacco smoke in mice alters microbiome and mucin gene expression (Allais, Kerckhof et al. 2016). Murine-models of opioid use has suggested a role of gut microbiota in the progression of morphine-dependence and disruption of gastrointestinal function (Kang, Mischel et al. 2017, Lee, Vuong et al. 2018, Plein and Rittner 2018, Wang, Meng et al. 2018). In addition to these studies linking microbiota to altered intestinal permeability, inflammation, and metabolic processes in opioid-treated mice (Kang, Mischel et al. 2017, Lee, Vuong et al. 2018, Wang, Meng et al. 2018). Intermittent morphine exposure increased bacterial genus *Ruminococcus,* intestinal permeability, and pro-inflammatory microglia (Lee, Vuong et al. 2018). Volatilized cocaine – commonly referred to as “crack” –

alters gut microbiota in mice, including reduced alpha diversity, decreases in bacteria in family *Ruminococcaceae* and *Veillonallaceae*, and increased in *Lachnospiraceae*, *Prevotellaceae*, and *Peptococcaceae* (Scorza, Piccini et al. 2018). Methamphetamine administration during conditioned place preference training decreased the propionate-producing genus *Phascolarctobacterium* alongside reduced propionate levels in faecal matter (Ning, Gong et al. 2017). Animal studies investigating the effects of an antipsychotic drug, olanzapine, on the microbiota has also linked changes to altered metabolism (Davey, Cotter et al. 2013). Interestingly, a study in mice showed that chronic treatment with the major psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), changed gut microbiota and these changes contributed to reduction in energy intake and prevention of high fat diet-induced increases in body weight and adiposity (Cluny, Keenan et al. 2015).

Recurrent Changes in Gut Microbiota in Human Studies Related to Addiction

Microbiota	Substance Abuse	Psychological Conditions	Inflammation
Phylum			
↑↓ **Bacteroidetes**	**Tobacco** (Benjamin et al., 2012; Biedermann et al., 2013) **Alcohol** (Kosnicki et al., 2018; Mutlu et al., 2012)	**Depression** (Jiang et al., 2015; Naseribafrouei et al., 2014)	**Alcoholic Hepatitis** (Chen et al., 2011; Mutlu et al., 2012) **IBD** (Benjamin et al., 2012)
↑↓ **Firmicutes**	**Tobacco Cessation** (Biedermann et al., 2013)	**Depression** (Jiang et al., 2015)	**IBD** (Benjamin et al., 2012)
↑ **Proteobacteria**	**Alcohol** (Chen et al., 2011) **Tobacco** (Biedermann et al., 2013)	**Depression** (Jiang et al., 2015)	**Liver Cirrhosis** (Chen et al., 2011)
Family			
↑ **Enterobacteriaceae**	**Alcohol** (Leclercq et al., 2014; Llopis et al., 2015)	**Depression** (Jiang et al., 2015)	**Alcoholic Hepatitis** (Leclercq et al., 2014; Chen et al., 2011; Llopis et al., 2015)
↑↓ **Lachnospiraceae**	**Alcohol** (Chen et al., 2011)	**Depression** (Naseribafrouei et al., 2014)	**Alcoholic Hepatitis** (Chen et al., 2011) **IBD** (Honda & Littman, 2012; Morell Miranda et al., 2018)
↑↓ **Prevotellaceae**	**Alcohol** (Chen et al., 2011) **Tobacco** (Benjamin et al., 2012)	**Depression** (Kelly et al., 2016)	**Liver Cirrhosis** (Chen et al., 2011) **IBD** (Benjamin et al., 2012)
↑↓ **Ruminococcaceae**	**Alcohol** (Kosnicki et al., 2018; Leclercq et al., 2014)	**Negative affect during withdrawal** (Leclercq et al., 2014) **Bipolar, Depression and Anxiety** (Evans et al., 2016; Leclercq et al., 2014; Kleiman et al., 2015)	**Increased Cytokines** (Leclercq et al., 2014; Jiang et al., 2015) **IBD** (Morell Miranda et al., 2018)
Genus			
↑ ***Alistipes***	**Alcohol** (Llopis et al., 2015)	**Depression** (Jiang et al., 2015; Naseribafrouei et al., 2014)	**Alcoholic Hepatitis** (Llopis et al., 2015) **IBD** (Morell Miranda et al., 2018)
↓ ***Bifidobacterium***	**Alcohol** (Leclercq et al., 2014) **Tobacco** (Biedermann et al., 2014)	**Negative affect during withdrawal** (Leclercq et al., 2014)	**Increased Cytokines** (Leclercq et al., 2014)
Species			
↑↓ ***Clostridium spp.***	**Alcohol** (Kosnicki et al., 2018; Leclercq et al., 2014; Llopis et al., 2015) **Tobacco Cessation** (Biedermann et al., 2014)	**Negative affect during alcohol withdrawal** (Leclercq et al., 2014)	**Intestinal Inflammation** (Honda & Littman, 2012; Benjamin et al., 2012) **Increased Cytokines** (Leclercq et al., 2014; Jiang et al., 2015)
↓ ***Faecalibacterium spp.***	**Alcohol** (Leclercq et al., 2014)	**Negative affect during alcohol withdrawal** (Leclercq et al., 2014) **Bipolar and Depression** (Evans et al., 2016; Jiang et al., 2015; Kleiman et al., 2015; Morell Miranda et al., 2018)	**IBD** (Honda & Littman, 2012, Benjamin et al., 2012; Morell Miranda et al., 2018) **Increased Cytokines** (Leclercq et al., 2014)

Table 1.4.6*: Arrows indicate 1) ↑ increases, 2) ↓ decreases, or 3) ↑↓ increases and decreases in microbiota. Changes in bacteria seen across human studies of substance abuse, psychological conditions, and inflammation.*

1.4.7 Animal Models of Addiction

Modelling a complex disorder, such as addiction, in rodents is an example of the challenge in modelling a strictly human disorder in laboratory animals (Singer, Fadanelli et al. 2018). Therefore, many rat and mouse models are implemented to get a broader understanding of the multiple factors associated to addiction. There are three major models employed to investigate addiction in rodents: 1) passive administration of a substance of abuse to assess biological changes associate to the pharmacodynamics of the substance (Holmes, Fitzgerald et al. 2012, Peterson, McCool et al. 2015, Radke, Jury et al. 2017); 2) use of an addiction model analogous to behaviour matching the DSM-V criteria for addiction in humans which selects rats that compulsively self-administer a substance of abuse after a training period (Deroche-Gamonet, Belin et al. 2004, Jadhav, Magistretti et al. 2017); 3) behavioural phenotyping of rodents, predominantly rats, to reveal variations in neural processes known to be associated with addiction, such as dopaminergic activity (Flagel, Watson et al. 2008, Flagel, Akil et al. 2009, Gancarz, San George et al. 2011, Palmer and de Wit 2012, Versaggi, King et al. 2016).

1.4.7.1 Chronic-Intermittent Vapor Alcohol Exposure

Chronic intermittent vapor ethanol exposure (CIE) is a commonly employed model to examine the effects of prolonged, repeated ethanol exposure in rodents similar to patterns of intoxication seen in humans (Radke, Jury et al. 2017). This model is advantageous as it induces a high blood ethanol content (BEC) which most rodents would not achieve through self-administration and reduces BEC variability between subjects, as well as eliminating the confound of caloric effects from oral ingestion. Briefly, rodents are placed in sealed plexiglass chambers with vaporized ethanol pumped into each chamber for an extended portion of the day (12-16 hours) for a variable period of weeks (Peterson, McCool et al. 2015, Peterson, Jury et al. 2017). During ethanol exposure, BECs are achieved in the range of 150-200 mg/dL. To put this measurement in the context of human alcohol consumption, if an average 73kg (160lb) man consumed 5 shots (~44ml) of

whiskey (40% ethanol) in a 10-minute interval his BEC would be 140 mg/dL for the next 30min (assuming he did not consume more alcohol). If this man chose to drive following those 5 drinks, he would be 1.4x over the limit in the US and 2.8x over the limit in Ireland (aka serious trouble for the fellow). For this same man to achieve the 150-200 mg/dL BEC in the ethanol exposure paradigm, he would need to drink 7-8 shots of whiskey in an hour (half of a standard 700ml bottle sold in shops!).

1.4.7.2 Preclinical Model of DSM-V Alcohol Addiction Criteria

The Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-V) is produced by the American Psychiatric Association with a set of symptomatic criteria that must be met to diagnose a patient with a certain disorder. Defined as “Substance-Related and Addictive Disorders”, several criteria that must be met for diagnosis, include hazardous use, social/interpersonal problems with use, legal problems, withdrawal, craving, tolerance, use of larger amounts or longer periods of use, physical or psychological problems associated to use, and neglect of other activities for use (Grant, Goldstein et al. 2015). Some of these criteria have been translated into a preclinical model, where high-criteria ranking rats display enhanced motivation to self-administer drug measured on a progressive-ratio schedule, willingness to endure aversive conditions to self-administer drug, relapse behaviour measured as reinstatement of lever-pressing after an extended period of abstinence, and inability to abstain from lever-pressing during a signal period of drug unavailability (Deroche-Gamonet, Belin et al. 2004, Jadhav, Magistretti et al. 2017). In brief, all rats can freely self-administer the drug in a neutral environment during a “training period”. Following the period of free drug use, rats are tested for the outlined criteria, scores, and classed in to groups based on addiction severity scoring.

1.4.7.3 Addiction-Related Behavioural Tests

Behavioural tests related to addiction are carried out in rodent models of addiction to examine behavioural phenotypes indicative of addiction. Similar

behavioural phenotypes have been associated with humans at-risk of addiction.

1.4.7.3.1 Reward-Learning and Incentive-Salience

Reward learning behavioural tests include both operant and classical conditioning. Reward learning tasks that implement operant conditioning includes light reinforcement (LR) where light is the rewarding stimulus and choice reaction time (RT and 5CSRTT) which requires the rat to learn to nose poke based on light/tone cues for a water reward. Operant chambers are used to assess motivation and incentive-salience for a reward measured by lever presses either on a fixed-, random-, or progressive-ratio schedule, and can also incorporate aversive conditions such as crossing water or an electric-shock floor to lever press for a reward. Conditioned Place Preference (CPP) and Cocaine Cue Preference (CCP) are frequently used tests of a subject's vulnerability to the classical conditioning of a recreational drug. In both paradigms, an animal is introduced to a chamber with three floor types during multiple sessions where injection of a drug or saline is paired with a particular floor type. On the final day of testing, the subject is allowed to freely choose how much time is spent in the different floor types. Animals that choose to spend more time in the cocaine-paired floor type also show increases in drug self-administration, and alterations in dopamine activity (Meyer, Ma et al. 2012, Kiraly, Walker et al. 2016, Ning, Gong et al. 2017). Another behavioural test based on classical conditioning includes Pavlovian conditioned approach (PavCA) and reinforcement (CRF) wherein a lever cue is repeatedly paired with the presentation of a magazine dispensed food reward, following training rats are tested for lever or magazine associations which assesses the salience of the lever vs. magazine. Rats that assign incentive-salience to the lever cue (aka sign-trackers) have been shown to have greater propensity to self-administer substances of abuse and show alterations in striatal dopamine activity (Flagel, Watson et al. 2008, Flagel, Clark et al. 2011, Meyer, Lovic et al. 2012).

1.4.7.3.2 Impulsivity

Impulsivity is a behaviour mediated through the prefrontal cortex (PFC), which requires the executive ability to control behaviours and impulses by inhibiting signals in other cortical regions. A common test of impulsivity used in both human and animal studies is Delay Discounting (DD). Like the operant progressive-ratio (PR) test, a breaking point or indifference point is assessed by incrementally increasing time in-between subsequent rewards until the subject is no longer interested in the reward. However, in DD the reward increases with time. Thus, in DD a subject may choose a smaller reward for a shorter delay time or a larger reward with a longer delay time. The choice of increased reward vs. increased wait time is plotted and the area under the curve (AUC) is calculated by subject's selection. A lower AUC value in delay discounting is associated to increased impulsivity and addictive-behaviour (Reynolds, Richards et al. 2004, Perry, Larson et al. 2005, de Wit 2009). In the choice reaction time task, a measure of impulsivity is assessed by counting every instance that a rat attempts to poke its snout in the reward hole , prematurely, before the indication light/tone, defined as false alarms per opportunity.

1.4.7.3.3 Locomotor Behaviour

In the context of addiction-related behaviour, locomotor activity is measured in response to novelty and following drug administration. These locomotor measures provide an indication of dopaminergic activity in response to stimuli. Increased locomotor response to these novel stimuli are associated with enhanced dopamine release and increased addiction potential (Gancarz, San George et al. 2011). This locomotor response to novelty is usually conducted in a low-lit open field arena. Locomotor response to drug injection is often an additional measure taken in the conditioned place preference test.

1.5 Mining the Microbiome – Next-Generation Sequencing, Bioinformatics, and Biostatistics

At every step of microbiome analyses, careful planning and consultation with experts is required to properly execute microbiome analysis appropriate for experimental aims. Starting with sample collection and storage, DNA extraction, library preparation, sequencing, to selection of bioinformatic pipelines, classifier, reference database, and biostatistical analysis -- every step can potentially alter microbiome results (Clooney, Fouhy et al. 2016). While many microbiome researchers have called for a unification of methodology, consensus on 'proper' analysis seems to depend, in part, on experimental design and aims. The following sections will outline basic methodologies and touch on protocol and/or procedural disparities that may result in altered results.

1.5.1 "The Poop Phase"

Researchers of the gut microbiome, particularly those collecting and processing samples, inevitably realize the nature of their work. Thus, a jovial approach, ripe with witticism, assists in coming to terms with the samples necessary for this scientific research. However, execution of this phase in microbiome analysis must not be carried out flippantly.

1.5.2 Sequencing

Next-generation sequencing (NGS) is currently the common method used in microbiome analysis. The Illumina platform will be overviewed herein, however other platforms exist such as the Ion Torrent. Three Illumina sequencers are predominantly used for microbiome sequencing: 1) the least expensive MiSeq which is predominantly used for 16S rRNA gene sequencing, however is also capable of virome shot-gun metagenomic

(SGM) sequencing 2) the mid-cost NextSeq capable of SGM microbiome sequencing and deep 16S sequencing, 3) most expensive HiSeq used for SGM sequencing in large-sample studies and transcriptome sequencing. The Selection of the sequencing platform can alter microbiome results because different sequencing machines read a mixture of DNA differently, resulting in altered proportions of bacterial DNA read (Fouhy, Clooney et al. 2016). Cost is often the major consideration when selecting a sequencing method, however the method that provides the most relevant data to address experimental aims and sequencing depth (how much data per sample) should be the major deciding factors.

Consideration of primer selection for amplification of 16S rRNA gene region is necessary. For 16S rRNA gene sequencing on the MiSeq, the maximum read length is 2x300bp with read lengths of 2x250bp commonly selected. For higher sequencing reads per sample (i.e., more microbiome community data/depth) or larger sample pools, the NextSeq can be used. The NexSeq maximum read length is 2x150bp, affecting 16S rRNA gene primer selection, which is preferable for studies interested in accuracy at the genus level (Claesson, Wang et al. 2010). The Illumina Nextera library prep kit for the MiSeq includes a protocol for 16S rRNA gene primers which covers the V3-V4 hypervariable regions of the 16S rRNA gene, resulting in ~500bp amplicon suitable for sequencing at 2x250bp. This primer has been selected based on appropriate amplicon size and high bacterial coverage (~94.5%) and can amplify archaea (Klindworth, Pruesse et al. 2013). Importantly though, V3-V4 primers have shown low efficiency in classifying sequences at certain taxonomic levels and drastically bias relative taxa abundancies compared to other 16S rRNA gene primer regions (Claesson, Wang et al. 2010, Zheng, Tsompana et al. 2015). Primer selection can have a significant impact on resulting microbiome composition, depending on the hypervariable regions amplified on the 16S rRNA gene (Claesson and O'Toole 2010, Fouhy, Clooney et al. 2016).

1.5.2.1 Metagenomic vs 16S

Shot-gun metagenomic (SGM) and 16S rRNA gene microbiome sequencing both have benefits and limitations. 16S rRNA gene sequencing is limited to taxonomic characterization of bacteria and archaea, yet provides a greater resolution at higher taxonomic levels, including phylum, class, order, family, and genus (Zhernakova, Kurilshikov et al. 2016, Barton, Penney et al. 2017). SGM sequencing provides higher resolution at species and strain level, allowing for more accurate predictions of functionality, as well as covering genes of all organisms in the microbiome. Unlike 16S rRNA gene sequencing, SGM provides a more accurate representation of true community proportions, as sequencing is not biased by primer selection.

1.5.2.2 Bacteriome Sequencing

Microbiome processing of samples requires swift preservation of the collected samples. Placing samples on ice during collection is generally considered good practice. If the experimental design is focused on certain bacteria, understanding of oxygen exposure and freezing method must be considered (Fouhy, Deane et al. 2015). Anaerobic bacteria viability and abundance can be altered with prolonged exposure to oxygen. Freezing methods affect cell viability in many *Lactobacillus* species - for improved survival, methods includes a 6-hour period at low temperatures (~20C) before transferring to -80C freezer (Bâati, Fabre-Gea et al. 2000, Volkert, Ananta et al. 2008). With all microbiome samples, care must be taken to minimize exposure to light, most importantly ultraviolet, heat, air, and contaminants.

In animal models, caecum or freshly collected faeces may be used for bacteriome sequencing. While caecum may provide a more representative picture of bacterial abundance in the host, caecum can only be collected at time of culling and thus is not practical for longitudinal studies. Furthermore, most sample collection in clinical studies are faecal, unless a biopsy is performed. Certain bacteria, and not others, can differ between caecal and faecal samples from the same subject.

If a DNA extraction kit is used, all samples or randomized groups of samples should be extracted with the same kit(s). Minor variations in kit reagents and protocol, notably the Qiagen InhibitEX reagent which absorbs DNA degrading substances and heating of lysis buffer, can affect microbiome results. Vigorous vortexing or bead-beating can potentially sheer DNA, also altering results. Randomized samples should undergo extraction and library preparation in batches to eliminate the confound of batch-effects. Similarly, sample order should be randomized in the 96 well plates to control for time effect. Microbiome DNA extraction and library preparation must be performed in a sterile environment, using sterile equipment. Primer selection for amplification of the 16S rRNA gene hypervariable region will be discussed further in 1.6.2 Sequencing subsection. During 16S rRNA gene amplification, ideally, a separate sterile hood and pipettes should be used to prevent amplicon contamination in subsequent DNA sample extractions.

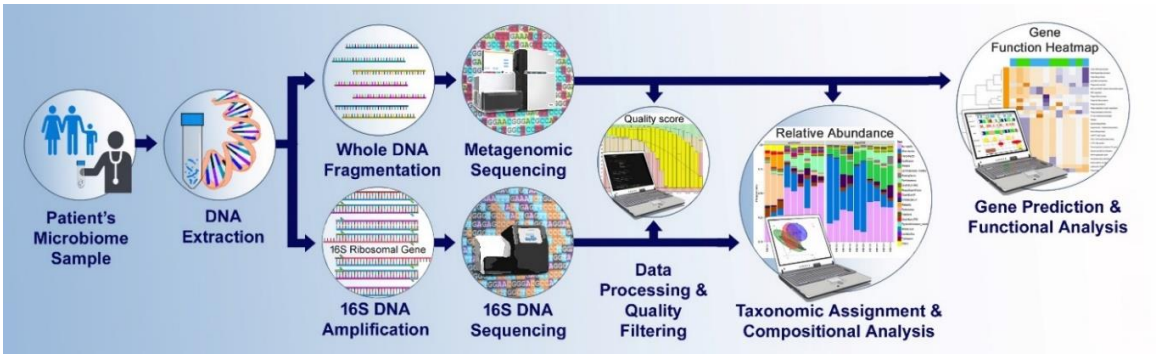

***Figure 2.5: How the microbiome is analysed**. Flow chart (left to right) of how a patient's sample is processed to obtain microbiota data. Following DNA extraction, the microbiome can either be sequenced by 16S ribosomal RNA gene amplification, which is specific for archaea and bacteria, or metagenomic sequencing which sequences all genes and captures predominantly bacterial DNA as well as fungi, virus, and archaea. Both methods then undergo quality filtering and bioinformatic analysis. With metagenomic sequencing, bacteria can be better identified at the species and strain level and functional genetics can be estimated. 16S sequencing provides a more robust profile of overall bacterial composition.*

1.5.2.3 Viral Sequencing

Sample collection for refined virome sequencing varies considerably from the bacteriome. Published protocol for the refinement of lytic bacteriophage virus from faecal samples requires 2-3grams of faecal matter (Reyes, Wu et al. 2013), a goliath task when a single fresh mouse dropping weight approximately 40mg. Hence, in studies collecting mouse stool at multiple timepoints throughout the study, with aims to sequence both bacteriome and virome separately, the only viable option is to collect dried pellets from bedding for virome analysis. Lytic bacteriophages are relatively stable under normal environmental conditions (Olofsson, Ankarloo et al. 2001, van Raaij, Schoehn et al. 2001) and their DNA/RNA is encapsulated in a protective protein barrier which prevents degradation in foreign environments; however relative abundancies likely vary compared to fresh faeces or caecum. Additionally, animals co-housed together in a single cage must be treated as a signal sample since faeces is collected from the entire cage. DNA extraction of refined phage/virome uses the ethanol-precipitation method to maximise DNA yield. Shot-gun metagenomic sequencing of phage/virome is performed without amplification, as this step can potentially bias results.

1.5.3 Bioinformatic Pipelines

Bioinformatic pipelines are the development and use of software programs to analyse genomic sequences in a biologically relevant manner. Pipelines vary between shot-gun metagenomic and 16S sequencing, as well as within these two sequencing methods. A simplified overview of a pipeline includes: i) filtering of raw sequence data; ii) joining or assembly of filtered sequences; iii) taxonomic assignment using a classifier and reference database. Various packages are available for performing steps in the pipeline. The redundancy of available packages is a major cause of variability across different pipelines for the same sequencing method. Additionally, packages performing the same task can have variations in algorithms used (Ahmed, Claesson et al. 2009, Clooney, Fouhy et al. 2016). As stated previously, ‘proper’ pipeline methodology can depend on experimental design and aims.

Aspects of the bioinformatic pipeline that influence microbiome results include a) classifiers and binning software (Ahmed, Claesson et al. 2009, Clooney, Fouhy et al. 2016, Walsh, Crispie et al. 2018), b) reference database (Ahmed, Claesson et al. 2009, Balvociute and Huson 2017), and c) threshold settings for filtering and accepting taxonomic assignments (Ahmed, Claesson et al. 2009). Classifiers and binning software implement various algorithms to compare sample sequences to the reference genomes in a database and determine taxonomic assignment at different levels (ex., Phylum, Family, Genus). Several reference databases are available to assign phylogeny to sequences. For 16S bacteriome sequences, Silva and Ribosomal Database Project (RDP) are two commonly used databases with Silva being one of the largest (Balvociute and Huson 2017). Thresholds are set to filter out low quality sequences and to control for false-positives during taxonomic assignment (Walsh, Crispie et al. 2018); changes in these thresholds can effect quality, reliability, and reproducibility of results.

1.5.4 Alpha and Beta Diversity

Differences in microbiome between groups can be assessed by alpha & beta diversity (Morgan and Huttenhower 2012). Measurements of diversity are a crucial part of microbiome analysis, as these measures are generally less sensitive to discrepancies in methodology (Ahmed, Claesson et al. 2009, Walsh, Crispie et al. 2018). Beta diversity measures similarities and differences in microbiome composition. Ordination plots are commonly used to measure beta diversity of the microbiome composition between samples or groups. Ordination plots are made from a distance matrix that is calculated from pairwise comparisons of the microbiome composition of each sample. Types of ordination plots include Principle Component Analysis (PCA), Principle Coordinate Analysis (PCoA), Multidimentional Scaling (MDS), and Canonical Correspondence Analysis (CCA), among others. Each dot in an ordination plot represents the entire microbiome of one sample. Dots are then coloured by group; an ellipsis can be calculated and drawn around the group. The location in space where a sample or group lies in the ordination plot is relative to other samples in the dataset. A statistical test can be

performed on the distance matrix to estimate significance of a grouping factor – most commonly referred to as ADONIS, ANOSIM, or PERMANOVA. Alpha diversity measures richness, abundance, and evenness of the microbiome in a single sample. Various formulas are used to measure alpha diversity, including: Shannon diversity index, Chao1 index, Observed Species, Simpson's diversity index, and Pielou's evenness. The most common alpha diversity index measures reported in the literature include Chao1, Observed Species. Shannon and Simpson. Example formulas are shown for Simpson and Shannon index, taken from the vegan document (Oksanen, Blanchet et al. 2019):

$$H = -\sum_{i=1}^{S} p_i \log_b p_i \qquad \text{Shannon–Weaver}$$

$$D_1 = 1 - \sum_{i=1}^{S} p_i^2 \qquad \text{Simpson}$$

Where p_i is the proportion of species *i* (or OTU in the case of 16S rRNA gene sequencing) and S is the total number of observed species (or OTUs). When all proportions for every specie is added together, it must equal 1 or 100%. Shannon uses the natural log numbers for the base (*b*), such as 2 or 2.718. Both Shannon and Simpson alpha diversity formulas measure richness and evenness. To exemplify the difference in the Shannon and Simpson a table is provided for two samples, where sample 1 is both richer and more even than sample 2:

	Sample 1	Sample 2
Spp 1	0.1	0.1
Spp 2	0.2	0.3
Spp 3	0.2	0.4
Spp 4	0.2	0.1
Spp 5	0.2	0.1
Spp 6	0.1	0
Sum P	1	1
Shannon	**2.521928**	**2.046439**
Simpson	**0.82**	**0.72**

The index values are different between Shannon and Simpson, however both have a higher index value for sample 1 relative to sample 2 but the magnitude of the difference is greater for the Shannon measure. For group comparisons, the median alpha diversity index is calculated per group and Mann-Whitney U test is performed to determine statistical significance. Alpha diversity measures are commonly depicted in bar plots. Unlike beta-diversity, the alpha diversity measure for one sample remains the same regardless of what it being compared to. Therefore, alpha diversity is a measure of diversity within a single site, and beta diversity measures across multiple sites.

1.5.5 Biostatistical Analysis

Taxonomic composition at a certain phylogenetic level (e.g., Phylum, Family, or Genus) depicts the composition of a single sample or group. This analysis allows for identification of specific organisms in the microbiome which may be contributing to a phenotype. Microbiome sequences are first normalized relative to their total sequence counts, each sample will have a total sequence sum of one (100%). Highly abundant taxa are assigned unique colours; all remaining taxa in low abundance are assigned to Other. Taxonomic composition is then represented in either a bar or pie chart. However, this graphical representation of community composition requires further analysis to report significance. Taxonomic comparisons are used to identify certain phylogenetic organisms in the microbiome that are significantly different between groups. The use of proper statistical tests in microbiome analysis usually consists of non-parametric tests. The median log-relative abundance of the phylogenetic organism is calculated per group and either Mann-Whitney or Kruskal-Wallis test is performed and corrected for False Discovery Rate (FDR) to determine significance. Heatmaps depict pairwise comparisons between individual samples and/or measures. Heatmaps can be organized in a manner that reveal trends in the data set. Common calculations used in heatmaps include correlation, similarity (e.g., Beta diversity), and deviation from the mean (Z-score). These heatmaps are commonly used to show correlations between microbiome and biological

measures, functional changes in metabolic pathways, and how similar the microbiome of a single sample is to others in the same group vs. another group.

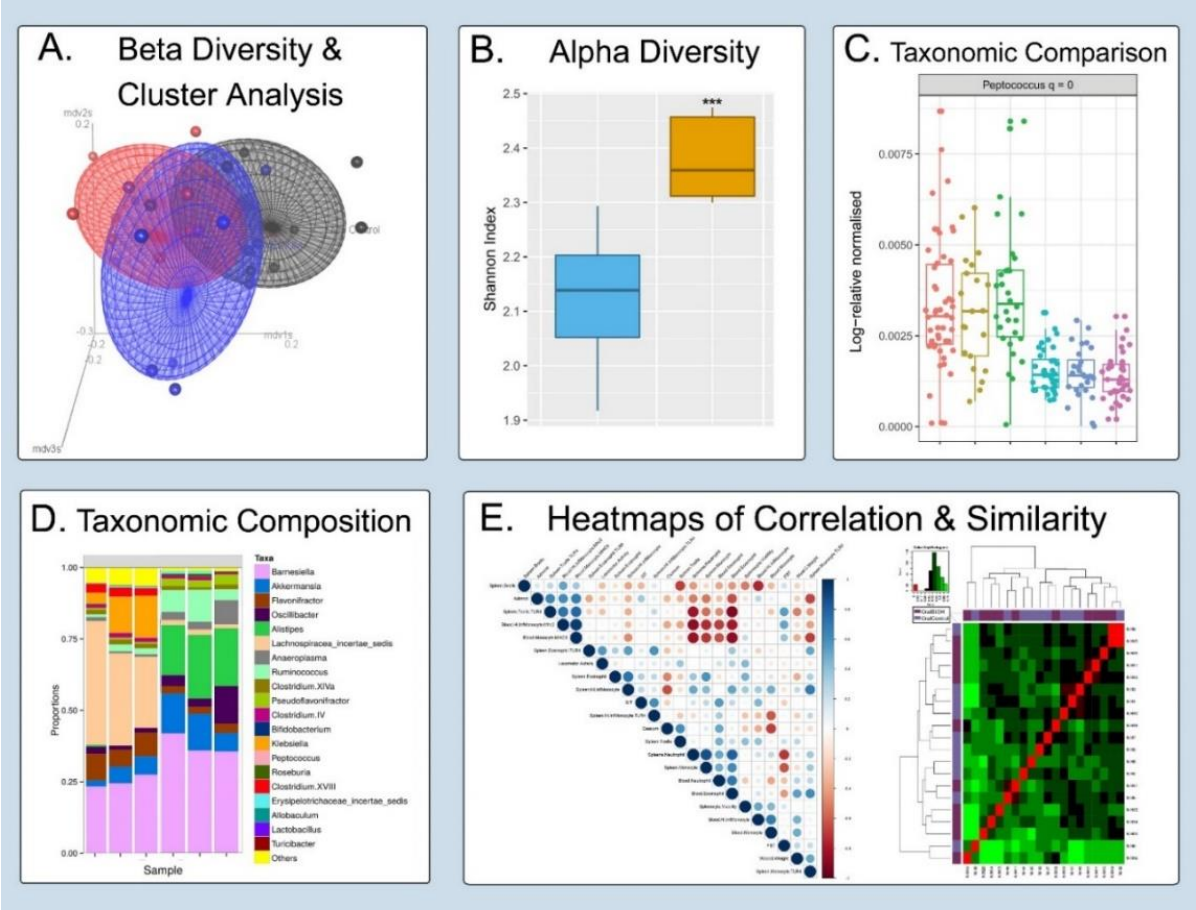

Figure 1.5.5: Ways to measure the microbiome. *Graphs are depicted that are commonly used to report finding from microbiome analysis. A) Beta-Diversity by ordination plots include Principle Component Analysis (PCA) or Principle Coordinate Analysis (PCoA). Each dot in an ordination plot represents the entire microbiome of one sample. The location in space where a sample or group lies in the ordination plot is relative to other samples in the dataset. B) Alpha diversity measures richness, abundance, and evenness of the microbiome in a single sample. Various formulas are used to measure alpha diversity, including: Shannon diversity index, Chao1 index, Observed Species, Simpson's diversity index, etc. Unlike beta-diversity, the alpha diversity measure for one sample remains the same regardless of what it being compared to. C) Taxonomic comparisons are used to identify certain phylogenetic organisms in the microbiome that are significantly different between groups. This analysis allows for identification of specific organisms in the microbiome which may be contributing to a phenotype. D) Taxonomic composition at a certain phylogenetic level (e.g. Phylum, Family, or Genus) depicts the composition of a single sample or group, normalized relative to their total sequence counts, each sample will have a total sequence sum of one (100%). E) Heatmaps depict pairwise comparisons between individual samples and/or measures. Heatmaps can be organized in a manner that reveal trends in the data.*

1.6 Primary hypothesis and aims of thesis

The primary goal of this work focused on characterizing changes in gut microbiome between 1) drug-exposed/addiction phenotype and 2) stress groups compared to normal controls. The hypothesis was that microbiome changes are associated to different behavioural phenotypes, either inherent or induced through drugs/stress. These changes in microbiome are linked to behavioural and biological measures attributed to a behavioural phenotype. The purpose of these studies was to investigate if a behavioural phenotype was associated to the 1) bacteriome and alcohol abuse/addiction and 2) virome and stress. We hypothesize that bacteriophage exert an effect on psychological health via the microbiota-gut-brain axis by altering the dynamics and composition of gut microbiota, and by affecting the immune system and intestinal permeability. This work represents a unique facet in microbiome research, investigating its implication in drug addiction and virome in stress.

AIM I: Does alcohol exposure alter gut microbiome composition when administered outside the gastrointestinal system? To answer this, a vapor alcohol administration mouse model was implemented to assess alterations in gut microbiome.

AIM II: Is the gut microbiota composition inherently different between subjects predisposed to addiction compared to those at lower risk? For this, we utilized a rat model of compulsive alcohol seeking which characterizes propensity to compulsively self-administer alcohol orally. This model has clinical relevance, as many tests of compulsive alcohol addiction simulate the DSM V criteria for alcohol abuse. Furthermore, low to high-risk phenotypes in this rat model fall along a spectrum, which is seen in many human psychological disorders. Furthermore, caecum samples from phenotype rats were collected after a 2-month abstinence period. Thus, microbiome results are more representative

of the inherent associations to behavioural phenotype than that of oral alcohol exposure.

AIM III: Is sex a significant factor that may account for some inherent microbiota variations associated to addiction? In our current research, microbiome analysis was carried out on a large cohort of female (N=101) and male (N=101) rats. These subjects underwent rigorous behavioural phenotyping, mainly comprised of tests that assess behavioural characteristics associated to addiction including impulsivity and dopamine-mediated behaviour. To assess variations in microbiota, samples were analysed using a goal/sign-tracking phenotype while accounting for sex.

AIM IV: Is the virome sensitive to chronic stress? Chronic psychosocial stress in mice was carried out to characterize changes in bacteriome and virome. Chronic stress is a contributing factor in many psychological disorders comorbid in addiction, such as anxiety and depression. Additionally, stress triggers compulsive addictive behaviour which further perpetuates the cycle of addiction. This exploratory study sought to breach the topic of gut virome in the context of the microbiome-gut-brain axis (MGB axis). Virome analysis focused on bacteriophage because of their direct influence on bacteria. Findings from this introductory research may spur future MGBA studies associated to stress, such as addiction.

2 Chapter

Drunk Bugs: Chronic Vapour Alcohol Exposure Induces Marked Changes in the Gut Microbiome in Mice

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2.1 Abstract

The gut microbiota includes a community of bacteria that play an integral part in host health and biological processes. Pronounced and repeated findings have linked gut microbiome to stress, anxiety, and depression. Currently, however, there remains only a limited set of studies focusing on microbiota change in substance abuse, including alcohol use disorder. To date, no studies have investigated the impact of vapour alcohol administration on the gut microbiome. For research on gut microbiota and addiction to proceed, an understanding of how the route of drug administration affects gut microbiota must first be established. Animal models of alcohol abuse have proven valuable for elucidating the biological processes involved in addiction and alcohol-related diseases. This is the first study to investigate the effect of vapour route of ethanol administration on gut microbiota in mice. Adult male C57BL/6J mice were exposed to 4 weeks of chronic intermittent vapourized ethanol (CIE, N=10) or air (Control, N=9). Faecal samples were collected at the end of exposure followed by 16S sequencing and bioinformatic analysis. Robust separation between CIE and Control was seen in the microbiome, as assessed by alpha (Shannon and Simpson index, $p<0.05$) and beta (ANOSIM, $p<0.001$) diversity, with a notable decrease in alpha diversity in CIE. These results demonstrate that CIE exposure markedly alters the gut microbiota in mice. Significant increases in genus *Alistipes* ($p<0.001$) and significant reductions in genera *Clostridium IV* and *XIVb* (Kruskal-Wallis, $p<0.001$), *Dorea* (Kruskal-Wallis, $p<0.01$), and *Coprococcus* (Kruskal-Wallis, $p<0.01$) were seen between CIE mice and Control. These findings support the viability of the CIE method for studies investigating the microbiota-gut-brain axis and align with previous research showing similar microbiota alterations in inflammatory states during alcoholic hepatitis and psychological stress.

2.2 Introduction

The gut microbiota includes a community of bacteria that play an integral part in nutrient metabolism and absorption in addition to gating host immune function (Luczynski, Neufeld et al. 2016). Recently, a growing body of evidence points to the presence of a microbiota-gut-brain axis. Indeed, preclinical studies have associated commensal bacteria to hypothalamic-pituitary-adrenal (HPA) signalling (Luczynski, Neufeld et al. 2016), and neurodevelopment processes (McVey Neufeld, Luczynski et al. 2016), such as myelination (Hoban, Stilling et al. 2016), in addition to various behavioural phenotypes (Luczynski, Neufeld et al. 2016). Currently, however, there remains only a limited set of studies focusing on microbiota change in substance abuse, including alcohol use disorder (Bull-Otterson, Feng et al. 2013, Leclercq, Matamoros et al. 2014, Llopis, Cassard et al. 2016). To date, no studies have investigated the impact of vapour alcohol administration on the gut microbiome.

Chronic alcohol abuse can cause damage to health including nutrient depletion, cognitive deficits, and alcoholic liver disease. Animal models of alcohol abuse have proven valuable for elucidating the biological processes involved in addiction, fetal alcohol syndrome, and alcohol-related diseases. A widely used murine model of chronic alcohol abuse is chronic intermittent ethanol (EtOH) (CIE) exposure because it resembles the prolonged, repeated patterns of alcohol abuse seen in humans (Lopez and Becker 2005, Koob 2013). Prior studies using the CIE model have reported changes in a range of neural indices and behavioural phenotypes, including increased EtOH self-administration (Holmes, Fitzgerald et al. 2012, DePoy, Daut et al. 2015, McCool and Chappell 2015, Peterson, McCool et al. 2015, Radke, Jury et al. 2015).

The CIE model could be advantageous for studying potential changes in the gut microbiome resulting from chronic EtOH exposure, as compared to other methods such as EtOH drinking, because 1) there is less potential for confounding effects of caloric intake 2) administration dose is consistent

among subjects, and 3) vapour administration circumvents the physiological effects of EtOH in the gastrointestinal tract, such as altered nutrient absorption. For these reasons, the current study employed the CIE method in order to investigate the consequences of chronic vapour EtOH on gut microbiome.

2.3 Methods

Adult male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) at 7 weeks of age. The C57 mouse strain was selected because previous work from this laboratory had established a compulsive-ethanol seeking phenotype in this strain (Radke, Jury et al. 2017). Mice were housed two per cage in a temperature (72±5°F) and humidity (45±15%) controlled SPF vivarium on a 12 hour light/dark cycle (lights on 06:00). Animals were allowed to acclimate to the facility for 1 week prior to CIE. Food and water was provided *ad libitum* for the duration of the experiment. Food, NIH-31 Harlan/Teklad Roden Chow, was obtained from Harlan Teklad (Indianapolis, IN, USA). Cages were changed the same day each week (Mondays) and fresh Teklad corn cob bedding (1/8") was also obtained from Harlan Teklad. Experimental procedures were approved by the NIAAA Animal Care and Use Committee and followed NIH guidelines.

Subjects were randomly assigned to either the air (Control) or CIE group; N=10 for each group. A previously described vapour inhalation procedure was employed (Lopez and Becker 2005, DePoy, Daut et al. 2015). Prior to each session of vapour ethanol exposure, test subjects received IP injections of 1.5 g/kg of 20% EtOH (v/v) with 71.6 mg/kg of an alcohol dehydrogenase (ADH) inhibitor, pyrazole (Sigma, St. Louis, MO, USA), in a combined volume of 10 ml/kg body weight, to initiate intoxication and stabilize blood EtOH concentrations (BECs). The average weight of mice was ~22 grams for both groups. During exposure, mice were removed from their home cages and singly housed in clean 60×36×60 cm cages (PlasLabs, Lansing,

MI, USA) and placed into Plexiglas vapour chambers. 95% EtOH was passed through a vapourization stone at 19-22 mg EtOH/L of fresh air and delivered at a rate of ~10 L/min. BECs were measured in sentinels using the Analox AM1 alcohol analyzer (Analox Instruments USA, Lunenburg, MA, USA) and achieved BECs of 175±25 mg/dL. The protocol for Control group was similar to the CIE. The Control group received a 68.1 mg/ kg IP injection of pyrazole and was exposed to air at a rate of ~10L/min in Plexiglas vapour chambers directly adjacent to the EtOH vapour chambers. Pyrazole dose was adjusted based on solubility for mice to receive equal concentrations, thus dosage was higher in the CIE treated group because the solubility of pyrazole (a hydrophobic heterocyclic compound) is lower in EtOH than in saline (0.9% NaCl). Vapour exposure occurred for 16 hours per day (17:00-09:00), 5 days a week (Monday-Friday) for 4 consecutive weeks. Mice were 8 weeks of age at the beginning of the study and 12 weeks of age at the end of the study.

Immediately following the final exposure, mice from both groups were euthanatized via cervical dislocation and cecal and colonic contents were harvested, pooled and diluted 40-fold (weight: volume) in sterile water. After centrifugation at 800 RPM, the supernatant was aliquoted under sterile conditions for storage at -80°C.

The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA from samples dilutions. Extracted DNA was further concentrated using a standard EtOH precipitation protocol. The prokaryotic 16S ribosomal RNA gene (16S rRNA) was amplified from extracted DNA using amplicon PCR for the V3 and V4 regions following the *Illumina 16S Sample Preparation Guide*. Using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), 16S rRNA amplicons were further prepared for 2x250bp sequencing on the Illumina MiSeq platform. The Illumina V3–V4 primers were selected for this study because they have a high coverage (94.5% bacteria)

while remaining in the amplicon size necessary to sequence at 2×250 bp (Klindworth, Pruesse et al. 2013).

All sequences in FASTQ files format were filtered using PRINSEQ. Sequences with length less than 150 nucleotides or with low quality at the 3' end were removed. Paired-end reads with a minimum overlap of 20 base-pairs were joined using FASTQ-join. Finally, all single files were processed to a final filtering sequence (mean quality score > 20). Sequences from one sample in the control group had to be removed due to low sequencing reads, which resulted in a low quality score (< 20). After filtering quality and length trimming, the average number of high-quality sequences generated per sample (Control N=9, CIE N=10) was 63,600 ±25,932 SD. The average number of OTUs per sample was 649±128 SD. The sequences were matched at operational taxonomic unit (OTU; 97% identity level) using closed-reference USEARCH v7.0 algorithm against The Ribosomal Database Project. Alpha and beta diversity was determined using QIIME. Additional alpha and beta diversity analyses were performed with the R package phyloseq.

Alpha diversity was computed based on Shannon and Simpson methods and was visualized via the phyloseq package. Family and genera-level analyses were carried out using the Kruskal-Wallis method with the phyloseq package. Corrected p-values (q-value) adjust for multiple testing according to the method of Benjamini and Hochberg (Benjamini and Hochberg 1995). Post hoc analysis of statistically significant taxa was performed to calculate log2 fold change in CIE relative to Control, p-values were estimated using negative binomial distribution (Gamma-Poisson).

2.4 Results

The Shannon and Simpson indexes were selected because the two are the most commonly used formulas for measuring evenness and richnessalpha

diversity of microbiota (Morgan and Huttenhower 2012). These indexes showed significant (Shannon and Simpson indexes, $p<0.05$) differences in alpha diversity, with mean diversity decreased in the CIE group, relative to controls (Figure 2.3A).

Sample distribution by sequenced OTUs using Principle Coordinate Analysis (PCoA) revealed phylogenetic separation between CIE and Control groups (Figure 2.3B). Beta diversity analysis comparing taxonomic similarities between individual samples was calculated with Bray-Curtis (Figure 2.3C). The heatmap shows clustering of samples based on microbiota composition. Samples are distributed by overall taxonomic OTU similarity, regardless of group designation. This method is used to assess if subjects cluster by exposure method, similar to a PCoA plot. Also similar to the PCoA plot, individual taxonomic components cannot be determined. In the Bray-Curtis heatmap, subjects clustered by exposure group (Figure 2.3C). Multivariate analysis of beta diversity between CIE and Control groups revealed significant differences in total sequenced OTUs (ANOSIM, $p<0.001$ or adonis, $p<0.001$) and at the genus level (ANOSIM, $p<0.01$) but no significant difference at the family level (ANOSIM, $p=0.157$).

At the family level, data revealed a significant difference in the Rikenellaceae family (Kruskal-Wallis, $p<0.001$) between groups, which remained statistically significant when corrected for multiple comparisons (Bonferonni, q-value<0.05) (Figure 2.4B). Rikenellaceae was significantly increased (log2 fold change>3.5, $p<0.0001$) in CIE relative to Control (Figure 2.4B).

At the genus level, data revealed significant differences in *Alistipes* (Kruskal-Wallis, $p<0.001$) and *Clostridium* IV and XIVb (Kruskal-Wallis, $p<0.001$) between CIE mice and Control, which remained statistically significant when corrected for multiple comparisons (Bonferonni, q-value<0.05) (Figure 2.4B). A higher abundance of *Alistipes* (log2 fold change>3.8, $p<0.0001$) was seen in the CIE group, with decreases in *Clostridium* IV (log2 fold change>-1.4, $p<0.001$) and *Clostridium* XIVb (log2 fold change>-2.0, $p<0.001$) (Figure 2.4).

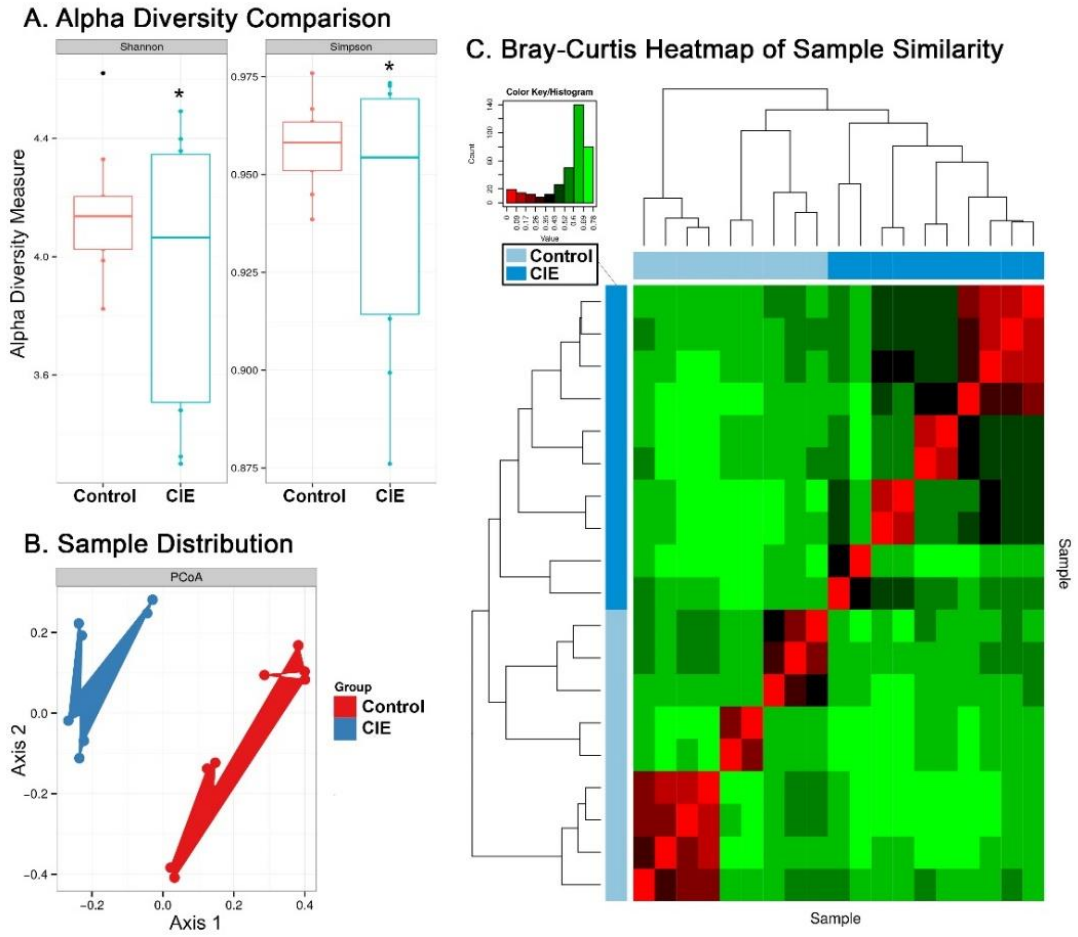


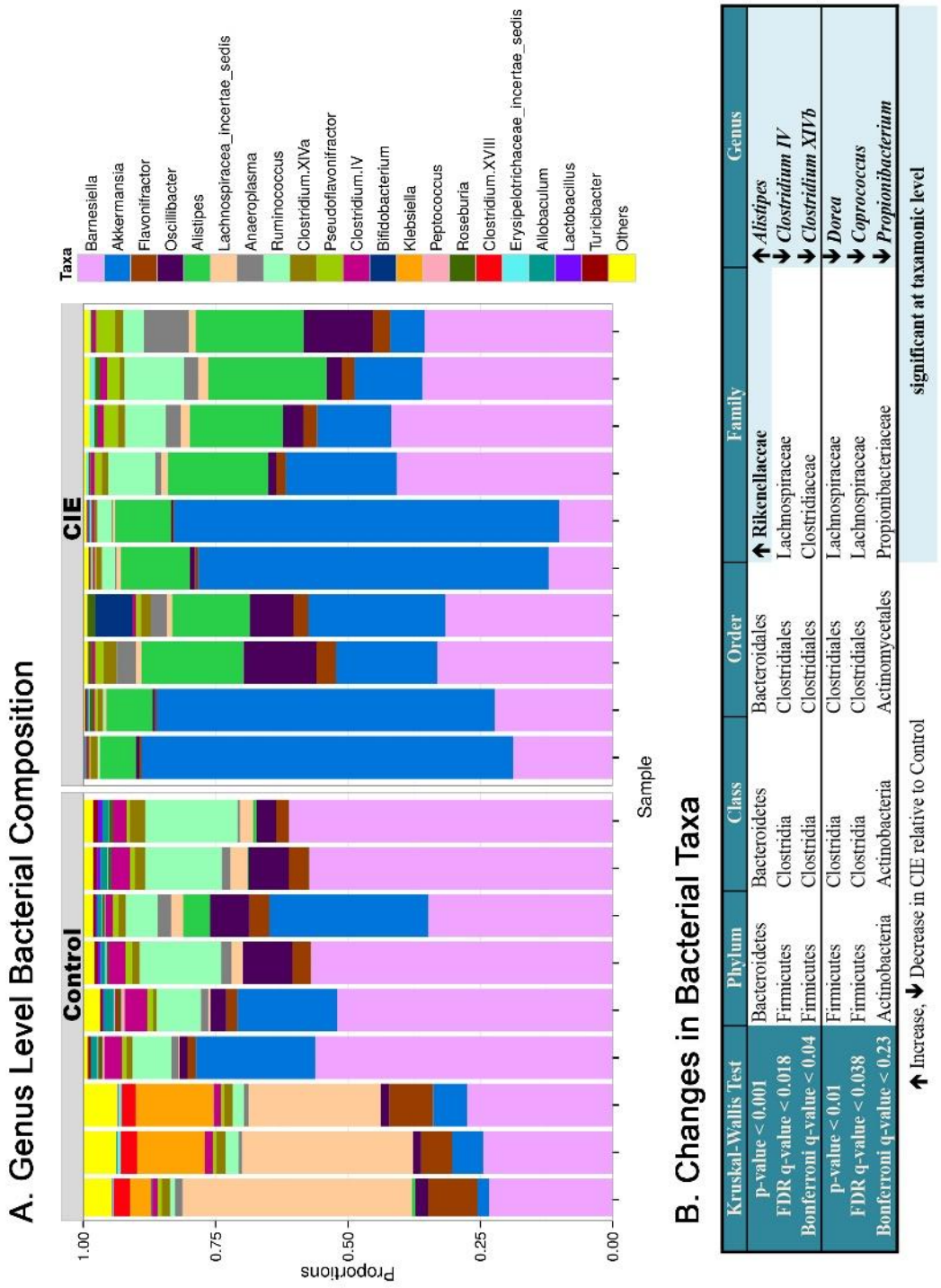
Figure 2.3: Effects of CIE vapour exposure on the microbiome.

(A) Shannon and Simpson Index alpha diversity measures of bacterial number and distribution by group. Group outliers indicated with a dot, significance ($p<0.05$) indicated with an asterisk. (B) PCA plot showing distribution of samples by OTUs. Clusters are coloured based on their respective experimental group. (C) Bray-Curtis heatmap of beta diversity clustering by sample. Axes indicate: i) individual sample per row (right y-axis) and column (bottom x-axis) and ii) dendrogram showing hierarchical relationships (not phylogenetic) by total OTUs composition with blue bar showing group assignment (left y-axis, top x-axis). Taxonomic information can not be determined from these figures.

Other changes were seen for *Dorea* (Kruskal-Wallis, p<0.01)*, Coprococcus* (Kruskal-Wallis, p<0.01), and *Propionibacterium* (Kruskal-Wallis, p<0.01), which did not remain statistically significant when corrected for multiple comparisons (Bonferonni, q-value=0.23) but did remain significant when corrected for false discovery rate (FDR, q-value<0.05)*. Akkermansia* was visibly increased in the CIE graph of bacterial composition (Figure 2.4A), but significance (Kruskal-Wallis, p<0.05) did not hold after corrections (Bonferonni q-value=1.00). Significant decreases in *Dorea* (log2 fold change>-2.5, p<0.01)*, Coprococcus* (log2 fold change>-1.6, p<0.05), and *Propionibacterium* (log2 fold change>-1.7, p<0.05) were observed in the CIE group relative to Control (Figure 2.4).

2.5 Discussion

Although disturbances in the microbiome are linked to a variety of health states and the action of a variety of pharmacological agents, there is a paucity of information on the impact of chronic EtOH on microbiome composition (Spanogiannopoulos, Bess et al. 2016). Here we found that the bacterial composition in mice exposed to CIE, as measured from Bray-Curtis and PCoA analyses, exhibited a clear separation from that seen in air-exposed Controls. These data add to a growing literature showing how changes in the microbiome can occur independently of direct administration of a substance into the gut (Bailey, Dowd et al. 2011). They also extend the findings of a previous report showing reductions in alpha diversity following chronic alcohol feeding (Bull-Otterson, Feng et al. 2013). Taken together with the current findings, these results demonstrate alterations in the gut microbiota in response to chronic EtOH across a range of routes of administration.



Kruskal-Wallis Test	Phylum	Class	Order	Family	Genus
p-value < 0.001	Bacteroidetes	Bacteroidetes	Bacteroidales	↑ Rikenellaceae	↑ *Alistipes*
FDR q-value < 0.018	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	↓ *Clostridium IV*
Bonferroni q-value < 0.04	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	↓ *Clostridium XIVb*
p-value < 0.01	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	↓ *Dorea*
FDR q-value < 0.038	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	↓ *Coprococcus*
Bonferroni q-value < 0.23	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	↓ *Propionibacterium*

↑ Increase, ↓ Decrease in CIE relative to Control

significant at taxamonic level

Figure 2.4: Effects of CIE vapour exposure on bacterial composition. *(A) Genus level bacterial composition by group. (B) Statistically significant taxonomic changes in CIE exposed relative to Controls. Statistically significant taxa highlighted in light blue and grouped into rows ordered based on p-value.*

In this study, *Alistipe*s was the only genus of bacteria that significantly increased with CIE. Increases in *Alistipe*s and reductions in *Clostridium cluster* IV have been found in mice receiving fecal microbiota transfer from alcoholic patients with severe hepatitis (Llopis, Cassard et al. 2016). An increased population of *Alistipe*s has also been observed in chronic fatigue syndrome, inflammation, inflammatory bowel syndrome, and depression (Naseribafrouei, Hestad et al. 2014, Jiang, Ling et al. 2015). We observed a decrease in genus *Dorea* in CIE relative to Control (Figure 2B). Conversely, a study by Leclerq and colleagues found increases in *Dorea* associated with high intestinal permeability in alcoholics. However, our current study found reductions in *Clostridium cluster* IV also seen in this aformentioned study (Leclercq, Matamoros et al. 2014). The genus *Clostridium cluster* IV is populated by bacterial species believed to confer an anti-inflammatory effect and therefore beneficial to the host (Llopis, Cassard et al. 2016). *Faecalibacterium prausnitzii* and *Clostridium leptum* are among these beneficial species within this genus and are reduced in studies of alcohol consumption and hepatitis (Leclercq, Matamoros et al. 2014, Llopis, Cassard et al. 2016). There is limited information about the functions of genra *Propionibacterium* and *Clostridium* XIVb within the gut microbiome though they are known to include species that produce short-chain fatty acids (Johns 1952, Kelly, Kennedy et al. 2015) which are implicated in behavior and cognition (Kennedy, Murphy et al. 2016). Similar reductions in *Dorea* and *Coprococcus* which have previously been observed in chronic social stress and correlated to increases in pro-inflammatory cytokines IL-6 and MCP-1 (Bailey, Dowd et al. 2011). Future studies are needed to examine the relative importance of these alterations to the pathophysiology of disease states.

Despite its advantage of mimicking the most common route of alcohol administration in humans, oral EtOH self-administration in rodents has several limitations, including: 1) dosage variability between test subjects, 2) inconsistent and unreliable self-administration in animals, 3) difficulty in

achieving and maintaining a desired BEC, and 4) experimental confounds such as caloric intake (McCool and Chappell 2015). Many of the issues with animal self-administration models or ethanol exposure are predominantly due to the fact that most animals do not enjoy the taste of alcohol nor the feeling of being inebriated While vapour exposure is not a common form of achieving intoxication in humans, this method is valuable in differentiating the underlying mechanisms involved in microbial changes caused by gastrointestinal EtOH exposure and other changes acting through the brain-gut-microbiota axis. In this study, we selected the vapour ethanol exposure method because it by-passes the gastrointestinal system. A limitation in using this CIE model of vapor exposure is the administration of pyrazole, a known hepatoxin, which may confound results by having a synergistic effect with ethanol and LPS that may further alter liver function, inflammation, and gut microbiome. No obvious signs of liver damage were observed in either the AIR or CIE mice during the necropsies that immediately followed the respective exposures. Selection of sequencing method and strain specific differences in microbiota composition may have also affected accurate identification of bacterial taxa and reproducibility of results within this study. However, current findings indicate changes in bacteria possessing functional traits that have been seen across a variety of related microbiome studies (Leclercq, Matamoros et al. 2014, Llopis, Cassard et al. 2016).

In conclusion, the current study reveals marked changes in the bacterial composition of faecal microbiota in C57BL/6J mice chronically exposed to EtOH vapours. These findings align with and extend a growing body of evidence of microbiota changes associated with inflammation and support the viability of the CIE method for future studies investigating the effects of EtOH on the microbiota-gut-brain axis. Furthermore, the robust effects of EtOH vapour suggest that other substances of abuse administered outside of the gastrointestinal system may potentially alter gut microbiota.

3 Chapter:

Gut microbiome correlates with altered striatal dopamine receptor expression in a model of compulsive alcohol seeking

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Veronica L Peterson (Co-first author) Contribution: Performed DNA extraction, microbiome analysis, interpreted results, created Figures, and wrote large parts of the manuscript.

3.1 Abstract

Identifying biological markers predicting vulnerability to develop excessive alcohol consumption may lead to a real improvement of clinical care. With converging evidence suggesting that the gut microbiome is capable of influencing brain and behaviour, this study aimed at investigating whether changes in gut microbiome composition is associated with conditioned responses to alcohol. We trained Wistar rats (N=59) to self-administer alcohol for a prolonged period before screening those exhibiting uncontrolled alcohol seeking and taking by modelling diagnostic criteria for alcohol use disorder (AUD): inability to abstain during a signalled period of reward unavailability, increased motivation assessed in a progressive effortful task and persistent alcohol intake despite aversive foot shocks. Based on addiction criteria scores, rats were assigned to either Vulnerable or Resilient groups. Vulnerable rats not only displayed increased impulsive and compulsive behaviours, but also displayed increased relapse after abstinence and increased sensitivity to baclofen treatments compared to resilient animals. Then, rats underwent a 2-month wash out period before sacrifice. Dorsal striatum was collected to assess dopamine receptor mRNA expression (N=15), and 16S microbiome sequencing (N=38) was performed on caecal contents. Multiple significant correlations were found between gut microbiome and impulsivity measures, as well as augmentations in striatal Dopamine 1 receptor (D1R) and reductions in D2R as vulnerability to AUD increased. Therefore, using a singular translational approach based on biobehavioral dispositions to excessive alcohol seeking without heavy intoxication, our observations suggest an association between gut microbiome composition and these specific “at risk” behavioural traits observed in our translationally relevant model.

3.2 Introduction

Unhealthy alcohol use is one of the world's leading causes of death and diseases. Recent reports underline that approximately 3.3 million deaths worldwide and 139 million disability adjusted life years are attributed to alcohol use (World Health Organization 2014). Besides this unacceptable human cost, alcohol use disorder (AUD) represents a growing economic burden worldwide, the total cost of which is estimated in the range of US$ 200-700 billion annually, both in Europe and in the United States (Baumberg 2006, Sacks, Gonzales et al. 2015, Barrio, Reynolds et al. 2017). Reduction of heavy drinking and relapse prevention currently represents the main therapeutic objectives in the treatment of alcohol use disorder, but the ratio of good responders remains much too low to be satisfying (Mann and Hermann 2010). Therefore, alternative approaches should be privileged. One strategy would consist of better identifying "problem drinkers" in the general population who are not yet manifesting major symptoms of heavy intoxication but are drinking at levels that increase risks for medical and psychosocial consequences (Saitz 2005). Growing evidence points out to a role of the microbiota-gut-brain axis in AUD, with excessive ethanol consumption altering the gut microbiome, increasing the intestinal permeability and exacerbating systemic inflammation, ultimately amplifying comorbidities classically observed in alcoholic patients (Leclercq, Cani et al. 2012, Bull-Otterson, Feng et al. 2013, Leclercq, De Saeger et al. 2014, Leclercq, Matamoros et al. 2014, de Timary, Leclercq et al. 2015, Gorky and Schwaber 2016, Leclercq, de Timary et al. 2017, Temko, Bouhlal et al. 2017).

Recent observations have begun to shed light on the inextricable connection between microbes and mammals, leading to the provocative postulate that humans would not have developed the current level of cognitive performance in absence of bacteria (Cryan and Dinan 2012, Montiel-Castro, Gonzalez-Cervantes et al. 2013, Dinan and Cryan 2015, Sampson and Mazmanian 2015). In support of this assertion, a current consensus has now established how the commensal microbiota of the intestine greatly influence all aspects

of physiology, including a fine tuning of brain function and behaviour (Hoban, Stilling et al. 2017, Dunphy-Doherty, O'Mahony et al. 2018). In this perspective, with the intestinal microbiome collectively encoding more than 3.3 million of non-redundant genes (Qin, Li et al. 2010), exceeding by far, the number encoded by the human host genome. Moreover, there is a growing appreciation of the role of the gut microbiome in regulating brain and behaviour in health and disease (Dinan and Cryan 2017, Sherwin, Dinan et al. 2017). Overall, the microbiota–gut–brain axis helps maintain homeostasis of the brain by controlling central physiological processes including neurotransmission, neurogenesis, neuroinflammation and neuroendocrine signalling (Clarke, Stilling et al. 2014). In particular, recent reports suggest that alcohol exposure triggers neuroimmune and inflammatory processes in the brain (Crews, He et al. 2007, Crews, Qin et al. 2013, Crews, Walter et al. 2017). Although the source of this neuroinflammation is not yet understood, growing evidence suggests that alterations in microbiota composition may contribute to neuroimmune processes and peripheral inflammation (de Timary, Leclercq et al. 2015, Gorky and Schwaber 2016, Rea, Dinan et al. 2016). Changes in the gut microbiome have been reported in both human alcoholic individuals and murine models of chronic alcohol exposure, with increased intestinal permeability (causing endotoxin to escape into the circulation and impact the host), increased abundance of pro-inflammatory gut microbes, like Proteobacteria species, and decreased abundance of normal commensal bacteria like Bacteroidetes (Mutlu, Gillevet et al. 2012, Bull-Otterson, Feng et al. 2013, Leclercq, de Timary et al. 2017, Peterson, Jury et al. 2017). However, if chronic excessive alcohol use seems to significantly impact the microbiota, it does not seem to be sufficient to cause gut dysfunction in all alcohol dependent patients, since altered microbiota composition was reported in only a subset of alcoholics. Further, alterations in microbial composition were not correlated to the duration of sobriety, suggesting alcohol-related dysbiosis is long-lasting and persists despite abstinent periods (Leclercq, Cani et al. 2012, Mutlu, Gillevet et al. 2012, Leclercq, De Saeger et al. 2014, Leclercq, Matamoros et al. 2014, Leclercq, de Timary et al. 2017). Meanwhile, gut microbial and peripheral metabolite level alterations remain narrowly linked to alcohol craving, anxiety, and

depression, considered important personality traits associated with the vulnerability to develop AUD (Leclercq, Cani et al. 2012, Leclercq, De Saeger et al. 2014, Leclercq, Matamoros et al. 2014, de Timary, Leclercq et al. 2015).

The inability to control drug taking in general, and conditioned responses, in particular, is a complex brain disorder that affects the most vulnerable individuals and worsens with recurring drug consumption. Therefore, understanding the heterogeneity in the behavioural characteristic of patients with AUD is warranted for developing personalized treatments. We recently claimed that most preclinical studies still defend pharmacology-centered views that do not really capture the inter-individual vulnerability to lose control over alcohol consumption (Jadhav, Magistretti et al. 2017). Considering that preclinical investigations about genetic/temperament predisposition to alcohol abuse require the development of an appropriate and relevant animal model, we adapted to rodents a few criteria used for screening AUD according to the DSM-V and contributed to recognize that addiction is a progressive and idiosyncratic disorder. The recent reports suggesting that personality traits associated with risk for drug addiction may be linked tothe microbiome-gut-brain axis (Bravo, Forsythe et al. 2011, Collins, Kassam et al. 2013, McVey Neufeld, Luczynski et al. 2016, Golubeva, Joyce et al. 2017) calls for further investigation to determine whether gut microbiome diversity and composition may be associated with behaviors related to alcohol use disorders. To this end, we assessed if microbiota composition was different in rats exhibiting a biobehavioural disposition to lose control over alcohol consumption by comparison with resilient animals. Moreover, we sought to investigate if changes in the microbiome correlated with alterations in striatal dopamine receptor levels that may underlie the observed behavioural changes in the rat model of AUD.

3.3 Material and methods

3.3.1 Animals

Male Wistar rats (N=59) were bred in-house at the Center for Psychiatric Neuroscience animal facility (breeders ordered from Charles River, France). They were approximately 7 weeks old and weighed 200–250 grams at the beginning of the experiment. They were kept in reversed 12-h light/dark cycle (lights off at 8.30 am) and housed in controlled temperature and humidity conditions. All experiments were performed in accordance with the Swiss Federal Act on Animal Protection and the Swiss Animal Ordinance and were approved by the cantonal veterinary office (authorization 3047 to B.B).

3.3.2 Behavioural phenotyping of alcohol use disorder

The procedure for screening addiction-like behaviours has been extensively described elsewhere (Jadhav, Magistretti et al. 2017). Briefly, rats were first monitored for assessing impulsive behaviours using a 5-choice serial reaction time task paradigm. They were then tested in an elevated plus maze (EPM) for measuring their anxiety-like behaviours. Then, rats were daily trained for 30 min to self-administer 0.1 ml of alcohol 10% weight/volume for 80 consecutive sessions, before being screened for addiction-like behaviour (see supplementary information for further detail). Test sessions aiming at identifying rats at risk of losing control over alcohol intake operationalized 3 diagnostic criteria for AUD: inability to abstain during a signalled period of reward unavailability, increased motivation assessed in a progressive effortful task and persistent alcohol intake despite aversive foot shocks. Each rat was considered positive for one addiction-like criterion if its score reached the 66th-99th percentile of the total distribution. The addiction score was calculated as the sum of the standardized scores of each of the addiction-like criteria (Deroche-Gamonet, Belin et al. 2004). A total of 60 rats were trained (1 outlier exhibiting very high lever pressing behaviour was excluded), with those identified as positive for 2-3 criteria, defined as Vulnerable were

grouped together (N=19), and those with 0 and 1 criterion grouped and named Resilient (N=40). To further validate our model, rats were first exposed to a conflict situation in which they had to bear electrical foot shocks prior to get access to ethanol. Second, we tested baclofen responses in both groups of rats in order to assess whether the reinforcing and motivational properties of alcohol in resilient and vulnerable rats were differentially sensitive to an anti-alcohol effect treatment. Further details on the behavioural procedure are provided in Supplementary Information.

3.3.3 Ceacal microbiome collection and sequencing

All samples from the Vulnerable group (N=19) and an equivalent subset from Resilient group (N=19) were used for microbiome analysis. The selected Vulnerable rats belonged to the top 33% of the population for the three behaviours and the Resilient rats belonged to the lowest 33% of the population. Caecum was collected following two months of abstinence under aseptic conditions and snap-frozen on dry ice. Protocols for microbiome sequencing were used as previously described (Peterson, Jury et al. 2017). Briefly, caecal contents from frozen caecum (stored at -80ºC) was extracted under a sterile hood. The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA from caecal contents using the manufacturer's handbook (Second Edition 2012) *Isolation of DNA from Stool for Pathogen Detection* protocol. Samples were prepared for 16S sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), as described in the Illumina 16S library preparation workflow. 16S bacterial rRNA gene was amplified using primers targeting the V3-V4 hypervariable region (Forward: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; Reverse:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Sigma Aldrich Ireland ltd., Wicklow, Ireland). The Illumina V3–V4 primers were selected for their high coverage (94.5% bacteria) while remaining in the amplicon size necessary for sequencing

(Klindworth et al., 2013). 16S rRNA amplicons were sequenced on the Illumia MiSeq platform (Teagasc, Morrepark, Ireland).

3.3.4 Microbiome Sequence Processing

Sequence reads in FASTQ files were joined using FLASH and analysed with QIIME(Quantitative Insights Into Microbial Ecology, v1.9.1). Sequence quality was checked, and chimeras removed, remaining sequences were clustered into Operational Taxonomic Units (OTUs) using USEARCH (Version 7.0-64bit). The average number of high-quality sequences generated per sample was 150,707 ± 69,666 SD. Taxonomy was assigned to OTUs using Silva version 123. Alpha diversity indices were calculated with Qiime.

3.3.5 Gene expression analysis

Samples from 0Crit (N=8) and 3Crit (N=7) group were selected for gene expression analysis. Whole brains were extracted and rapidly sliced into 2 mm-thick coronal sections in a rat brain stainless steel matrix. Slices containing dorsal striatum were used for micro-punch dissection (0.98 mm diameter micro-punch, Stoelting, Dublin, Ireland). RNA was extracted with an RNeasy Plus Minikit (Qiagen, Valencia, CA, USA) and converted into cDNA by reverse transcription reaction using TaqMan Reverse Transcriptase Reagents (Applied Biosystem, Foster City, CA, USA). Real-time PCR amplification was performed with an ABIPRISM 7500 cycler and SYBER green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) using specific sets of primers (Microsynth AG, 9436 Balgach, Switzerland). Forward and reverse primers for the tested genes are the following: β-actin = forward: 5′-GCTTCTTTGCAGCTCCTTCGT-3′, reverse: 5′-ATATCGTCATCCATGGCGAAC-3′; D1 receptor = forward: 5’-GGAGGACACCGAGGATGA-3’, reverse: 5’-ATGAGGGACGATGAAATGG-3’, D2 receptor = forward: 5’-TGGGTCAGAAGGGAAGG-3’, reverse: 5’-GATGATAAAGATGAGGAGGGT-3’. All samples were analysed in triplicates. Relative gene expression was measured with the comparative ΔΔCt method24 and normalized with β-actin transcript levels.

3.3.6 Statistical Analysis

For behavioural & mRNA Analysis, data was tested for normality (Shapiro-Wilks test) and equality of variances (Levene's Test). Data are expressed as mean ± standard error (SE). Parametric data were analysed by one way- and two-way ANOVAs followed by Bonferroni corrections, respectively. Homoscedastic, parametric measures were evaluated with two sample T-test. Unpaired T test was used to analyse the anxiety data. Nonparametric measures were evaluated with Mann–Whitney test. Baclofen response was calculated with a Wilcoxon sign-ranks test. The level of significance was set at 0.05, and analyses were performed using IBM SPSS Statistics. Microbiota analysis was performed in R (v3.3.3) and RStudio (v1.0.136). Plots were generated in R using ggplot2 package (v2.2.1). Mann–Whitney test was used to assess statistical significance in alpha diversity indices and taxonomic comparisons between groups. Beta diversity was visualized and analysed by OTU counts normalized using the wisconson function from vegan community ecology package (v2.4-3). Adonis (PERMANOVA, permutations=999) vegan function assessed beta diversity significance between groups. Spearman correlation was performed on genus and family level bacterial abundance, behavioural measures, and dopamine receptor mRNA relative-expression levels. Since dopamine mRNA expression data was only available for 15 subjects due to the remainder of cortical samples used for other analyses not reported in this manuscript, correlations to microbiome only included these 15 subjects. In all other behaviour correlations, all samples were used (N=38). All correlations and taxonomic comparisons were corrected for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg 1995), with the critical value for false discovery rate (Q) set at 0.25. A less stringent Q value was selected due to the exploratory nature of this study and because the total number of tests (n>23,000) created a restrictively conservative FDR-corrected p value at Q=0.05. Log2 fold ratio calculated mean genus-level change in abundance for the 3.Vulnerable group relative to Resilient group.

3.4 Results

3.4.1.1 Identification of rats at risk of alcohol use disorder

After 80 sessions of operant conditioning (fixed ratio 1, time out 4 sec to get 0.1 mL of 10% w/v ethanol, Figure 3.3), rats underwent a procedure for screening evidence for addiction-like behaviour. A rat was considered positive for an addiction-like criterion when its score was in the 66th to 99th percentile of the distribution. Hence, of the total 59 rats, we obtained 4 groups, 26 rats with 0 criterion, 14 rats with 1 criterion, 12 rats with 2 criteria and 7 rats with 3 criteria (Figure 3.3).

One-way ANOVAs revealed significant differences in the inability to abstain during a signalled period of reward unavailability ($F_{3,55} = 17.436$, $p < 0.0001$, Figure 3.3), in the motivation to seek for ethanol in a progressive effortful task ($F_{3,55} = 23.23$, $p < 0.0001$, Figure 3.3), in the persistence in ethanol seeking despite aversive foot shocks ($F_{3,55} = 22.55$, $p < 0.0001$, Figure 3.3), and finally in the vulnerability to relapse after a period of abstinence ($F_{3,55}=6.13$, $p=0.0012$, Figure 3.3). Further statistical analyses are provided in Supplementary Information.

The addiction scores, calculated as the sum of the standardized scores of each of the addiction-like criteria, were significantly different from each other ($F_{3,55} = 67.20$, $p<0.0001$), and were linearly increasing from 0crit to 3crit rats (Figure 1 F). We therefore clubbed 0 and 1 crit rats together and named them Resilient, while 2 and 3 crit rats were grouped and named Vulnerable. Further statistical analyses (available in the Supplementary Information) showed increased compulsive behaviour and increased sensitivity to baclofen treatments (Figure 3.3H, I and J), as well as increased impulsivity measures taken from the 5 Choice Serial Reaction Time Task (5CSRTT) (Figure 3.3K) in Vulnerable rats compared to Resilient. Factor analysis revealed that the three addiction-like criteria, the reinstatement and the pre-existing trait of impulsivity loaded on one construct accounting for 50% of the variance, and therefore measuring one single underlying factor. Overall,

these series of observations served as a strong rationale for identifying rats with a loss of control-prone phenotype, without heavy ethanol intoxication given their history of brief exposures to alcohol used in this procedure.

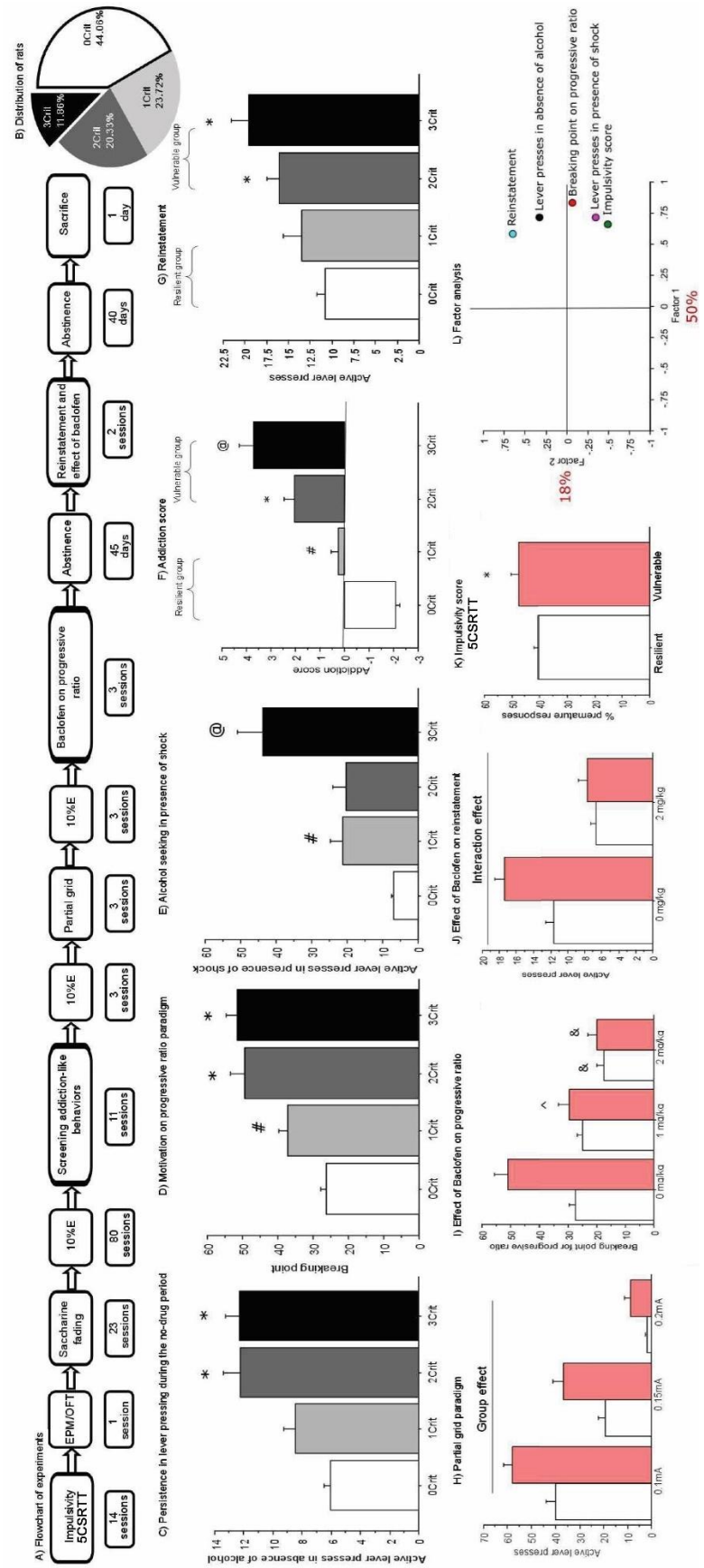

Figure 3.3: Identification of rats at risk of alcohol use disorder. *(A) Flowchart of the experimental procedure. (B) Distribution of ratsinto criteriom*

*group 0, 1,2, and 3. (C) Persistence in lever pressing during no-drug period. * Significant compared to 0Crit and 1Crit rats. (D) Motivation on progressive ratio. * Significant compared to 0Crit and 1Crit rats. # Significant compared to 0Crit rats. (E) Alcohol seeking in presence of shock. # Significant compared to 0Crit rats. @ Significant compared to 0Crit, 1Crit and 2Crit rats. (F) Addiction Score measure. *Significant compared to 0Crit and 1Crit rats. #Significant compared to 0Crit rats. @Significant compared to 0Crit, 1Crit and 2Crit rats. (G) Reinstatement of ethanol seeking following a period of abstinence. * Significant compared to 0Crit and 1Crit rats. (H) Partial grid paradigmby intensity of the shock. (I) Effect of Baclofen on progressive ratio (J) Effect of Baclofen on Reinstatement. ^ Significant compared to 0Crit rats. (K) Impulsivity on 5CSRTT. (L) Factor Analysis of all the five variables included loaded on one construct (Persistence in drug seeking during the no-drug period, Excessive motivation for alcohol seeking, Resistance to punishment, reinstatement, and impulsivity).*

3.4.2 Behavioural profiling of selected resilient and vulnerable rats

Nineteen Resilient (eighteen 0Crit and one 1Crit rats) and nineteen Vulnerable (twelve 2Crit and seven 3Crit rats) animals among the 59 rats initially screened were selected for microbiome analyses (see supplementary information). A brief presentation of their respective behaviours is summarized on Figure 3.3.2.1.

Vulnerable rats exhibited increased alcohol seeking behaviours, in the presence of shock (Mann–Whitney $U = 14$, $p<0.001$, Figure 3.3.2.1A) and absence of ethanol (Mann–Whitney $U = 15$, $p<0.001$, Figure 3.3.2.1B), and increased motivation assessed in a progressive ratio schedule of reinforcement (Mann–Whitney $U = 11$, $p<0.001$, Figure 3.3.2.1C). Addiction score and reinstatement of a lever pressing behaviour after a period of abstinence were significantly higher in Vulnerable rats compared to Resilient (Mann–Whitney $U = 0$, $p<0.001$, Figure 3.3.2.1D and T-Test $t(36)=-4.50$, $p<0.001$, Figure 3.3.2.1E, respectively). Vulnerable rats' response to baclofen treatment (1mg/kg) was enhanced, with a breaking point for ethanol seeking significantly reduced compared to resilient rats (Mann–Whitney $Z=-2.24$, $p<0.05$, Figure 3.3.2.1F).

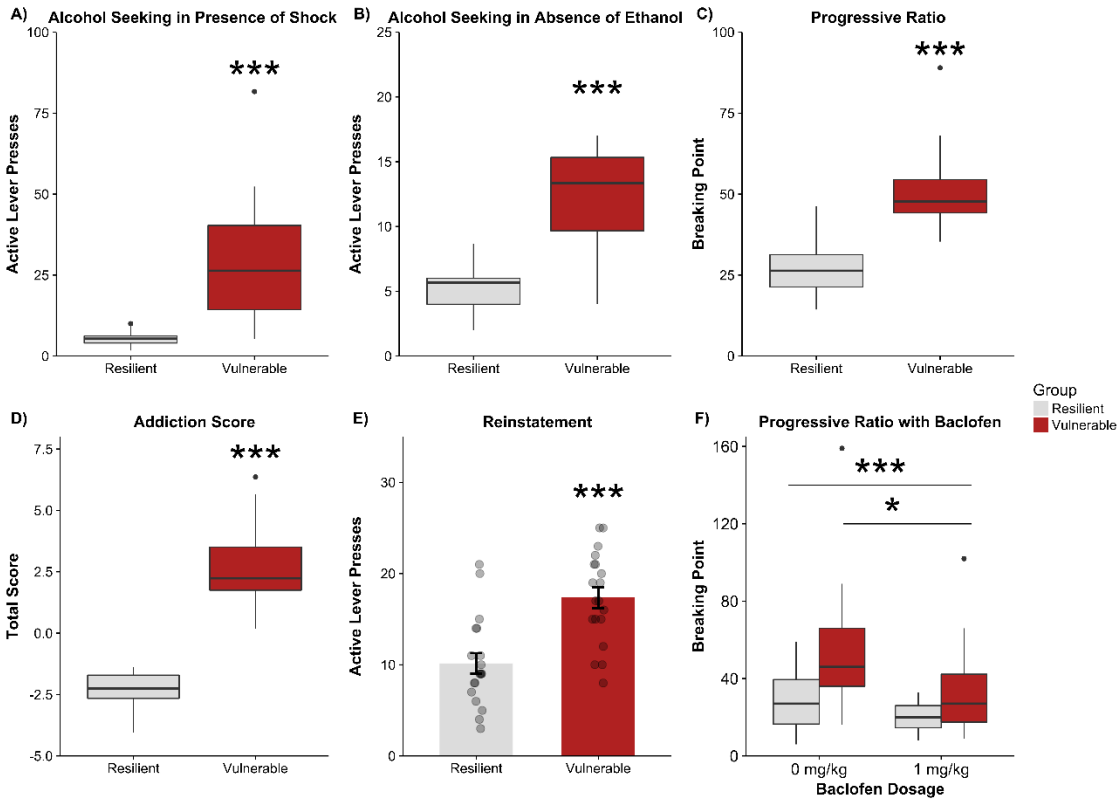

Figure 3.3.2.1: *Selection of most Resilient (grey, n=19, combining eighteen 0Crit and 1 1Crit rats) and most Vulnerable (red, n=19, combining twelve 2Crit and seven 3Crit rats) animals among the 59 rats screened, according to (A) Alcohol seeking in presence of shock (B) Persistence in lever pressing during no-drug period, (C) Motivation on progressive ratio schedule, (D) Addiction Score, (E) Reinstatement and (F) Response to baclofen assessed in a progressive ratio schedule. Resilient group (grey) is plotted on left, Vulnerable group (red) on right. Significance codes: *** p<0.001, ** p<0.01, * p<0.05.*

Vulnerable rats exhibited enhanced motor impulsivity, reflected by the percentage of premature responses in a 5-choice serial-reaction time task (5CSRTT) (T-test, $t(36)=-2.74$, $p<0.01$, Figure 3.3.2.2A). They did not display any anxiety-like behaviour on the elevated plus maze (EPM) (Mann–Whitney $U = 136$, $p>0.05$, Figure 3.3.2.2B), but increased novelty induced locomotor activity (Mann–Whitney $U = 99$, $p<0.05$, Figure 3.3.2.2C) compared to resilient animals.

Total ethanol consumed over the entire 80 self-administration sessions was analysed for subjects used in microbiome analysis. There was no significant difference between groups ($t(36)=-1.73$, $p>0.05$). Although the Vulnerable group had significantly higher body weight compared to Resilient ($t(36)=-2.38$, $p<0.05$), cecum weight was not significantly different between groups ($t(36)=0.40$, $p>0.05$).

Finally, given the importance of the dopaminergic system in the striatum and the pivotal role it plays in the reward circuitry, we investigated the expression of the D1receptor and D2 receptor in the dorsal striatum. D1 receptor expression was significantly higher ($t(13)=-2.88$, $p<0.05$) and D2 receptor expression significantly lower in the Vulnerable group ($t(13)=5.54$, $p<0.001$) compared to the Resilient group (Figure 3.3.2.2E-F).

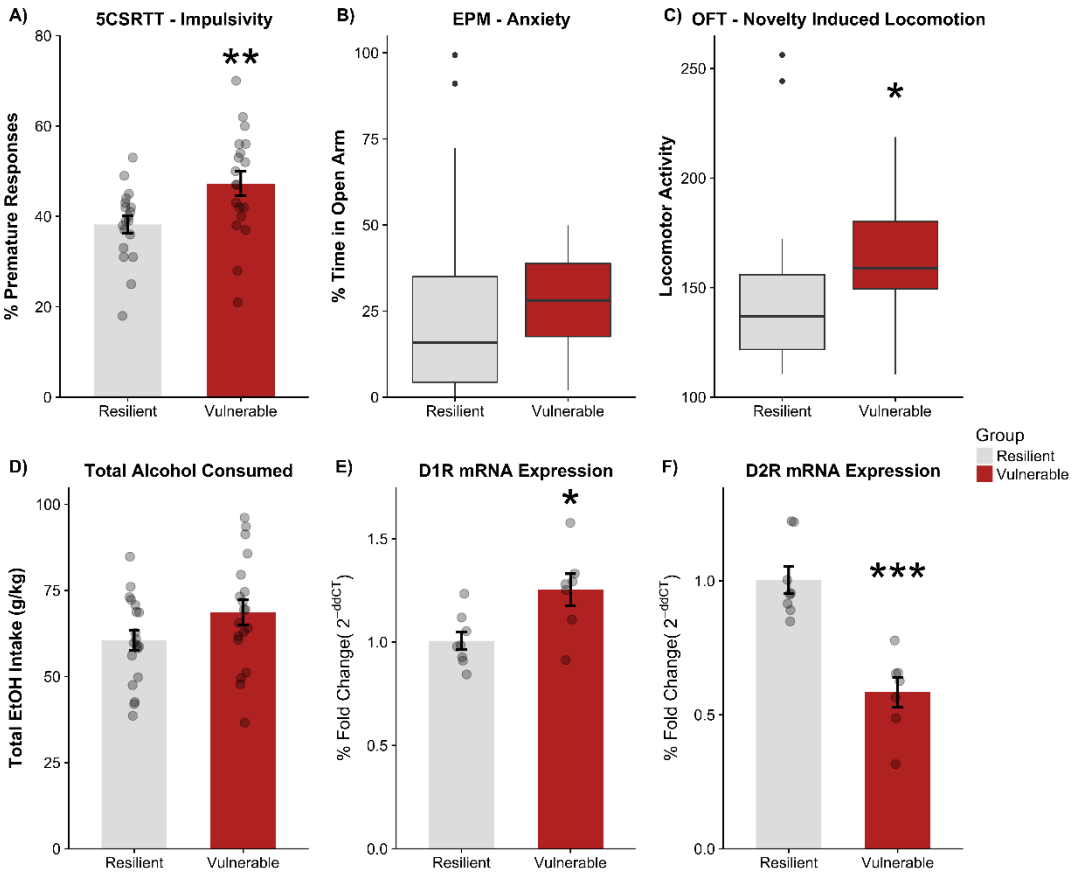

Figure 3.3.2.2: *Selection of most Resilient (grey, n=19, combining eighteen 0Crit and 1 1Crit rats) and most Vulnerable (red, n=19, combining twelve 2Crit and seven 3Crit rats) animals among the 59 rats screened, according to a posteriori analyses revealed that Vulnerable rats, compared to Resilient rats, exhibited (A) increased impulsivity (B) exploration in an elevated plus maze (C) increased novelty induced locomotion (D) non-significant increase in alcohol intake after prolonged conditioning (E) increased D1R mRNA (F) and decreased D2R mRNA expression in the dorsal striatum. In bar graphs all samples are plotted as grey dots. In box-and-whisker plots outliers are indicated with a black dot. Resilient group (grey) is plotted on left, Vulnerable group (red) on right. Significance codes: *** p<0.001, ** p<0.01, * p<0.05.*

3.4.3 16S microbiome analyses in caecal contents of resilient and vulnerable rats

3.4.3.1 Alpha and Beta Diversity

Alpha and beta diversity analysis revealed no significant difference between Vulnerable and Resilient group, but a trend towards increased richness and evenness in the Vulnerable group (Figure 3.3.3.1A-B).

3.4.3.2 Taxa Level Relative Abundance

Compositional comparisons at the phylum, family, and genus level showed no significant difference between Vulnerable and Resilient group after FDR correction ($p>0.05$). At phylum level, a trend towards increased Firmicutes and decreased Actinobacteria in Vulnerable group ($p>0.05$) were seen. Comparisons at the family level revealed trends of increased *Ruminococcaceae* and decreased *Bacillales Family.XI* and *Deferribacteraceae* in Vunerable group. Additionally, changes in many genera of *Lachnospiraceae* and *Ruminococcaceae* were observed, however did not pass FDR significance testing ($p>0.05$) (Figure 3.3.3.2).

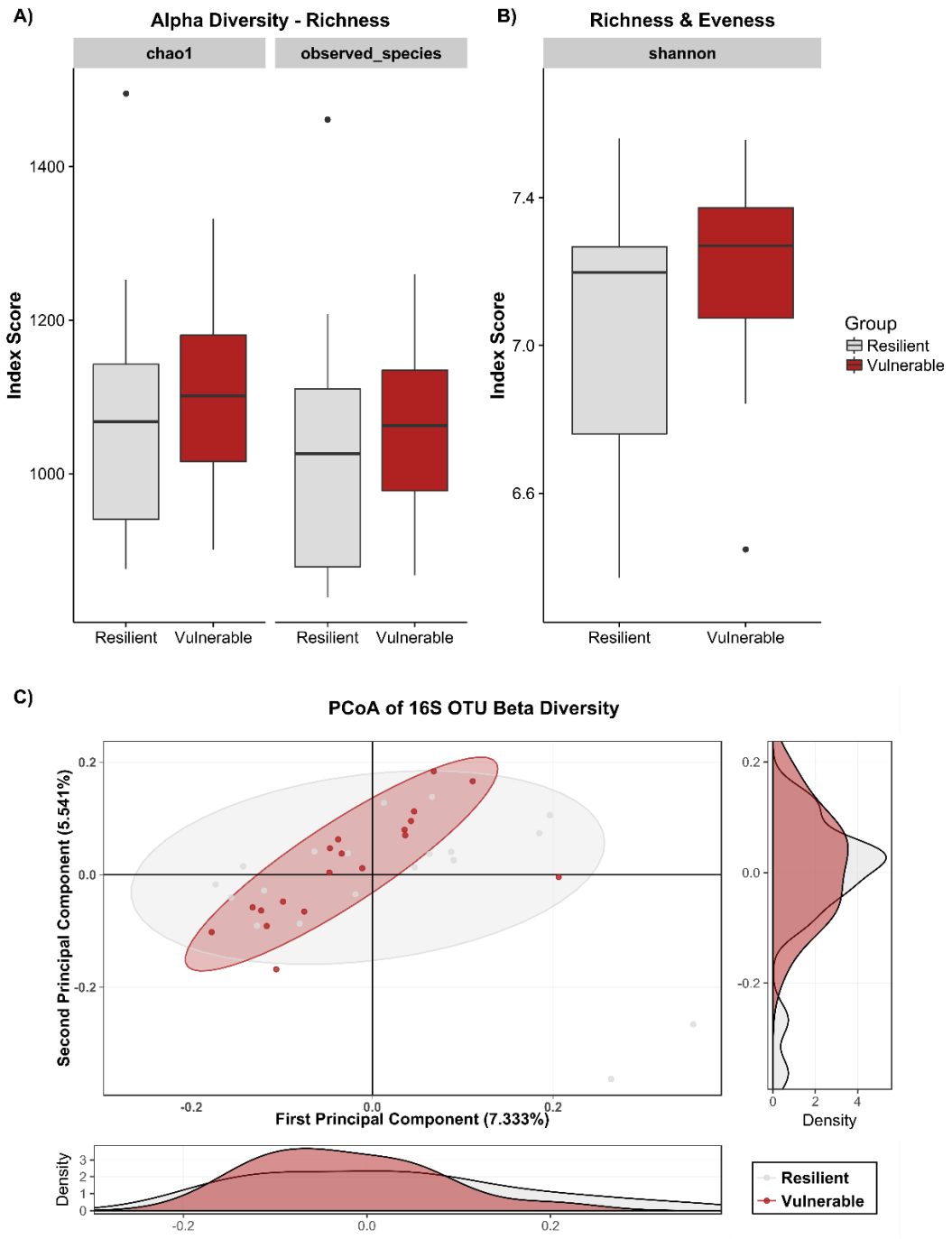

Figure 3.3.3.1: Microbiome Diversity. *A) Alpha diversity index measures of bacterial richness (chao1 index and observed_species index). B) Alpha diversity index measure of bacterial richness and evenness (shannon index). In box-and-whisker plots outliers are indicated with a black dot. C) PCoA ordination plot of orthogonal taxonomic unit (OTU) beta diversity. Percent explained variance reported on first (x-axis) and second (y-axis) principal component axis. Density of cluster indicated for the y-axis (right) and x-axis (bottom) by group.*

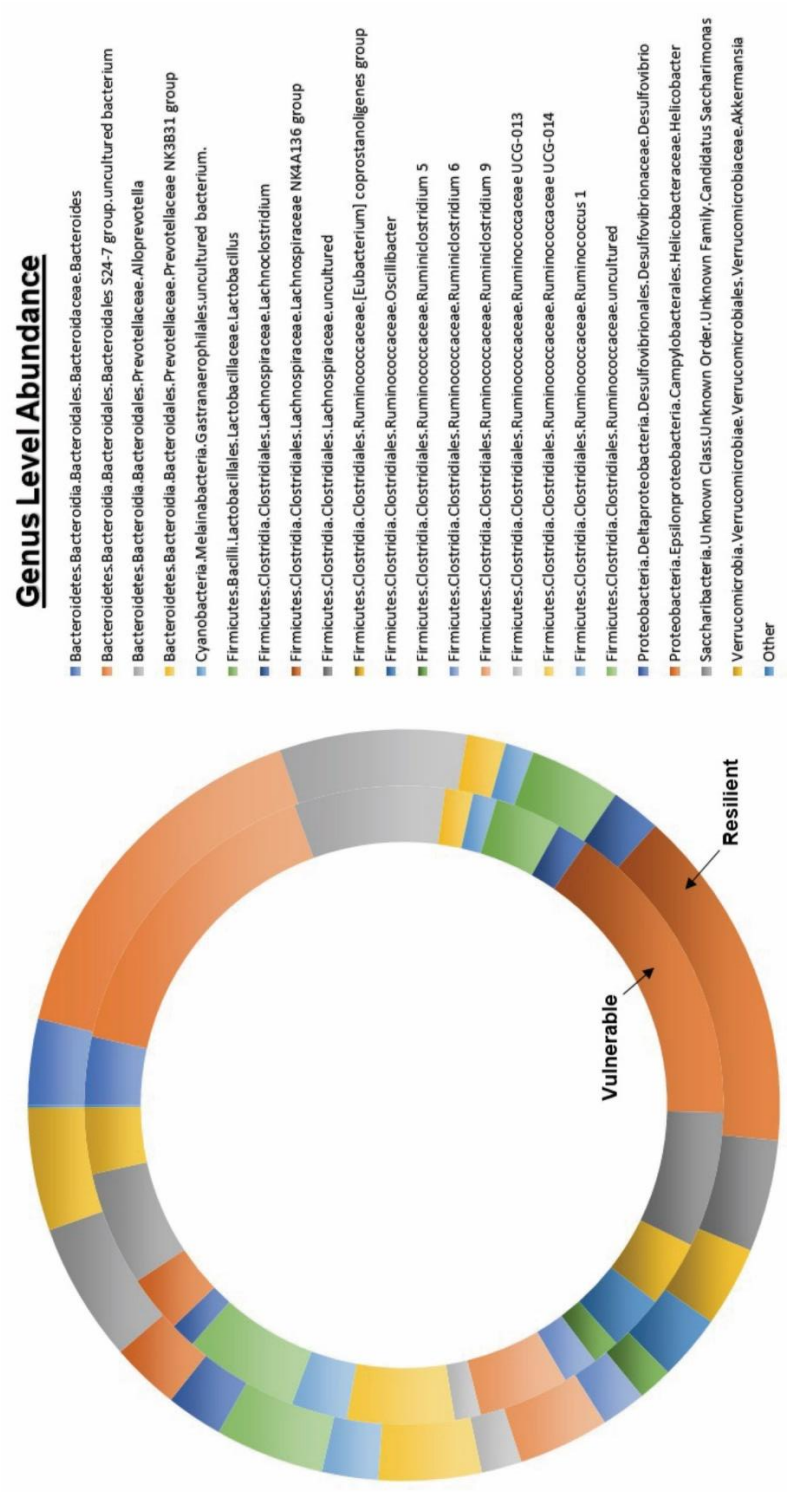

Figure 3.3.3.2: Genus Level Relative Abundance. *The mean of the 22 most abundant bacteria are plotted by group, with the inner ring representing Vulnerable and outer ring representing Resilient group. Legend displays bacterial name with taxonomic designation at each level down to genus; bacteria are listed by order of appearance starting with Bacteroides (blue) at top.*

3.4.3.3 Microbiome, Behaviour, and mRNA expression Correlation Analysis

The strongest correlations were seen between D2R mRNA expression and low abundance bacteria belonging to phylum Firmicutes (|rho|>0.55, p<0.05), comprised mainly of positive correlations. The largest decrease in Vulnerable relative to Resilient group was observed in genus *Veillonella* (log2 ratio=-4.6), which was negatively correlated to D1R mRNA expression (rho<-0.58, p<0.05). Other significant correlations to D1R mRNA expression include genera *Gemella* (rho<-0.61, p<0.05), and two from family *Ruminococcaceae* (rho<-0.57, p<0.05) (Figure 6).

AUD behaviour showed a significate positive correlation to bacteria in order Clostridiales (rho>0.35, p<0.05), including many genera from family *Ruminococcaceae* and *Lachnospiraceae*. Significant, negative correlations were seen between AUD behaviour and genera *Desulfovibrio* (rho<-0.45, p<0.01), *Gemella* (rho<-0.43, p<0.01), uncultured *Coriobacteriaceae* (rho<-0.40, p<0.05), and *Hydrogenoanaerobacterium* (rho<-0.36, p<0.05). The 5CSRTT measure of impulsivity showed significant positive correlations to two genera of *Lachnospiraceae* (rho>0.34, p<0.05), *Lachnospiraceae* uncultured and *Lachnospiraceae* UCG-005, and significant negative correlation to bacteria in family *Ruminococcoceae* (rho<-0.35, p<0.05), an uncultured bacterium in the *Eubacterium coprostanoligenes group*. Anxiety measures (percentage time on open arms) from the EPM test were significantly positively correlated to the genus level bacteria *Lachnospiraceae* UCG-006 (rho>0.41, p<0.01) and *Papillibacter* (rho>0.34, p<0.05), in family *Ruminococcoceae*. On the other hand, EPM anxiety measure was negatively correlated to genus *Anaerofilum* (rho<-0.42, p<0.01), in family *Ruminococcoceae*. OFT measure of novelty induced locomotion negatively correlated to genera *Lachnospiraceae* UCG-007 (rho<-0.44, p<0.01) and an uncultured bacterium in the *Ruminococcoceae Eubacterium coprostanoligenes group* (rho<-0.40, p<0.05). Total ethanol (EtOH) consumption (TotEtOH) significantly correlated with *Gemella* (rho<-0.37, p<0.05) (Figure 3.3.3.3).

Most correlations are seen in low abundance bacteria (mean abundance < 0.001%) however most of these low abundance genus level bacteria are present in the majority of samples (Supplementary Table 3). At the family level, *Bacillales Family XI* is most frequently correlated to behaviour measures. Both at the genus and family level this bacterium is in very low abundance and only present in 9 of the 38 samples. Family level correlations, relative abundance and presence in samples of bacteria correlating to addiction measures is listed in Supplementary Tables 3-5.

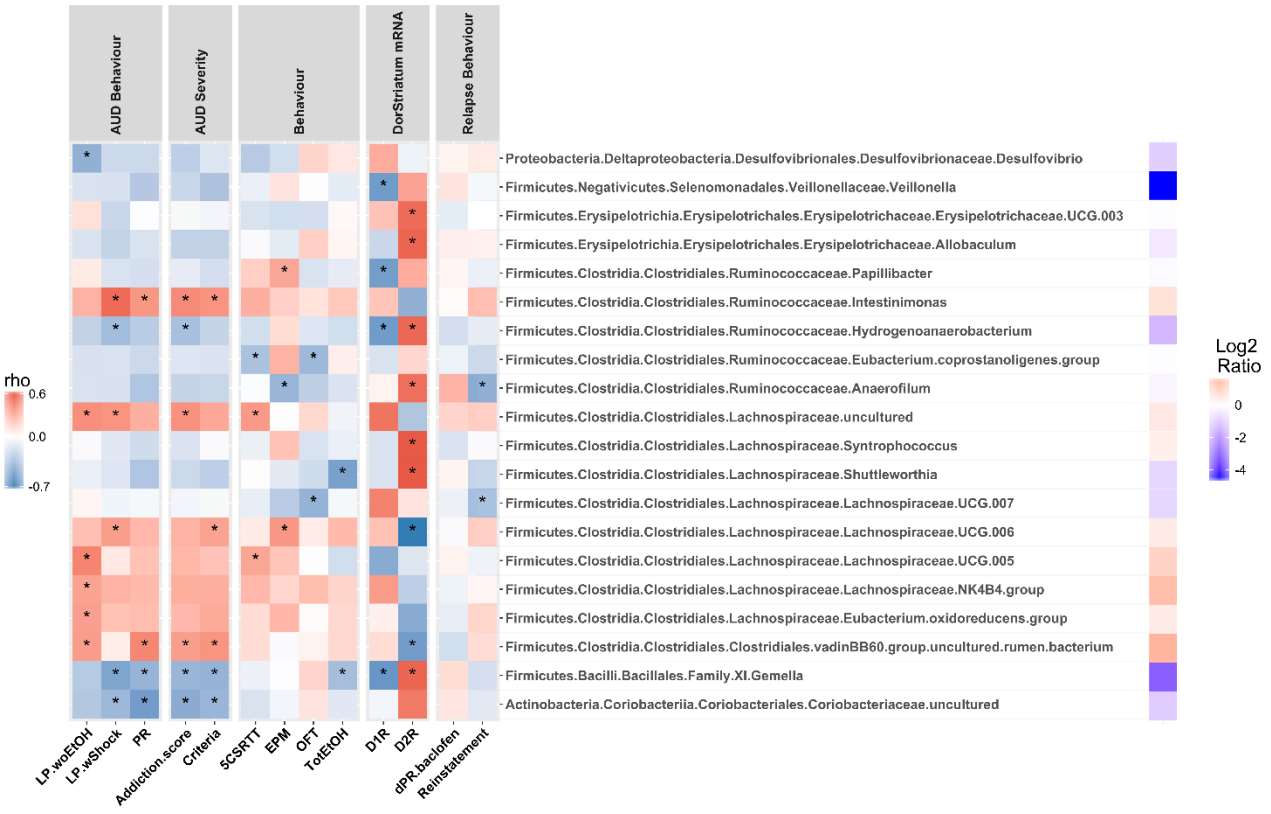

Figure 3.3.3.3: *Left panel - Correlations (positive in red, negative in blue) between genus level bacteria (y-axis) and addiction measures (x-axis); Right panel – Log2 fold change ratio showing changes in Vulnerable group relative to Resilient (increases in red, decreases in blue) for genera corresponding to correlation heatmap (left panel). Abbreviations (left to right x-axis): active lever presses without ethanol (LP.woEtOH), active lever presses with shock (LP.wShock), progressive ratio breaking point (PR), addiction score (Addiction.score), criteria designation 0Crit-3Crit (Criteria), percentage of premature responses in 5 choice serial reaction time task (5CSRTT), percent time in open arm in elevate plus maze (EPM), locomotor activity in open field test (OFT), total ethanol consumed over 80 sessions (TotEtOH), dopamine 1 receptor mRNA expression (D1R), dopamine 2 receptor mRNA expression (D2R), change in progressive ratio breaking point between 0mg/kg dosage and 1mg/kg dosage baclofen (dPR.baclofen), active lever presses during reinstatement (Reinstatement). * indicate correlations that pass FDR at Q=0.25 ($p<0.05$).*

3.5 Discussion

A link between alterations in microbiota and alcohol-related behavioural changes has remained relatively unexplored. Here we show for what is to our knowledge the first time microbiota composition is associated to addiction measures in a realistic animal model of AUD. Moreover, low abundance bacteria coincided with changes in central gene expression.

Converging evidence suggests that for some alcoholics (probably 30–50% of the total), ethanol consumption alters the gut microbiome by depleting protective bacteria, increasing intestinal permeability and releasing inflammation factors like bacterial peptidoglycans and lipopolysaccharide, which ultimately amplifies the psychopathology of alcoholism (Leclercq, Cani et al. 2012, Leclercq, De Saeger et al. 2014, Leclercq, Matamoros et al. 2014, de Timary, Leclercq et al. 2015, Gorky and Schwaber 2016, de Timary, Starkel et al. 2017). However, authors suggested that alterations in microbiota composition could be responsible for the ‘leaky gut’ upon alcohol consumption, as no increase in permeability was observed in alcoholic patients which were resilient to microbiota changes, despite their alcohol consumption (de Timary, Leclercq et al. 2015). With alterations in microbial composition reported in only a subset of alcoholic patients and not correlated to the duration of sobriety, alcohol-related microbial imbalance is considered a long-lasting consequence that persists despite abstinent periods (Mutlu, Gillevet et al. 2012). Also, personality traits associated with the vulnerability to develop AUD have been consistently linked to gut microbial and peripheral metabolite level alterations. In particular, alcoholic patients without overt microbiota disturbances showed less severe levels of depression, anxiety and craving which almost disappeared after nearly 3 weeks of withdrawal, whereas these clinical signs persisted in abstinent patients with concurrent microbial changes (Leclercq, Cani et al. 2012, Leclercq, Matamoros et al. 2014, de Timary, Leclercq et al. 2015).

This intriguing observation poses the question of whether the gut microbiome

composition could represent a biological marker of the vulnerability to develop AUD. Intriguingly, we report here, for the first time to the best of our knowledge, that many of the biobehavioural traits associated with a loss of control-prone phenotype, without heavy ethanol intoxication, correlate with microbiome composition.

It is important to note that rats in this study underwent a 2-month period of abstinence before microbiome analyses in caecal contents and measures of mRNA in striatal areas of the brain. Samples were received after a prolonged wash-out period because the aim of this study was to assess inherent differences in microbiome composition between groups of low- and high-risk addiction, this wash-out period reduces the confound of ethanol or baclofen affecting microbiome composition. Therefore, measures reported here do not correlate with acute ethanol intoxication, but rather reflect long-lasting behavioural traits, i.e., the loss of control-prone phenotype observed in vulnerable rats versus the temperate behavioural profile reported in resilient animals. Our observations are in line with those reported above regarding a role for microbiota composition in negative reinforcement processes driving alcohol consumption (de Timary, Leclercq et al. 2015, de Timary, Starkel et al. 2017). However, our study presents two limitations that need to be addressed in the near future, 1) faecal analyses before alcohol training would inform on pre-existing compositional differences in microbiome in rats developing uncontrolled alcohol seeking behaviour over time, and 2) measures of peripheral markers would inform on systemic inflammation occurring in vulnerable rats compared to resilient ones. Nevertheless, this is the first study reporting that microbiome composition is associated to addictive behavioural traits as opposed to acute effects of drug exposure.

Here we took advantage of normal variation in behavioural traits relevant to addiction to stratify an outbred cohort into either Vulnerable or Resilient. Not surprisingly, rats with impulsive traits were at higher risk of developing AUD, and this pre-existing impulsive trait shifted towards a compulsive-like behaviour after extensive instrumental conditioning, associated with increased relapse rates after a period of protracted abstinence. Of particular

relevance, these animals still exhibited higher mRNA expression of D1 receptors and lower mRNA expression of D2 receptor within the dorsal striatum after a prolonged period of abstinence. The striatum is mainly composed of medium spiny neurons (MSN), typically divided into those expressing dopamine receptor D1, forming the so-called direct pathway, and those expressing D2 receptor (indirect pathway). Whereas D1-MSNs mediate reinforcement and reward, D2-MSNs have been associated with aversion and avoidance. A current consensus suggests that D1-MSNs may facilitate the selection of rewarding actions encoded in the cortex, while D2-MSNs may help to suppress cortical patterns that encode maladaptive or nonrewarding actions. Therefore, positive reinforcement learning would be modulated by signalling within the D1 direct pathway while negative reinforcement learning would be modulated by signalling within the D2 indirect pathway (Volkow, Wang et al. 2013, Cox, Frank et al. 2015, Soares-Cunha, Coimbra et al. 2016). As a consequence, it is postulated that dopamine-related impulsive phenotype partly relies on impaired negative feedback learning (Dagher and Robbins 2009). Functionally, in humans, the A1 (T) allele of the dopamine D2 receptor/ankyrin repeat and kinase domain containing 1 (DRD2/ANKK1) TaqIA (rs1800497) single nucleotide polymorphism has been associated with reduced striatal D2 receptor availability (Eisenstein, Bogdan et al. 2016), and a recent large-scale meta-analysis confirmed the association between the ANKK1/DRD2 Taq1A polymorphism and alcoholism (Wang, Simen et al. 2013). Therefore, the lower expression of striatal D2 receptors, concomitant with higher expression of D1 receptors, in Vulnerable rats long after their last alcohol consumption confirms the construct validity of our model, and questions on the significance of those persistent brain adaptations occurring concomitantly with gut microbiota composition.

The most profound correlations were seen in D2R mRNA expression corresponding to the inhibitory, indirect pathway. Significant correlations revealed changes in low abundance genera *Lachnospiraceae* UCG-006, *Syntrophococcus*, *Shuttleworthia*, *Gemella*, *Allobaculum*, uncultured rumen bacterium from *Clostridiale vadinBB60 group*, and

Hydrogenoanaerobacterium associated to reductions in D2R. This novel finding indicates that gut microbiota composition may contribute to inhibitory innervations in brain circuits associated to addiction. The capability of gut microbiota to influence inhibitory circuits is not surprising given the fact that administration of *Lactobacillus rhamnosus* (JB-1) reduces anxious behaviour by altering cortical GABAergic innervations (Bravo, Forsythe et al. 2011).

Many genus level bacteria in order Clostridiales, family *Ruminococcaceae* and *Lachnospiraceae*, were positively associated to AUD severity, and also correlated to decreased D2R mRNA expression. Correlations between genus level bacteria and addiction measures would indicate that although these genera are not significantly different by group, subtle variations in abundance may potentially coincide with differences in addictive behaviour. While such a correlation opens a debate and requires further investigation on the mechanism linking gut microbiota to cortical D2R mRNA expression, recent evidence offers a partial explanation with the demonstration that gut microbiota regulate microRNA expression in the amygdala and prefrontal cortex. In particular, antibiotic treatment was shown to decrease miR-206-3p, a microRNA implicated in the regulation of brain-derived neurotrophic factor, essential in synaptic plasticity (Hoban, Stilling et al. 2017). Rare are the studies showing that altered microbiome impacts reward seeking behaviours, but a recent study reports that microbiome-depleted animals (following antibiotic treatment) exhibited an enhanced sensitivity to cocaine reward (Kiraly, Walker et al. 2016). Therefore, a link most likely exists between the microbiota, the brain and the vulnerability to drug abuse. Interestingly, this finding indicates that supplementation of these low abundance bacteria may have potential for treatment in AUD, but future studies are required to investigate if probiotic/prebiotic intervention targeting the gut-brain axis (aka. psychobiotics) is capable of reducing alcohol-seeking behaviors (Dinan, Stanton et al. 2013, Hoban, Stilling et al. 2017).

The lack of significant differences in microbiome composition may be due to the 2-month abstinence period, however this wash-out period was chosen to ensure observed differences were not due to drug administration. Non-

significant trends in altered microbiome composition between Vulnerable and Resilient group were seen in bacteria from family *Ruminococcaceae* and *Lachnospiraceae*. These findings are in line with alcohol studies showing reductions in bacteria from family *Ruminococcaceae*, increases in bacteria from family *Lachnospiraceae*, and increased alpha diversity associated to alcohol severity and altered intestinal permeability (Leclercq, Matamoros et al. 2014, Llopis, Cassard et al. 2016). Interestingly, it has been shown in patients with hepatic encephalopathy that the levels of *Ruminococcaceae* correlate negatively to inflammation (Bajaj, Hylemon et al. 2012). Behavioral traits, such as impulsivity, predispose individuals to addiction and other neuropsychiatric conditions, such as Attention Deficit Hyperactivity Disorder (ADHD) and autism. Previous work showed that reductions in genera *Instestimonas* (family *Ruminococcaceae*) and *Desulfovibrio* (family *Desulfovibrionaceae*) were associated with gastrointestinal dysfunction, altered metabolism, as well as anti-social, anxious, and compulsive behaviours in a mouse model of autism (Golubeva, Joyce et al. 2017).
With current pharmacotherapies largely unsatisfactory, discovering novel alternatives to prevent AUD becomes a priority. Hence, identifying biological markers predicting vulnerability to develop excessive alcohol consumption may lead to a real improvement of clinical care. In this study, we report that gut microbiome composition is associated with specific “at risk” behavioural traits in a translationally relevant model of alcohol use disorder. These preclinical observations open a debate on the possible role of gut microbiome in predisposing individuals to AUD and offers a perspective on understanding alcohol addiction the etiology of which remains partially unknown. While addressing addiction-related associations to gut microbiome composition is probably not a panacea, it offers itself as an important underappreciated additional component in favour of better identifying those at risk of losing control over their alcohol intake.

3.6 Supplementary information

3.6.1 Material and methods

3.6.2 Apparatus:

3.6.2.1 Self-administration (SA) chambers

Twelve operant chambers (305 x 241 x 210 mm, Med Associates, St. Albans, Vermont, USA) were used for the experiment. The chambers were housed in larger sound attenuated cubicle, equipped with exhaust fans for air renewal, also used for masking the background noise. The floor was made of a grid capable of delivering electrical shock. Each operant panel contained two retractable levers 60 mm above the grid and 35 mm equidistant from the midline, with a white light diode mounted 30 mm above each lever. Between the two levers was the delivery section which delivered 0.1mL of the fluid by means of a dipper.

3.6.2.2 Chambers for testing impulsivity

Six operant chambers (305 x 241 x 292 mm) were used for the 5-CSRTT experiment (Med Associates Inc., St-Albans, Vermont, USA). Each chamber was enclosed in wooden cubicles equipped with an exhaust fan for ventilation. Each cage contained a stainless-steel grid floor spaced by 18mm, allowing waste collection in a removable tray containing sawdust. Front and back wall of the cage were in Plexiglas, while left and right wall were made of steel. Five nosepoke cavities (25x25 mm) were located on the left side of the cage, each spaced by 25mm. A food tray located 20 mm above the grid was available on the right side. Nosepoke cavities and food tray were equipped with a light and an infrared beam to monitor activity. Each cage was also equipped with a house light fixed on the ceiling, and a tone device. All the operant cages were linked to a common interface, and to a computer that controlled experimental procedures through Med associates software (Med-PC IV).

3.6.3 Impulsivity- 5 Choice serial reaction time task

Before the rats were exposed to alcohol, their impulsivity profile was ascertained by testing them on the 5 Choice serial reaction time task (5CSRTT). Rats were food restricted and maintained at 90% of their initial weight. For the whole experiment, sucrose food pellets (Dustless precision pellet 45 mg, rodent purified diet, BioServ, Frenchtown, NJ, USA) were given as a “reward” for correct responses, i.e., when the rat correctly poked its nose into the hole that had the illuminated light. Sucrose-laced food pellets were selected as sucrose provides higher motivational value (incentive salience) than normal, low sucrose, food.

3.6.3.1 5CSRTT Training Procedure

Rats were first trained to nose poke in the food tray to get a “reward” pellet. The training session stopped when rats collected 50 pellets of after 30 min. Then, on each trial, rats were trained to nose poke first in the food tray to start a trial and later nose poke in one of the five holes, randomly illuminated in order to get a “reward” pellet (recorded as a correct response). Nose poking in a different hole was recorded but had no consequence. The trial session stopped when rats collected 40 pellets of after 30 min. The next training phase was similar to the previous ones, but a 5-sec inter-trial interval (ITI) delay preceded the random illumination. Nose poking in a non-illuminated hole was recorded as an incorrect response, triggering a 5-sec time out period signalled by the illumination of the house light. Then, a 5 second tone was introduced to indicate the 5-sec delay before the random illumination. At the end of the tone, one of the 5 holes was briefly illuminated for 2 seconds. Any response during the tone, i.e., before the holes being illuminated, was recorded as a premature response but had no consequence. The final training session stopped when rats collected 50 pellets of after 30 min. These training sessions follow the standard time interval of 30 minutes for measuring attention (Robbins 2002), however if an

animal consumed 40-50 pellets before the 30 minutes the session was stopped to prevent behavioural confounds due to overconsumption of sucrose laced food pellets, which can cause obesity and alterations in metabolic function.

3.6.3.2 5CSRTT Test Procedure

In the final test, rats were tested in the 5CSRTT paradigm during which each premature response was punished by a time out of 5 seconds and the illumination of the house light. The final test was 30 minutes or 100 trials (potential to receive 100 “reward” pellets), whichever came first. Absence of response during the 5 sec post-illumination was recorded as an omission. The correct, incorrect, premature and omission responses were recorded.

Premature responses were calculated as a percentage: [premature responses / (correct + incorrect + omission + premature responses)]*100%

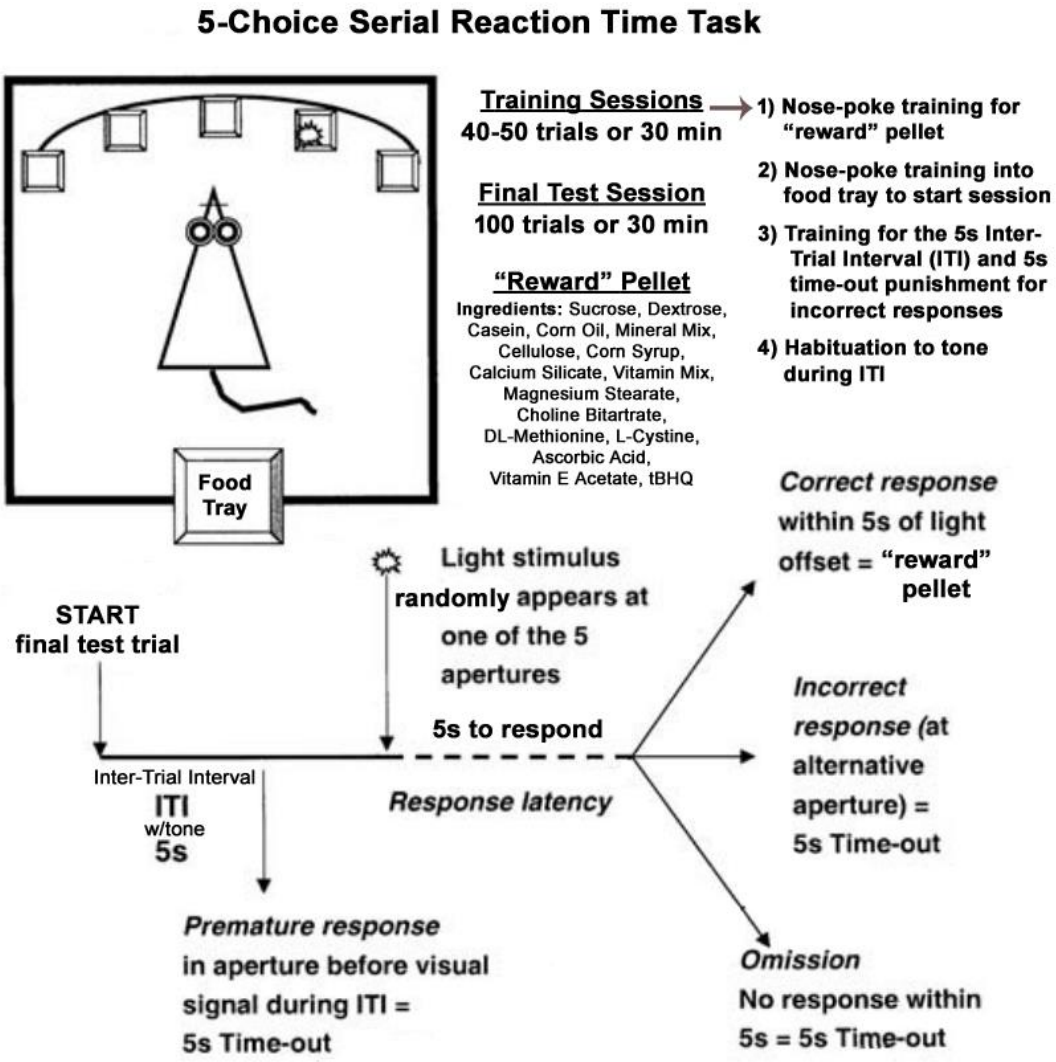

Figure 1: Process of the 5-Choice Serial Reaction Time Task (5CSRTT) for training and final testing. Figure adapted from previous publication (Robbins 2002).

3.6.4 Anxiety-related behaviors

Rats were tested in an Open Field Test (OFT) and in an Elevated Plus Maze (EPM) paradigm for evaluating anxiety-like behaviours after the 5CSRTT procedure. Both experiments were conducted under a dim light (10-15 Lx) during the active phase. Animal tracks were recorded by a digital video camera mounted above the maze and connected to a computer running a tracking-software (ANY-maze Video Tracking System v.4.99 – Stoelting Co., Wood Dale, IL, USA).

3.6.4.1 Open Field Test (OFT)

Rats were placed in a round arena (140 cm of diameter, 30 cm of depth) and their motor and exploratory activities (i. e., rearing) were monitored for 60 min.

3.6.4.2 Elevated Plus Maze (EPM)

The elevated plus maze consisted of two opposite open arms (50cm L x 10cm W x 42.5cm H) and two opposite closed arms (50cm L x 10cm W) arranged in a cross and elevated 50 cm above the floor. In the center, a small platform (10cm x 10cm) gave access to all arms. Rats were gently placed in the center of the maze face to a close arm and their behavior was monitored for 5 min. The time spent on open arms was used as an index of anxiety.

3.6.5 Animal's training for alcohol self-administration

Laboratory rodents do not voluntarily consume alcohol to intoxication, in part because of taste aversion. Higher levels of consumption could be achieved by masking the taste of alcohol with saccharine (Roberts, Heyser, & Koob, 1999), which was faded out as alcohol concentrations increased (Dayas, Liu, Simms, & Weiss, 2007). Rats were trained under a Fixed Ratio 1 - Time Out 4sec schedule of reinforcement for a total of 105 sessions, 30-min per weekday (25 sessions of saccharine fading + 80 sessions of ethanol self-administration). During these baseline conditions, pressing the right (active) lever delivered 0.1 mL of ethanol (10%w/v in tap water, prepared from a 94% (vol/vol) ethanol solution) in the liquid delivery section and illuminated the diode above the active lever. The left lever was inactive, presses were recorded but had no consequence.

3.6.6 Screening for addiction-like behaviour

Between test sessions aiming at scoring addiction-like behaviours (3 daily consecutive sessions each time), rats underwent 2 consecutive sessions of

basic training during which they were trained again under the same baseline conditions (Jadhav et al., 2017).

3.6.6.1 Inability to abstain during a signalled period of reward unavailability

Rats underwent 3 daily consecutive sessions; each one consisted of an 8 minute-period of reward availability, followed by a 4 minute-period of signalled unavailability. Repeated three times, this sequence resulted in a total of 36 min. The period of unavailability was signalled by lighting up the self-administration chamber house light and interrupting the cubicle fan. The light diode above the active lever remained off after lever presses, and alcohol was not delivered. The average number of active lever presses during the signalled unavailability periods indicated the persistence in drug seeking during the no drug period.

3.6.6.2 High motivation for alcohol seeking

Rats were required to progressively increase the number of active lever presses between two successive rewards based on the progression sequence given by the following formula: response ratio = (5e(reward× 0.2)) − 5. Hence, the progressive-ratio schedule followed the progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, etc. Each session lasted 90 min, or automatically shut down following 20 consecutive minutes of inactivity on the active lever. The maximal number of active lever presses performed to reach the final ratio was defined as the breakpoint, a value reflecting animals' motivation to get the reward. The breaking point across 3 consecutive daily sessions was averaged and considered as marker of motivation for alcohol seeking.

3.6.6.3 Resistance to punishment

In this paradigm, each lever press delivered 0.1 mL of 10% w/v ethanol by means of a dipper, followed by mild electric foot shocks (0.22 mA for 0.5 second) through the grid of the SA chamber when the dipper retracted. This was conducted for 3 consecutive daily sessions, and the average

number of active lever presses across these 3 consecutive trials was considered as a marker of resistance to punishment, reflecting a compulsive reward seeking and taking behavior.

3.6.7 Partial grid paradigm

In this paradigm, the rats must press the active lever once to gain access to 0.1mL of 10% ethanol. The grid was divided into thirds, 2/3rd of the grid closest to the lever and delivery section delivered shock continuously, 1/3rd of the grid farthest from the lever was not electrified. Test rats had to bear shock before and during lever presses to administer ethanol. The rats were exposed to this paradigm for 30 mins for 3 sessions of increasing shock strength of 0.1mA, 0.15mA and 0.2mA. The number of active lever presses was recorded.

3.6.8 Reinstatement paradigm

At the end of all behavioral paradigm tests, rats were subjected to approximately 45 days of forced abstinence after which they were re-exposed to the self-administration chambers. The rats were given access to the same conditions as the training sessions, where one active lever presses esulted in lighting up of the cue light above the lever, however ethanol was not delivered. The number of active lever presses during this period of 30 minutes measured propensity to relapse.

3.6.9 Results

3.6.9.1 Persistence in lever pressing during the no-drug period

The mean number of lever presses for each group were 6.03 ± 0.43 (0Crit), 8.43 ± 0.79 (1Crit), 12.28 ± 1.16 (2Crit) and 12.33 ± 0.93 (3Crit), respectively. A one-way ANOVA showed a significant difference between groups ($F_{3,55} = 17.436$, $p < 0.0001$). Post hoc Bonferroni's test revealed that, during the no-drug period, the 3Crit rats exhibited higher lever presses compared to 0Crit ($p < 0.0001$) and 1Crit ($p<0.01$) groups, but not 2Crit ($p>0.05$). The 2 criteria rats differed from 0 criteria rats ($p < 0.001$) and 1 criteria rats ($p <0.01$). The 0 and 1 criterion rats had similar performances ($p>0.05$).

3.6.9.2 Increased motivation for alcohol seeking and drinking in an effortful condition

The average breaking points for the four groups were 26.34 ± 1.37 (0Crit), 37.21 ± 2.43 (1Crit), 49.33 ± 4.06 (2Crit) and 51.57 ± 3.27 (3Crit). One-way ANOVA showed a statistically significant difference among the groups ($F_{3,55} = 23.23$, $p < 0.0001$). Post hoc Bonferroni's tests revealed that the 3Crit rats displayed an increased motivation for ethanol seeking compared to 0Crit ($p < 0.0001$) and 1crit ($p>0.01$) groups, but not compared to 2Crit ($p>0.05$). The 2Crit rats displayed a higher breaking point compared to 0Crit ($p < 0.001$) and 1Crit ($p<0.01$) groups. Finally, even the 1Crit rats exhibited a higher breaking point compared to the 0Crit rats ($p < 0.001$).

3.6.9.3 Resistance to punishment

The average lever presses for each group when each lever press was associated with a mild shock were 6.84 ± 0.6 (0Crit), 21.23 ± 3.6 (1Crit), 20.36 ± 3.58 (2Crit) and 43.62 ± 7.28 (3Crit) (Fig. 2D). One-way ANOVA showed a statistically significant difference among the groups ($F_{3,55} = 22.55$, $p < 0.0001$). Post hoc Bonferroni's tests that 3Crit rats accepted more shocks than 2Crit ($p<0.0001$), 1Crit ($p < 0.0001$), and 0Crit rats ($p<0.01$). Whereas 2Crit rats were not different from 1Crit rats ($p>0.05$), they exhibited resistance to punishment as compared to the 0Crit rats ($p<0.01$). The 2Crit rats had higher lever presses as compared to 0Crit rats ($p<0.001$), but not 1Crit rats ($p>0.05$). Finally, even the 1Crit rats had higher lever presses than 0Crit rats ($p<0.001$).

3.6.9.4 Addiction score and vulnerability to relapse after protracted abstinence

The scores for the four groups were -2.06 ± 0.16 (0Crit), 0.23 ± 0.28 (1Crit), 2.04 ± 0.4 (2Crit) and 3.68 ± 0.6 (3Crit). A one-way ANOVA revealed a significant difference between groups ($F_{3,55} = 67.20$, $p<0.0001$). A post hoc Bonferroni's test showed that each group was significantly different every

other group. Pearson's correlation analysis showed that the three criteria scores were highly correlated with the addiction score (persistence in drug seeking in absence of alcohol [r=0.77, p<0.0001], excessive motivation for alcohol seeking and drinking [r= 0.836, p<0.0001] and resistance to punishment [r=0.536, p<0.0001]).

Rats were subjected to a 45-day period of forced abstinence, followed by which the rats were exposed to the reinstatement paradigm. A one-way ANOVA revealed a significant difference between criterion groups ($F_{3,53}=6.13$, $p<0.01$). A post hoc Bonferonni's test showed that the 3Crit and 2Crit rats had higher active lever presses compared to 0Crit ($p<0.001$) and 1Crit ($p<0.01$). A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,56} = 16.62$, $p<0.0001$) and a significant effect in intensity of shock ($F_{2,116} = 105.89$, $p<0.001$). Once the four groups were identified, we analysed their lever pressing behavior during the training sessions.

	Session 6-10	**Session 21-25**	**Session 41-45**	**Session76-80**
0Crit	39.20 ± 3.59	30.26 ± 2.10	30.56 ± 1.88	43.67 ± 2.74
1Crit	41.40 ± 4.48	37.77 ± 2.58	44.82 ± 4.64	46.31 ± 3.08
2Crit	35.25 ± 2.95	37.86 ± 3.59	37.41 ± 3.62	49.32 ± 4.78
3Crit	41.05 ± 3.44	41.17 ± 3.16	50.40 ± 3.69	64.46 ± 2.94

Table1: Evolution of lever pressing behavior *over the course of the operant conditioning*

A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{3,55} = 4.93$, $p=0.0042$), a significant effect of number of training sessions ($F_{3,177} = 14.03$, $p<0.001$) and a significant effect of group X number of training session interaction ($F_{9,165} = 2.05$, $p=0.0361$). A post hoc Bonferroni's test to compare the difference between

subjects at a particular training session revealed that all the groups were comparable to each other at training session 6-10 and training sessions 21-25. The number of lever presses between groups were significantly different at session 41-45 (0crit vs 3crit, p=0.0004). Also, the number of lever presses between groups were significantly different at session 76-80 (0crit vs 3 Crit, p=0.0007 and 1crit vs. 3crit, p= 0.0053). A post hoc Bonferroni's test to compare the differences within a group of rats, showed that for all the groups the active lever presses at session 76-80 were significantly higher than the lever presses at session 6-10 ($p<0.01$) and at session 21-25 ($p<0.01$). This shows at all the groups showed a steady increase in their lever pressing behaviour as compared to their baseline lever pressing behaviour. However, since the interaction effect is significant, it shows that magnitude of change was higher in the 2crit and 3crit rats.

	Average active lever presses for saccharine only	**Average active lever presses for ethanol only (Session 6-10)**	**Average active lever presses for ethanol only (Session 41-45)**	**Average active lever presses for ethanol only (Session 76-80)**
Persistence in drug seeking during the no-drug period	-0.04	-0.085	0.23	0.206
Excessive motivation for alcohol seeking and drinking	0.126	-0.013	0.497*	0.446*
Resistance to punishment	0.192	0.062	0.536*	0.27*

Table 2*:Table of lever press averages for different conditions. *p < 0.05 Significant using Pearson's correlational analysis (2 tailed).*

One argument against the model could be that some rats are inherently good at pressing the lever and the final output observed with the 3 behaviours is a reflection of their motor abilities and not their conditioning for lever pressing for alcohol. Hence, we ran a correlational analysis between the three criteria

scores used to define the addiction vulnerability and the lever presses for saccharine and for ethanol (at 3 time points: session 6-10, session 41-45 and session 76-80). As can be seen from Table 2, the lever presses for saccharine were not correlated with the three criteria scores. Similarly, the lever pressing for ethanol at the beginning of the training did not correlate with the three criteria scores. The persistence in lever pressing never correlated with the lever pressing at any time point. The motivation and resistance to punishment started showing significant correlations with the lever pressing for ethanol only after prolonged training.

3.6.9.5 Effect of Baclofen

Baclofen, a GABA-B receptor agonist, was tested on the progressive ratio paradigm to determine whether it could decrease the motivation for ethanol (Figure 1I). Two doses of baclofen were tested (1mg/kg and 2 mg/kg). A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,56} = 11.09$, $p<0.01$), a significant effect of dosage ($F_{2,116} = 25.71$, $p<0.0001$) and a significant interaction effect ($F_{112,173} = 10.46$, $p<0.0001$). A group-wise post hoc Bonferroni's test showed that both the doses of baclofen reduced ethanol intake in the Vulnerable group, while only the 2 mg/kg dose had an effect in the Resilient group.

Additionally, the effect of baclofen in the reinstatement paradigm was tested using the more effective 2mg/kg dosage (Figure 1J). A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,55} = 9.25$, $p<0.01$), a significant effect of intensity of dose of baclofen ($F_{1,55} = 67.81$, $p<0.0001$) and a significant interaction effect ($F_{55,113} = 7.55$, $p<0.01$).

3.6.9.6 Factor analysis-figure

A factor analysis was conducted for the three addiction-like criteria, reinstatement and impulsivity to determine whether they loaded on the same underlying construct (Figure 1L). The eigenvalue was kept as 1. The sampling adequacy score were also all around 0.75 as measured by the

KMO test (Persistence in drug seeking during the no-drug period: 0.768, Excessive motivation for alcohol seeking: 0.732, Resistance to punishment: 0.793, reinstatement: = 0.786 and impulsivity: 0.802) which indicates that five variables included are suited for testing factor analysis. All the five variables included loaded on one construct (Persistence in drug seeking during the no-drug period: r = 0.719, Excessive motivation for alcohol seeking: r = 0.832, Resistance to punishment: r = 0.715, reinstatement: r = 0.658 and impulsivity: r=0.65) accounting for 50% of the variance, further supporting that the three addiction-like criteria, reinstatement and the pre-existing trait of impulsivity are measures of a single underlying factor.

3.6.9.7 Microbiome Correlation Tables

Significant correlated genera presence and abundance	Relative Abundance (%)		All Samples		Resilient	Vulnerable
Taxa	Median	Mean	Presence	Presence%	Presence	Presence
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio	1.076%	1.569%	38	100.0%	19	19
Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Veillonella	0.000%	0.012%	10	26.3%	7	3
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Erysipelotrichaceae.UCG.003	0.002%	0.005%	26	68.4%	13	13
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Allobaculum	0.000%	0.010%	19	50.0%	11	8
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Papillibacter	0.123%	0.130%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	0.103%	0.108%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium	0.000%	0.001%	18	47.4%	9	9
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Eubacterium.coprostanoligenes.group	2.351%	2.893%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Anaerofilum	0.006%	0.007%	35	92.1%	19	16

Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured	4.017%	4.539%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Syntrophococcus	0.006%	0.010%	35	92.1%	18	17
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Shuttleworthia	0.014%	0.028%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.007	0.001%	0.002%	24	63.2%	12	12
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006	0.077%	0.083%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005	0.142%	0.178%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4B4.group	0.016%	0.025%	37	97.4%	18	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Eubacterium.oxidoreducens.group	0.125%	0.155%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	0.009%	0.018%	34	89.5%	15	19
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	0.000%	0.001%	9	23.7%	8	1
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured	0.109%	0.121%	38	100.0%	19	19

***Table 3**: Relative abundance and sample presence of genus level bacteria shown in correlation results - Figure 6 of manuscript.*

Taxa	Behaviour	rho	Pvalue	q
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	LP.wShock	-0.52572	0.000731	0.031114
D_0__Bacteria.D_1__Proteobacteria.D_2__Deltaproteobacteria.D_3__Desulfovibrionales.D_4__Desulfovibrionaceae	LP.woEtOH	-0.46947	0.002944	0.079496
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__4.15.D_4__uncultured.bacterium	TotEtOH	0.43216	0.005322	0.121537
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	Criteria	-0.4	0.005596	0.124331

		4089		
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	PR	-0.43581	0.006239	0.132315
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	Addiction.score	-0.4305	0.006976	0.140972
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	D1.mRNA	-0.61419	0.014854	0.222395
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Peptococcaceae	OFT	-0.39562	0.016925	0.240916
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Defluviitaleaceae	dPR.baclofen	0.38396	0.017318	0.244105
D_0__Bacteria.D_1__Firmicutes.D_2__Negativicutes.D_3__Selenomonadales.D_4__Acidaminococcaceae	Addiction.score	-0.3804	0.018452	0.254555
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	D2.mRNA	0.59585	0.019077	0.255258

***Table 4**: Family level correlations to behavioural measures. Green highlights indicate correlations that pass FDR with a q value set to 0.25.*

	Relative Abundance (%)		All Samples		Resilient	Vulnerable
Taxa	Median	Mean	Presence	Presence %	Presence	Presence
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	0.000 %	0.001 %	9	23.684 %	8	1
D_0__Bacteria.D_1__Proteobacteria.D_2__Deltaproteobacteria.D_3__Desulfovibrionales.D_4__Desulfovibrionaceae	1.189 %	1.714 %	38	100.000%	19	19
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__4.15.D_4__uncultured.bacterium	0.000 %	0.001 %	10	26.316 %	3	7
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Peptococcaceae	0.737 %	0.752 %	38	100.000%	19	19
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Defluviitaleaceae	0.004 %	0.004 %	32	84.211 %	15	17

***Table 5**: Relative abundance and sample presence of family level bacteria shown in correlation results - Table 4 above.*

4 Chapter:

Sex, Drugs, and the Microbiome: Goal/Sign-Tracking Phenotype Reveals Associations between Behavior and Microbiome in a Sex-Dependent Manner in the Rat

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4.1 Abstract

Introduction: Multiple factors contribute to the etiology of addiction, including genetics, sex, and a number of addiction-related behavioral traits. One behavioral trait where individuals assign incentive salience to food stimuli ("sign-trackers", ST) are more impulsive compared to those that do not ("goal-trackers", GT), as well as more sensitive to drugs and drug stimuli. Furthermore, this GT/ST phenotype predicts differences in other behavioral measures. Recent studies have implicated the gut microbiota as a key regulator of brain and behavior, and have shown that many microbiota-associated changes occur in a sex-dependent manner. However, few studies have examined how the microbiome might influence addiction-related behaviors. To this end, we sought to determine if gut microbiome composition was correlated with addiction-related behaviors determined by the GT/ST phenotype. *Methods:* Outbred male (N=101) and female (N=101) heterogeneous stock rats underwent a series of behavioral tests measuring impulsivity, attention, reward-learning, incentive salience, and locomotor response. Cecal microbiome composition was estimated using 16S rRNA. Behavior and microbiome were characterized and correlated with behavioral phenotypes. Robust sex differences were observed in both behavior and microbiome; further analyses were conducted within sex using the pre-established goal/sign-tracking (GT/ST) phenotype and partial least squares differential analysis (PLS-DA) clustered behavioral phenotype. *Results:* Overall microbiome composition was not associated to the GT/ST phenotype. However, microbial alpha diversity was significantly decreased in female STs. On the other hand, a measure of impulsivity had many significant correlations to microbiome in both males and females. Several measures of impulsivity were correlated with the genus *Barnesiella* in females. Female STs had notable correlations between microbiome and attentional deficient. In both males and females, many measures were correlated with bacterial the family *Ruminococcaceae* and *Lachnospiraceae*. *Conclusions:* These data demonstrate correlations between several addiction-related behaviors and the microbiome specific to sex.

4.2 Introduction

Addiction is a complex disorder; many factors modulate addiction severity and treatment efficacy. Extensive research has identified socioeconomic status (Heilig, Epstein et al. 2016), childhood trauma (Enoch, Hodgkinson et al. 2010, Schwandt, Heilig et al. 2017), genetics (Palmer and de Wit 2012), sex (Becker and Hu 2008), and psychological comorbidities as factors that contribute to addiction risk and severity (Grant, Goldstein et al. 2015). Due to the complex etiology of addiction, treatment is a trial and error process, frequently requiring a combination of therapies (Heilig, Goldman et al. 2011). More research is necessary to investigate how genetic and environmental factors contribute to addiction. Additionally, new factors such as the effect of diet, microbiome, and social interventions require greater attention. In particular, manipulation of the gut microbiome offers an intriguing target for new addiction treatments (Temko, Bouhlal et al. 2017).

The gut microbiota consists of a myriad of bacteria, archaea, fungi, and viruses colonizing the host gastrointestinal tract and influencing many host systems, such as metabolism (Barton, Penney et al. 2017, Ryan, Patterson et al. 2017), immune function (Kau, Ahern et al. 2011, Kundu, Blacher et al. 2017), hypothalamic-pituitary-adrenal axis response, and brain (Collins, Kassam et al. 2013, Hsiao, McBride et al. 2013, Mayer, Tillisch et al. 2015, Sherwin, Dinan et al. 2017). The gut microbiota is defined as the community of microbes residing in the intestines; the microbiome is the genomic DNA from all microbes in that community. In the context of affective disorders, both animal and human studies have strongly linked the composition of the gut microbiota to the behavioral aspects of these disorders (Bercik, Verdu et al. 2010, Bravo, Forsythe et al. 2011, Collins, Kassam et al. 2013, Kelly, Borre et al. 2016, Zheng, Zeng et al. 2016). Furthermore, microbiota has been linked to psychiatric disorders that involve an array of behavioral abnormalities, such as autism spectrum disorder (Hsiao, McBride et al. 2013, Mayer, Padua et al. 2014, Golubeva, Joyce et al. 2017). Previous research

has indicated a link between gut microbiome, addiction, and drugs of abuse (Yan, Fouts et al. 2011, Leclercq, Matamoros et al. 2014, Scheperjans, Pekkonen et al. 2015, Kiraly, Walker et al. 2016, Ning, Gong et al. 2017, Peterson, Jury et al. 2017).

Drugs of abuse activate ‘the reward pathway’, which includes cortical innervations in the ventral tegmental area (VTA), striatum, and prefrontal cortex (PFC). Behavioral measures that have been developed in rodents to explore this pathway, include: locomotor response to novelty (Flagel, Clark et al. 2011), measures of impulsivity (Reynolds, Ortengren et al. 2006), attention (Gancarz, Robble et al. 2012), and reward-stimulus learning (Flagel and Robinson 2017). In the case of reward-stimulus learning, the unconditioned stimuli (USs) and the conditioned stimuli (CSs) can activate this reward pathway (Schultz, Dayan et al. 1997). Individual differences in the activation of this pathway promote differential behavioral responsivity to CSs (Flagel, Clark et al. 2011); "sign-tracking" rats (STs) approach CSs more than their "goal-tracking" counterparts (GTs)(Flagel, Watson et al. 2007, Meyer, Lovic et al. 2012). Additionally, STs and GTs are differentially sensitive to the motivational properties of several abused drugs (Saunders and Robinson 2010, Yager, Pitchers et al. 2015, Yager and Robinson 2015, Versaggi, King et al. 2016). Furthermore, STs are more impulsive as measured by tests of impulsive action and choice (Tomie, Grimes et al. 2008, Lovic, Saunders et al. 2011, King, Palmer et al. 2016, Meyer and Tripi 2018). To conclude, this GT/ST phenotype predicts differences in other behavioral measures (Nasser, Chen et al. 2015). Interestingly, sign-tracking behavior is more frequent in female than male rats (Pitchers, Flagel et al. 2015, King, Palmer et al. 2016). Impulsivity has also been associated with increases in addiction-related behavior, as well as attention deficit disorder in both rodents (Gancarz, San George et al. 2011, King, Palmer et al. 2016) and humans (Sanchez-Roige, Fontanillas et al. 2018).

The relationship between sex hormones, microbiome and behavior is gaining attention (Amato, Leigh et al. 2014, Chaban, Links et al. 2014, Schnorr, Candela et al. 2014, Jasarevic, Morrison et al. 2016). During development factors like genetics and hormones, contribute to sexual dimorphism and associated to sex-differences in microbiome (Jasarevic, Morrison et al. 2016). Clinical and pre-clinical research has shown differences in microbiota composition associated with altered metabolism of essential vitamins and nutrients from the diet, with increased metabolic function seen in females compared to males (Amato, Leigh et al. 2014, Bolnick, Snowberg et al. 2014, Zhernakova, Kurilshikov et al. 2016). Worldwide, illicit drug use is significantly lower in females compared to males (World Health Organization 2012). However, females self-administer drugs more readily than males and are more vulnerable to addiction to a variety of licit and illicit drugs (Becker and Hu 2008, Yang, Han et al. 2017).

This study, to our knowledge, is the first to investigate sex-specific relationships between addiction-related behavioral measures and the microbiome in a large dataset. The pre-established GT/ST phenotype was characterized for this study, as it predicts differences in other addiction-related behavioral measures.These results are presented alongside a clustered behavioral phenotype constrained by goal/sign-tracker phenotype. Differences in behavioral measures and microbiome was characterized for each phenotype, within sex.

4.3 Methods

4.3.1 Animals

Male and female heterogenous stock (HS) rats were bred at Medical College of Wisconsin and then shipped to the University at Buffalo for behavioral testing. This National Institutes of Health (NIH)-derived outbred rat colony shows broad phenotypic and genotypic variation (Parker, Chen et al. 2014),

making it an ideal choice for the study of individuals differences. Rats (N=202) were housed in pairs in plastic cages (42.5 cm × 22.5 cm × 19.25 cm); males and females housed in the same room in alternating cages in testing order. In the event of odd numbers of rats, rats were housed individually. Animals were kept in reversed 12-h light/dark cycle and housed in controlled temperature and humidity conditions. Lights were on in the colony room from 19:00 pm to 07:00 hours. Behavioral testing occurred 6 days/week between of 08:30 and 12:30 hours during the dark phase of the light cycle. Food (#8604, Harlan Inc., Indianapolis, IN) was available *ad libitum* in the home cage. Animals were treated in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo, The State University of New York.

4.3.2 Behavioral tests

Behavioral testing was carried out on rats beginning at 63 days of age (see Table 1). All behavioral tests were conducted in the dark phase of the light cycle. Epochs for behavioral tests consisted of consecutive 3-minute intervals across a test session.

4.3.2.1 Locomotor Response to Novelty (Loco)

To assess locomotor response to novelty, rats were placed in a 24x45cm clean plastic standard laboratory cage. A Hamilton Kinder motor monitor frame contained infrared photo detectors which measured locomotor activity by beam breaks. Rats were placed into the test cages for one hour. Only the first 18 minutes of the 1-hour test session were used for analysis. Each rat was tested only once. Measures used for this test included total locomotor activity (Loco.Activity), epoch with the greatest activity (Loco.MaxAct), total distance travelled (Loco.Distance), epoch with the greatest distance travelled (Loco.MaxDist), time in center (Loco.Center), total number of rears (Loco.Rear), epoch with the greatest number of rears (Loco.MaxRear).

Age	Procedure
0-	Rats reared in Medical College of Wisconson
21	Rats arrive in Buffalo, NY (21 days old).
60	Rats housed until they are young adults (60 days of age)
63	Locomotor Response to Novelty
86	Light Reinforcement
129	Choice Reaction Time Task
154	Delay Discounting
171	Pavlovian Conditioned Approach (1+5 days), then Conditioned Reinforcement (1 day)
185	Cocaine Cue Preference
200	Rats sacrificed; cecal samples collected

Table 1: Study design flow chart *– Age in days (first column), Procedure (second column).*

4.3.2.2 Light Reinforcement (LR)

In-house constructed operant chambers were used for testing (see Supp. Methods Fig 2). The visual stimulus (VS) reinforcer used in the experiment was the onset of the light located in the middle of the back wall of the test chamber. Onset of the VS reinforcer produced an illuminance of 68 lx, as measured from the center of the test chamber. The VS reinforcer was illuminated for 5 s each time it was presented. Each test chamber was housed in a Coleman Cooler (Model # 3000000187), which blocked external audiovisual sources of stimulation. The pre-exposure phase consisted of six 18 min sessions. Light reinforcement testing took place immediately after pre-exposure testing with one day off in-between. The light reinforcement phase consisted of six 18 min sessions. During light reinforcement testing, rats were placed in dark experimental chambers and snout pokes into the aperture designated as ‘active’ resulted in 5 s illumination according to a variable interval 1-minute (VI1) schedule of reinforcement. Measures from light reinforcement testing included total number of light reinforcers (LR.Reinforcers), total number of InActive responses during test (LR.InActive), total number of Active and InActive responses during test

(LR.Total), epoch with greatest total responses (LR.TotMax) and epoch with greatest InActive responses (LR.InActMax).

4.3.2.3 Choice Reaction Time Task (RT)

Locally constructed experimental chambers were used for the choice reaction time task. The test panel had two water dispensers located on either side of a centrally located snout-poke hole (see Supp. Methods Fig 3). The water dispenser and stimulus lights were arranged so that they were level with the rat's eyes when the rat's snout interrupted an infrared beam in the center snout-poke hole. Rats were placed into the test cages for 18 minutes for each test session. Rats initiated trials by holding their snout in the center snout hole until the left stimulus light was turned on (hold time). Once the hold time criterion was reached and the imperative stimulus was presented, the rat had 3 seconds to respond by removing its snout and inserting it into the left feeder hole (reaction time), or the trial ended and the trial was counted as an omission. If the rat made a correct response, the rat received a water reinforcer (30 µl) and the trial ended. A false alarm was recorded when the rat pulled its snout out of the center hole to respond to the left water feeder hole prior to the onset of the imperative stimulus. Premature initiations are defined similar to a false alarm, except that the rat pulls out of the center snout poke hole before the imperative stimulus occurs and then puts its snout back into the center hole without going to the left water feeder hole. The final 3 test sessions were used for analysis. Measures for this test included total number of correct responses (RT.Corr), mean reaction time (RT.MeanRT), per opportunity (trial) premature initiations (RT.PerOPInit), per opportunity false alarms (RT.POFA), and total omissions (RT.Omissions).

4.3.2.4 Delay Discounting (DD)

Delay Discounting was measured using a sequential patch depletion procedure. This procedure mimics naturally occurring choice problems confronting animals while foraging in resource scarce environments (i.e., travel delays and patch depletion). Water was restricted and only made

available for 20 minutes following testing. Behavior was measured in in-house constructed operant chambers (see Supp. Methods Fig 2). In the laboratory patch depletion procedure rats drink water at both the left and center water feeders. Rats receive successively smaller amounts of water every 4 s by remaining at the same feeder. The amount of water is initially 150 µL and is then decreased by 20% after each delivery from the same feeder. The rats can reset the amount of water to the initial maximum of 150 µL by switching to the alternative water feeder. However, changing to a new patch results in a delay to activation of the new feeder (travel time delay). During the delay, water is not available at either feeder. A change in patch is indicated by a snout poke into the alternative non-active feeder (patch). The indifference point (IDPt) is defined as the amount of water available at the current feeder (or patch) when the rat chose to switch to the new patch. Test sessions last for 10 minutes or until the rats consumed a cumulative total 5 ml of water, which ever occurred first. The area under the curve (AUC) was calculated for successive session measures. Behavioral measures from this test included indifference point area maximum (DD.IDPMax) and under the curve (DD.IDPtAUC), patch change rate area under the curve (DD.PCRateAUC), and average reinforcer rate (DD.RFRate).

4.3.2.5 Pavlovian Conditioned Approach (PavCA) and Conditioned Reinforcement (CRF)

To examine individual differences in the propensity to attribute incentive salience to reward cues, HS rats were first exposed to a Pavlovian conditioning paradigm wherein a cue (lever) is repeatedly paired with presentation of a reward (food). Before animals undergo the standard PavCA procedure, they receive ~25 banana-flavored food pellets (Envigo, #F0059) in their homecages for 2 days. Then they undergo one day of magazine training, during which they are placed into Med-associates conditioning chambers and food pellets are delivered on a VI 30 s (1-60 s) schedule. For the subsequent 5 PavCA conditioning days, rats receive 25 CS-->US conditioning trials, presented on a VI 90 s (30-150 s) schedule. During each trial, an 8-s presentation of an illuminated lever CS preceded the delivery of

a food pellet. On the day following the final session of PavCA, we perform conditioned reinforcement (CRF) in which rats can nose poke for presentations of the lever CS. All variables are derived from lever presses and magazine entries, including latencies and probability. Measures for PavCA included index scores on day 4 and 5 [see (Meyer, Lovic et al. 2012) for a description of this index]; briefly, scores range from -1 to 1 with negative numbers indicating magazine directed responses (goal-tracking), and positive numbers indicating lever-CS directed responses (sign-tracking). CRF measures used were total number of lever presses (CRF.LeverPresses), total number of active nose-poke port entries (CRF.ActivePort) and total number of CS lever presentations (CRF.Reinforcers).

4.3.2.6 Cocaine Cue Preference (CCP)

To examine the individual differences in approach to a cocaine-associate cue, HS rats were tested for their response to a cocaine-paired tactile cue [see (Meyer, Ma et al. 2012) for details]. Briefly, testing chambers were constructed with black acrylic walls (47.5 cm length x 15.5 cm width x 30 cm height) with black spray-painted textured floors that were either stainless steel rods (“grid”) or perforated steel (“hole”), or half grid and half hole. After one day of habituation to the chamber and one "pre-test" day to determine grid-hole preferences, rats were given four conditioning trials. Each trial was two days, one saline-paired day and one cocaine-paired day. On saline-paired days, rats were given an injection of saline (i.p.) and placed into a chamber with either a uniform grid or hole floor (whichever was their preferred floor). On the cocaine-paired day, rats are given an injection of cocaine (10 mg/mL; Nat. Inst. Of Drug Abuse, Bethesda, MD) and placed in a chamber with the opposite uniform floor (i.e., non-preferred floor). On the last "post-test" day, rats were given a saline injection, and presented with both cocaine- and saline-paired floors. The time spent on each floor was analyzed to determine cocaine cue preference. The primary dependent measures are change in time spent on the cocaine-paired floor from pre-test to post-test (CCP.PreTest.Time.CS - CCP.PostTest.Time.CS = CCP.dtCS).

Secondary measures include cocaine change in locomotor activity on trails 1 and 4 (CCP.T1.Cocaine.Dist CCP.T4.Cocaine.Dist).

4.3.3 Cecal microbiome collection and sequencing

Cecum was collected and snap-frozen on dry ice. Protocols for 16S rRNA microbiome sequencing were used as previously described (Peterson, Jury et al. 2017). Briefly, cecal contents from frozen cecum (stored at -80ºC) were extracted under a sterile hood. The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA from cecal contents using the manufacturer's handbook (Second Edition 2012). Samples were prepared for 16S sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), as described in the Illumina 16S library preparation workflow. 16S bacterial rRNA gene was amplified using primers targeting the V3-V4 hypervariable region (Forward: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; Reverse: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Sigma Aldrich Ireland ltd., Wicklow, Ireland). The Illumina V3–V4 primers were selected for their high coverage (94.5% bacteria) while remaining in the amplicon size necessary for sequencing to sequence at 2 × 250 bp (Klindworth, Pruesse et al. 2013). 16S rRNA amplicons were sequenced on the Illumina MiSeq platform, multiplexed on 4 separate runs (~50 samples per run) (Teagasc, Moorepark, Ireland). No negative control was used in processing of DNA or sequencing.

4.3.3.1 Microbiome Sequence Processing

All sequences in FASTQ files format were filtered using PRINSEQ. Sequences with length less than 150 nucleotides or with low quality at the 3' end were removed. Paired-end reads with a minimum overlap of 20 base-pairs were joined using FASTQ-join and analyzed with QIIME (Quantitative Insights Into Microbial Ecology, v1.9.1). Sequence quality was checked and chimeras removed, remaining sequences were clustered into Operational

Taxonomic Units (OTUs ; 97% identity level) using USEARCH (Version 7.0-64bit). The average number of high-quality sequences generated per sample was 153,561 ± 84,269 SD. Taxonomy was assigned to OTUs using Silva version 123. Alpha diversity (Observed, Chao1, Shannon, Simpson) indices were calculated with QIIME.

4.3.4 Statistical Analysis

Data was analyzed in R (v3.3.3) and RStudio (v1.0.136). Plots were generated in R using ggplot2 package (v2.2.1). All testing was corrected for multiple comparisons using the qvalue R package (v2.6.0). For OTU correlations to behavior, q-value confidence interval was set to 0.15. For within-sex correlations, the q-value of 0.15 was accepted due to the exploratory nature of this study. In this dataset, q=0.15 indicates that of the 30 reported significant correlations within sex, only 4-5 of them may be false positives. For all other analyses, q-value was set to 0.05.

4.3.4.1 Behavioral Analysis

A total of 54 behavioral measures were selected for analysis based on relevance to addiction. Behavioral differences were assessed by behavioral cluster and goal/sign-tracking phenotype within sex using Kruskal-Wallis and Wilcoxon test. Behavioral measures significantly different by group were plotted by z-score to visualize trends between grouping phenotypes. Factor analysis was performed to test influence of weight and age at time of dissection, generation, and goal/sign-tracker phenotype using the ADONIS (PERMANOVA) function of the vegan (2.4-3) R package.

4.3.4.2 Sign-tracker/goal-tracker classification

Rats were classified as sign/goal-trackers based on PavCA Index Score (= [PavCA Score (Day 4) +PavCA Score (Day 5)]/2; see Meyer et al. 2012 for details (Meyer, Lovic et al. 2012). Subjects were classified as sign-tracker (ST) (PavCA Index Score between +0.5 and +1), goal-tracker (GT) (PavCA Index

Score between -0.5 and -1), and intermediate (IN) (PavCA Index Score between -0.49 and 0.49), based on the classification method previously described (Meyer, Lovic et al. 2012).

4.3.4.3 Behavioral Cluster Analysis

All behavioral measures (total=54) were used for cluster analysis using the KODAMA package (v1.4). Behavioral data within sex was normalized using probabilistic quotient normalization and centered to zero. Data was clustered using PLS-DA (partial least squares differential analysis) grouped by goal/sign-tracking phenotype and using multiple levels of cross-validation. Entropy was tested on point values for each cross-validation level to find optimal cluster. For both the female and male behavioral data set, the lowest entropy cluster was found with PLS-DA set to parameter (f.par) 100. Hierarchical cluster analysis was performed to select optimal number of clusters and to assign samples to cluster. Results were then plotted using PCA and colored based goal/sign-tracking phenotype.

4.3.4.4 *Microbiome analysis*

Microbiome was assessed by behavioral cluster and goal/sign-tracking phenotype within sex. Kruskal-Wallis and Wilcoxon test were used to assess statistical significance in alpha diversity indices and taxonomic comparisons between groups. Beta diversity was calculated using Euclidian distance visualized and analyzed using the vegan community ecology package (v2.4-3). Adonis (PERMANOVA) function from vegan assessed beta diversity significance by generation, behavioral phenotype, sign/goal-tracker phenotype (GT, ST, IN), age and weight at time of dissection. For Spearman correlations and taxonomic comparisons, Phylum, Family, Genus, and OTUs (Operational Taxonomic Units) were filtered by median >0.01%. For Spearman correlations this filtering resulted in sequences only present in >50% of samples and normalized to relative abundance. Correlations were performed between OTU-level bacterial abundance and behavioral measures, subset by grouping phenotype within sex. For correlation analysis, all

behavioral measures were classified into 3 major categories: reward-learning, impulsivity, and locomotion (Suppl. Table 1A). For Spearman correlations within sex, the qvalue R package (v2.60) was used to select false discovery rate. Due to the exploratory nature of this study, the q-value of 0.15 was accepted as it allowed reporting of interesting trends while still maintaining a low rate of false positives (4-5 total).

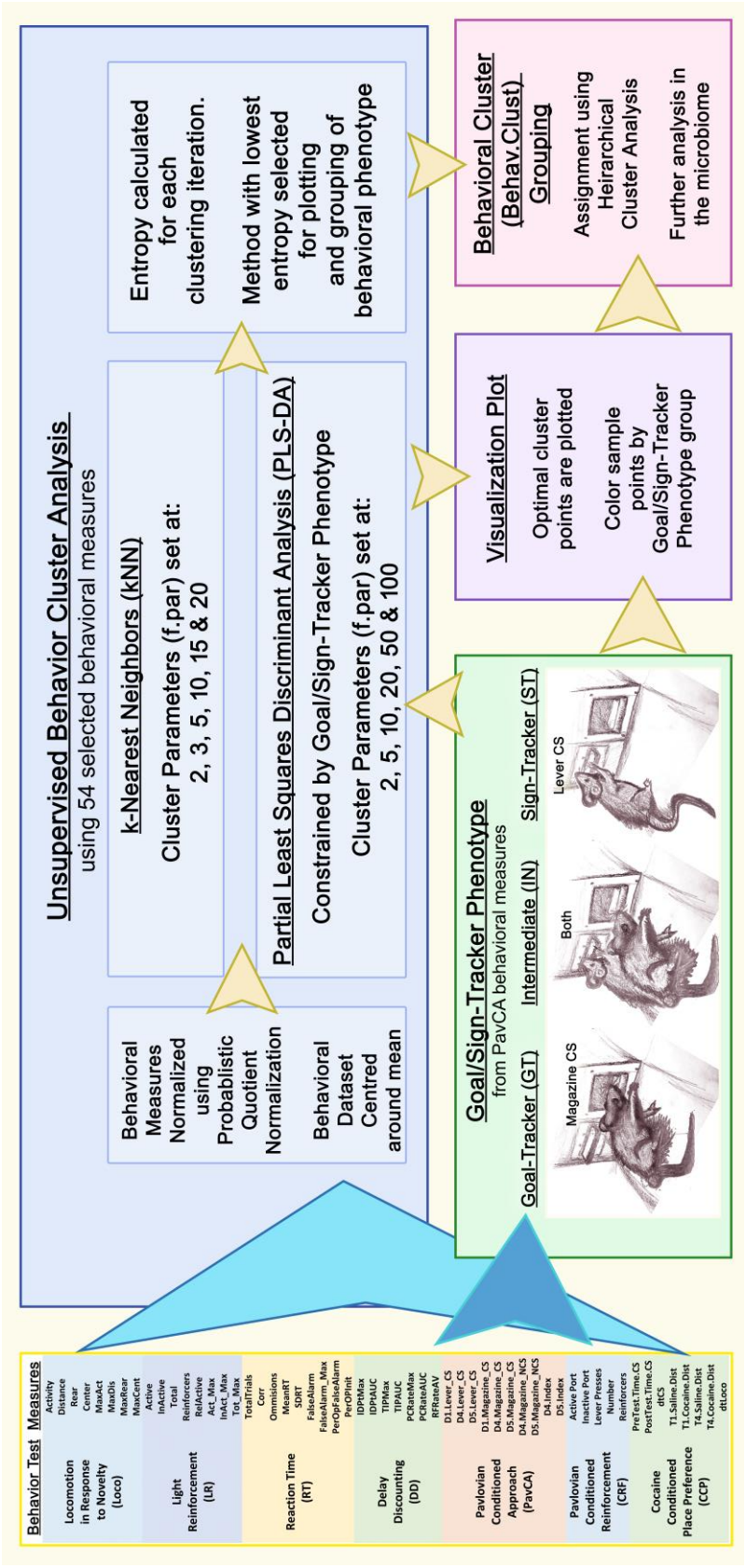


Figure 1 – Flow Diagram of how behavioral measures are used *to create goal/sign-tracking phenotype and behavioral cluster phenotype.*

4.4 Results

4.4.1 Behavioral cluster analysis

Preliminary analysis showed that all non-PavCA behavioral measures clustered by the goal/sign-tracking phenotype (GT/ST)(Fig. S1). Thus, we sought to evaluate the goal/sign-tracking phenotype, within sex, alongside a novel group cluster constrained by this pre-established grouping. Partial least squared differential analysis (PLS-DA) of all 54 behavioral measures (Fig. 1) within sex show a separation along the x-axis (Fig. 2A & 3A). In both males and females, the sign-tracking phenotype clusters in the negative (left) side of the x-axis (Fig. 2A & 3A). Behavioral clusters that contained the sign-tracking phenotype were labeled F.Behav.Clust.1 for female sup-population and M.Behav.Clust.1 for males. Samples clustering to the right of the x-intercept were labeled F.Behav.Clust.2 and M.Behav.Clust.2, for females and males, respectively.

4.4.2 Behavioral differences by behavioral cluster within sex

Factor analysis of 54 behavioral measures revealed that weight at the end of the experiment was the only significant contributing factor in both females ($R2=0.036$, $p=0.028$) and males ($R2=0.037$, $p=0.018$). Goal/sign-tracker phenotype, generation, and age at time of dissection were not significant factors ($p>0.05$) contributing to variations in all behavioral measures.

All 54 behavior measures were tested within sex by goal/sign-tracker phenotype (GT, IN, ST) and behavioral cluster (Behav.Clust.1, Behav.Clust.2). Females tested by goal/sign-tracking phenotype, 15 behavioral measures were significantly different (Kruskal-Wallis, $p<0.05$), with 13 of these measures due to differences in GTs compared to STs (Wilcoxon, $p<0.01$). In males tested by goal/sign-tracking phenotype, 11 behavioral measures were significantly different (Kruskal-Wallis, $p<0.001$), with 8 of these measures due to differences in GTs compared to STs

(Wilcoxon, $p<0.001$). Behavioral measure comparisons by behavioral cluster revealed 18 significant measures in females and 28 significant measures in males (Wilcoxon, $p<0.05$). Z-score was used to visualize behavioral comparisons by groups, behavioral cluster and GT/ST phenotype, within sex (Fig. 2B & 3B). See supplementary material for behavioral comparisons between sex (Supp. Section 1.2 and Fig. 1B).

4.4.3 Group differences in Pavlovian Conditioned Reinforcement (CRF)

Within both sexes and grouping phenotypes, significant differences were seen in measure for nose pokes in reinforcing port (CRF.Active.Port) and number of lever presses (CRF.Lever.Presses). In both male and female, significant differences in CRF.Active.Port were seen in goal-tracking (GT) phenotype compared to sign-tracking (ST) (Wilcoxon, $p<0.001$) and behavioral cluster grouping (Behav.Clust.1 vs. Behav.Clust.2) (Wilcoxon, $p<0.001$). Differences in CRF.Lever.Presses were explained by GT compared to ST group (Wilcoxon, $p<0.001$) and behavior cluster (Wilcoxon, $p<0.001$), in both sexes. Additionally, number of nose pokes into active port for presentation of lever reinforcer (CRF.Number.Reinforcers) was significantly different in both male and female by GT compared to ST phenotype (Wilcoxon, $p<0.001$).

4.4.4 Group differences in Cocaine Cue Preference (CCP)

Only the male behavioral cluster showed significant differences in cocaine-induced locomotor activity in Trial 1 measured by distance travelled (CCP.T1.Cocaine.Dist) (Wilcoxon, $p<0.001$). This difference was explained by the increases in M.Behav.Clust.1 compared to M.Behav.Clust.2 (Fig. 2B).

4.4.5 Group differences in Choice Reaction Time Task (RT)

Reaction time (RT) measures of standard deviation of reaction time per epoch (RT.SDRT) and number of failed responses defined as omissions (RT.Omissions) were significantly different by female goal/sign-tracking phenotype. For both measures, this result was explained by increases in IN group compared to GT (Wilcoxon, $p<0.01$) (Fig. 3B).

4.4.6 Group differences in Locomotor Response to Novelty (Loco)

Similar to measures from CCP, M.Behav.Clust.1 had significant increases in distance travelled (Loco.Distance) compared to M.Behav.Clust.2 (Wilcoxon, $p<0.01$). This was also seen in comparisons between male behavioral clusters in measures of exploratory rearing and maximum rears (Loco.Rear and Loco.MaxRear) (Wilcoxon, $p<0.05$), locomotor activity and maximum activity (Loco.Activity and Loco.MaxAct, $p<0.05$).

4.4.7 Group differences in Light Reinforcement (LR)

Measures from the LR task were significantly different by behavioral cluster in both males and females. Total number of light onset reinforcers (LR.Reinforcers), total active and inactive responses (LR.Total), total active responses (LR.Active), and maximum active responses in an epoch (LR.Act_ Max) were significantly different (Wilcoxon, $p<0.01$). These measures were decreased in F.Behav.Clust.1 compared to F.Behav.Clust.2, while they were increased in M.Behav.Clust.1 compared to M.Behav.Clust.2 (Fig. 2B & 3B).

4.4.8 Group differences in Delay Discounting (DD)

In female behavioral cluster, F.Behav.Clust.1 had significant increases in changes time in patch with experimenter imposed delay across epochs (DD.TIPAUC) compared to F.Behav.Clust.2 (Wilcoxon, $p<0.05$) (Fig. 3B). In the male behavioral cluster, M.Behav.Clust.1 was significantly increased in measures of patch change rate area under the curve (DD.PCRateAUC) and indifference point area under the curve (DD.IDPtAUC) compared to M.Behav.Clust.2 (Wilcoxon, $p<0.01$) (Fig. 2B).

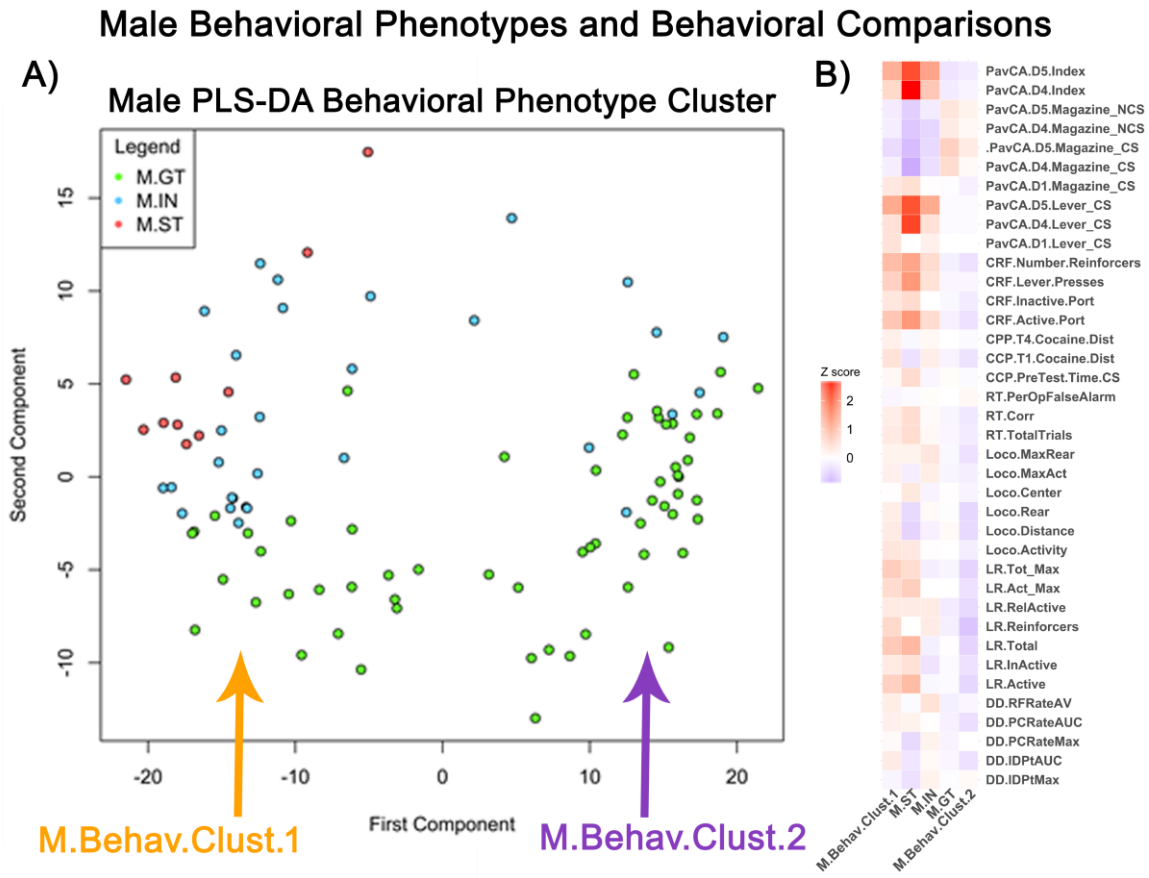


Figure 2 – Male Behavioral Phenotypes and Behavioral Comparisons *– A) Behavioral Phenotype Cluster visualized in principal coordinate analysis (PCA) of PLS-DA clustered behavioral measures. Each dot represents an individual rat, distance from one dot to another represents overall differences in behavioral measures.Goal-trackers (GT) colored green, intermediate (IN) blue, sign-trackers (ST) red. B) Z score indicate increases (red) or decreases (blue) in behavioral measures by behavioral cluster group and goal/sign-tracker phenotype group compared to entire male population.*

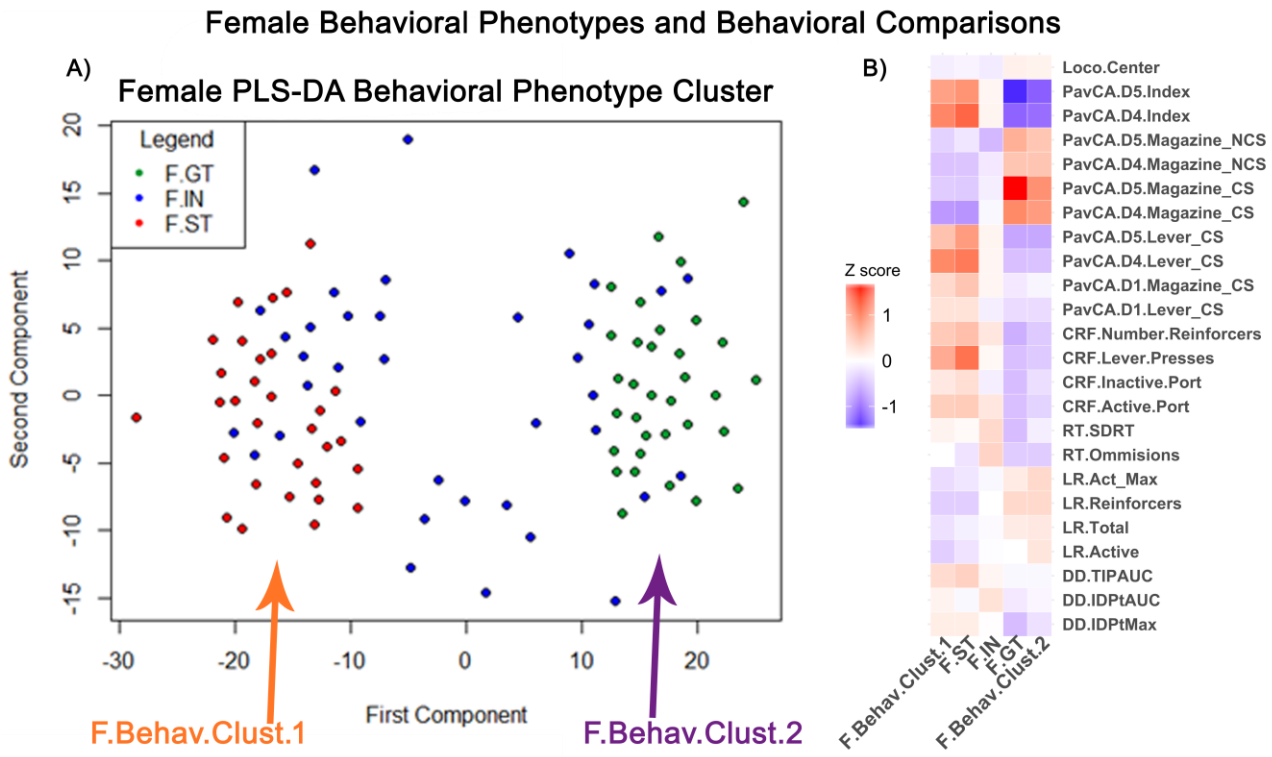


Figure 3 – Female Behavioral Phenotypes and Behavioral Comparisons *– A) Behavioral Phenotype Cluster visualized in principal coordinate analysis (PCA) of PLS-DA clustered behavioral measures. Each dot represents an individual rat, distance from one dot to another represents overall differences in behavioral measures.Goal-trackers (GT) colored green, intermediate (IN) blue, sign-trackers (ST) red. B) Z score indicate increases (red) or decreases (blue) in behavioral measures by behavioral cluster group and goal/sign-tracker phenotype group compared to entire female population.*

4.4.9 Microbiome diversity

In males, microbiome beta diversity was significant by generation ($R^2=0.039$, $p<0.001$) and age at end of experiment ($R^2=0.021$, $p<0.001$). Females were only significant by generation ($R^2=0.019$, $p<0.001$). No significant differences were seen for weight, sign/goal-tracker phenotype, or behavioral cluster in either males or females.

Alpha diversity analysis by female behavioral cluster revealed significant decreases in Shannon (Wilcoxson, $p<0.001$) and Simpson (Wilcoxson, $p<0.001$) index measures in F.Behav.Clust.1 compared to F.Behav.Clust.2 (Fig. 5A & 5C). Females ST group also had significant reductions in Shannon measure of alpha diversity compared to GT (Fig. 5B) There were no significant differences in alpha diversity measures in males (Fig. 4).

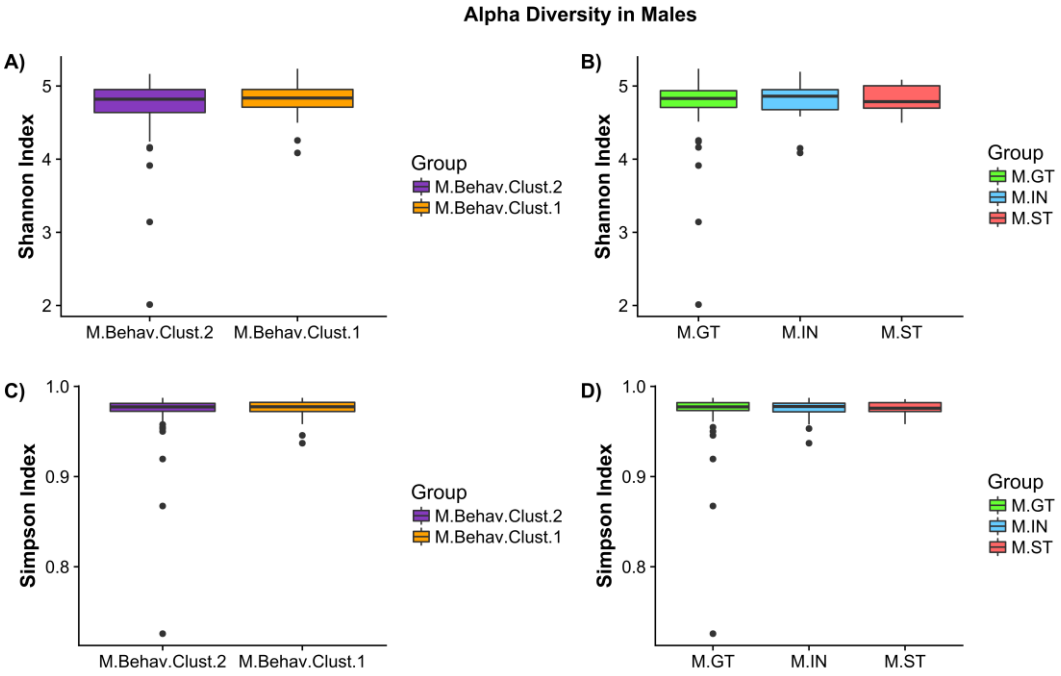


Figure 4 – Male Alpha Diversity by Phenotype Group *– A) Shannon index measure of alpha diversity by behavioral cluster (M.Behav.Clust 1 = orange, M.Behav.Clust.2 = purple). B) Shannon index by goal/sign-tracking phenotype: goal-tracker (M.GT = green), intermediate (M.IN = blue), and sign-tracker (M.ST = red). C) Simpson index measures of male alpha diversity by behavioral cluster. D) Simpson index of male goal/sign-tracker phenotype.*

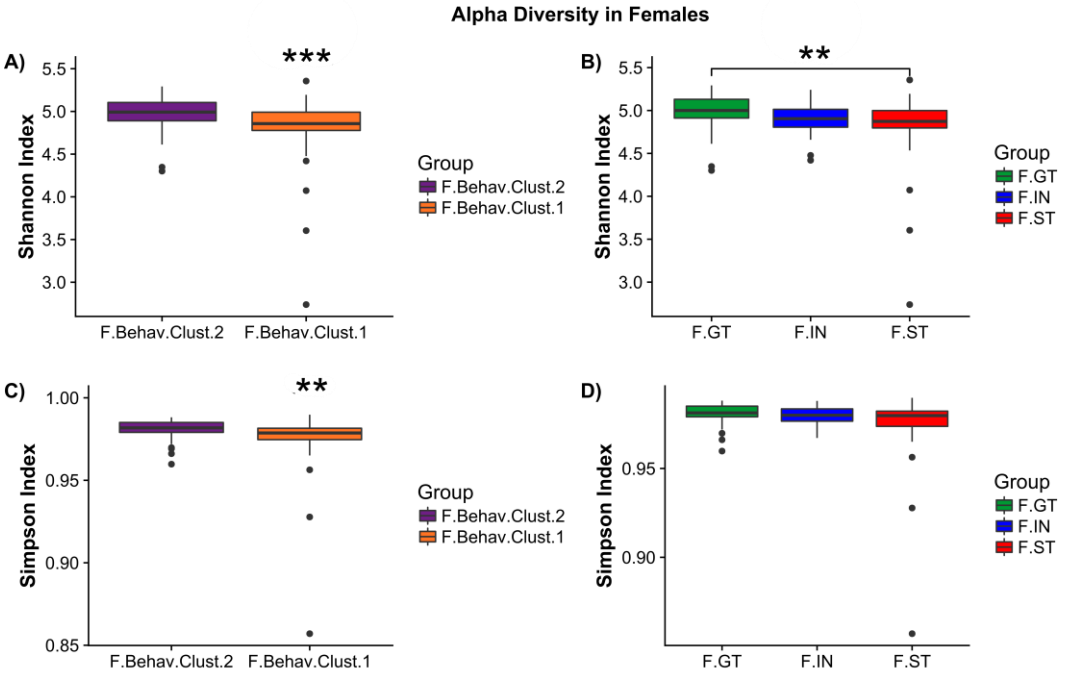


Figure 5 – Female Alpha Diversity by Phenotype Group *– A) Shannon index measure of alpha diversity by behavioral cluster (F.Behav.Clust 1 = orange, F.Behav.Clust.2 = purple). B) Shannon index by goal/sign-tracking phenotype: goal-tracker (F.GT = green), intermediate (F.IN = blue), and sign-tracker (F.ST = red). C) Simpson index measures of female alpha diversity by behavioral cluster. D) Simpson index of female goal/sign-tracker phenotype. Asterisks indicate significance: '***' p<0.001, '**' p<0.01*

4.4.10 Bacterial differences by groups within sex

No significant differences were seen in either phenotype group by sex at the Phylum, Family, Genus, or OTU level after FDR correction. In the female behavioral cluster, genus-level *Blautia* (Wilcoxson, q=0.062) and OTU-level *Papillibacter* OTU_128 and *Blautia* OTU_160 approached significance (Wilcoxson, q=0.055). All other results had an FDR q-value greater than 0.15.

4.4.11 OTU level bacterial correlations to behavior

4.4.11.1 Correlations by Male and Female Goal/Sign-Tracker Phenotype

In males, behaviors correlated to refined OTUs by GT/ST phenotype. The strongest correlation was between cocaine induced locomotor activity during trial 4 and *Tanneralla* OTU_157 (Spearman, rho=-0.976, p<0.001). Further correlations were seen in OTU-level bacteria belonging to family Lachnispiroceae and Ruminococcaceae in measures of reward learning (LR, PavCA, RT), in reaction time and light reinforcement measures, locomotion and impulsivity measures from delay discounting (Spearman, |rho|>0.722, p<0.001). An OTU-level bacteria from genus *Thermofilum* significantly correlated to impulsivity measures only in the male intermediate (IN) phenotype (Spearman, |rho|>0.709, p<0.001). Overall, the strongest correlations were seen in the male STs (Spearman, |rho|>0.939, p<0.001) (Fig. 6A).

In females, less significant correlations between microbiome and behavior by GT/ST phenotype were seen. A total of 9 significant correlations were observed, of these 6 were significant correlations within ST phenotype. A strong trend was seen in ST females with correlations between reaction time omissions and specific OTUs assigned to genera *Vampirovibrio, Coprococcus,* and *Flavinofractor* (Spearman, rho>0.729, p<0.001). Significant correlations were also found between impulsivity measures and

OTU bacteria assigned to genera *Clostridium XIVa* and *Barnesiella* (Spearman, rho>0.682, p<0.001) (Fig. 6B).

4.4.11.2 Correlations by Male and Female Behavioral Cluster

No significant correlations were seen between microbiome and behavior when analyzed by male behavioral cluster (Fig. 7A). In females, impulsivity measures and attention measures were significantly correlated with OTUs assigned to genus *Barnesiella* (Spearman, |rho|>0.620, p<0.001) (Fig. 7B).

4.4.11.3 Correlations by Male and Female

Spearman correlations were performed within the entire male and female sub-populations between behaviors and OTU-level bacteria (N=101, Males and Females) (Fig. 8). Impulsivity measures of indifference point area under the curve and patch change rate area under the curve was significantly correlated with *Lachnospiracaea_incertae_sedis* OTU_3469 (Impulsivity.DD.IDPtAUC and Impulsivity.DD.PCRateAUC, rho<-0.393, p<0.001) in males. In females, indifference point area under the curve and locomotor activity were negatively correlated to *Barnesiella* OTU_114 (Impulsivity.DD.IDPtAUC and Loco.Activity, rho<-0.407, p<0.001). In males, exploratory rearing was positively correlated to *Clostridium* XlVa OTU_3855; additionally, males had many OTU correlations to sensory preference for CPP floor before testing (see Supp. Materials). A positive correlation between reward learning and *Lachnospiracea incertae sedis* OTU_152 was observed in females (Reward.Learning.LR.Active, rho=0.407, p<0.001) (Fig. 8).

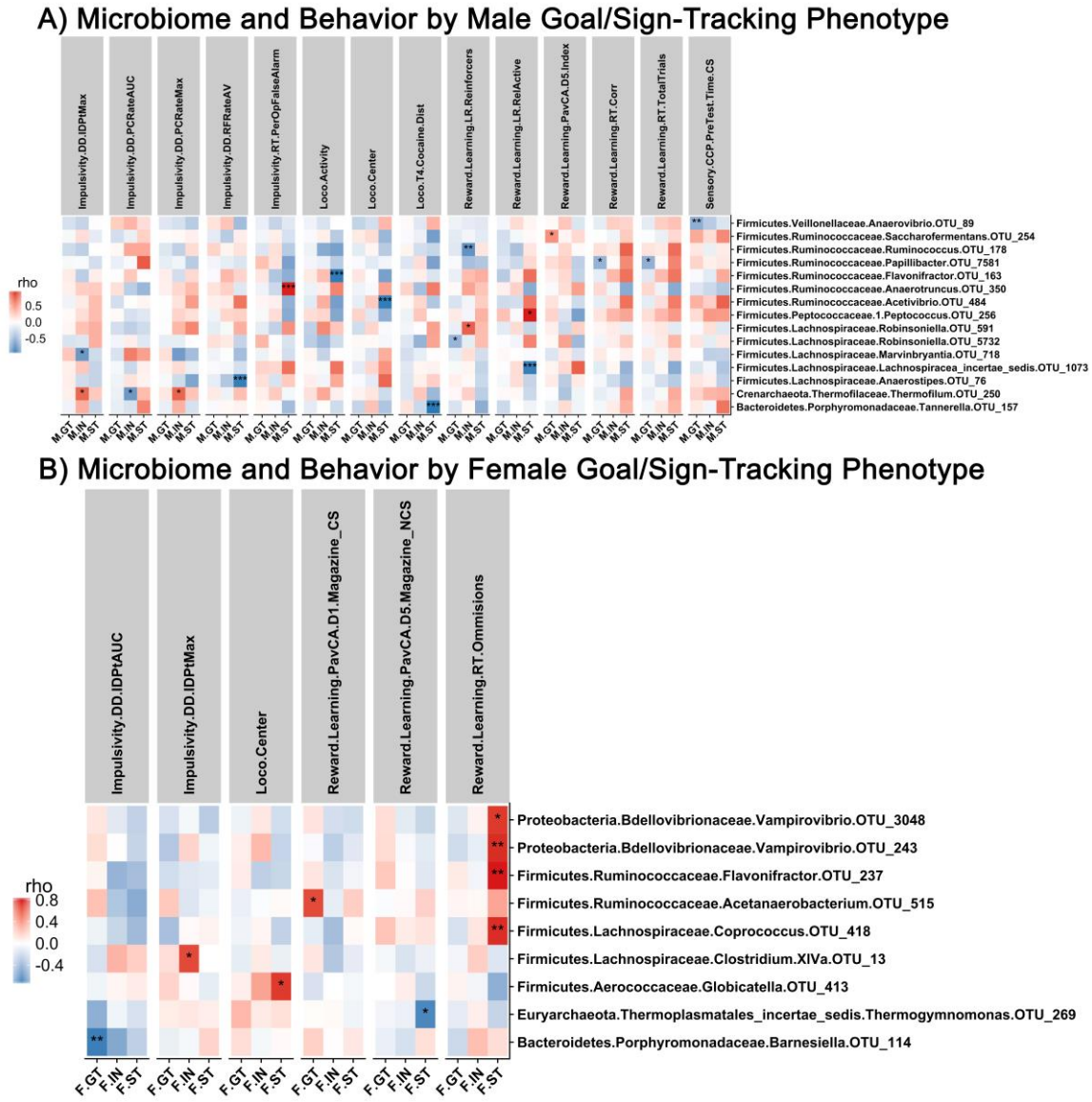


Figure 6 – Correlation Analysis in Male and Female Goal/Sign-Tracking Phenotype. *A) Correlations between OTU-level bacteria and behavior in male goal/sign-tracking phenotype. B) Correlations between OTU-level bacteria and behavior in female goal/sign-tracking phenotype. Positive correlations indicated in red, negative correlations indicated in blue. Significance that passes FDR indicated by asterisk: '***' q<0.05, '**' q<0.10, '*' q<0.15*

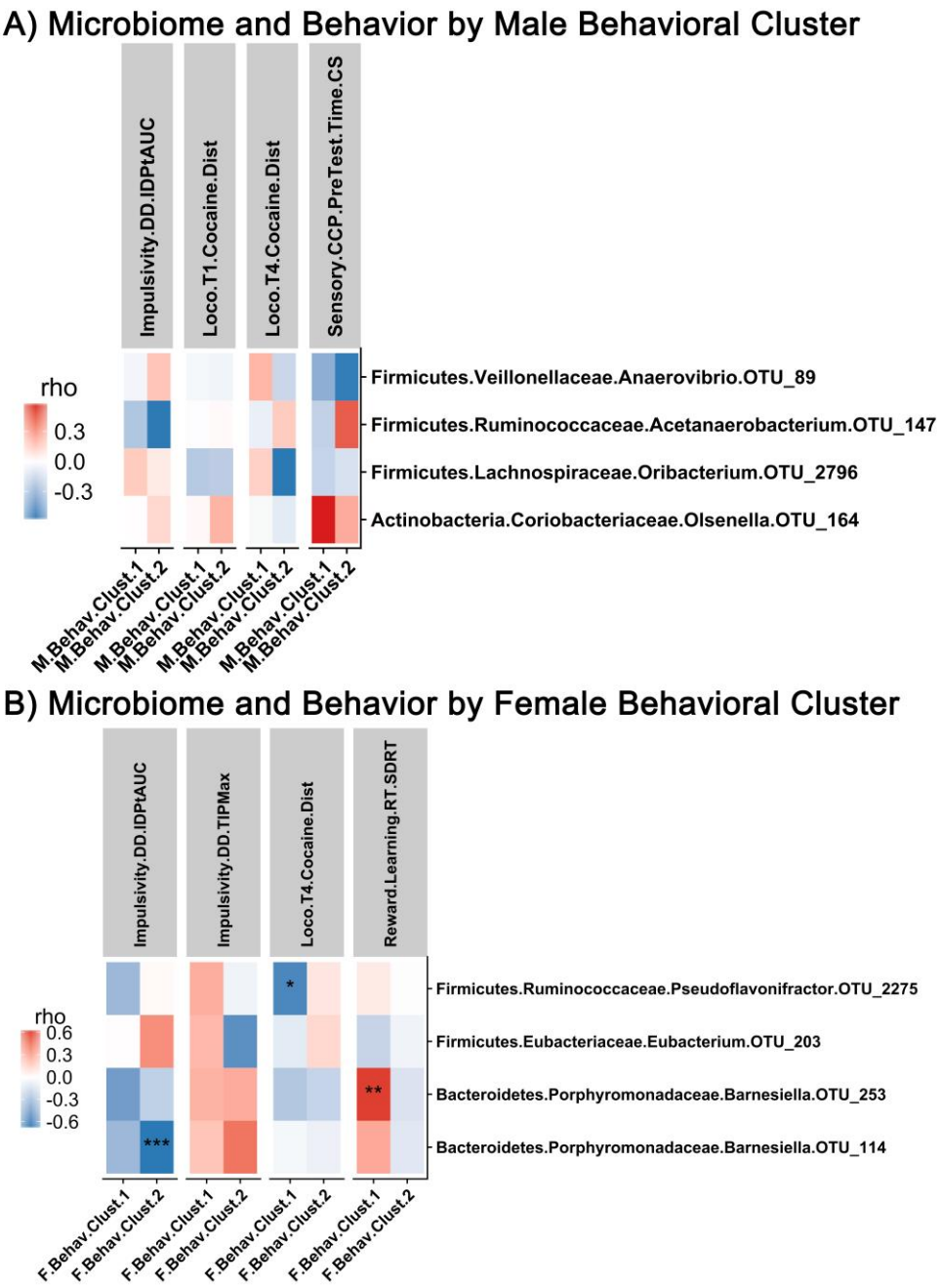


Figure 7 – Correlation Analysis in Male and Female Behavioral Clusters. *A) Correlation between OTU-level bacteria and behavior in male behavioral cluster phenotype. B) Correlation between OTU-level bacteria and behavior in female behavioral cluster phenotype. Positive correlations indicated in red, negative correlations indicated in blue. Significance that passes FDR indicated by asterisk: '***' q<0.05, '**' q<0.10, '*' q<0.15*

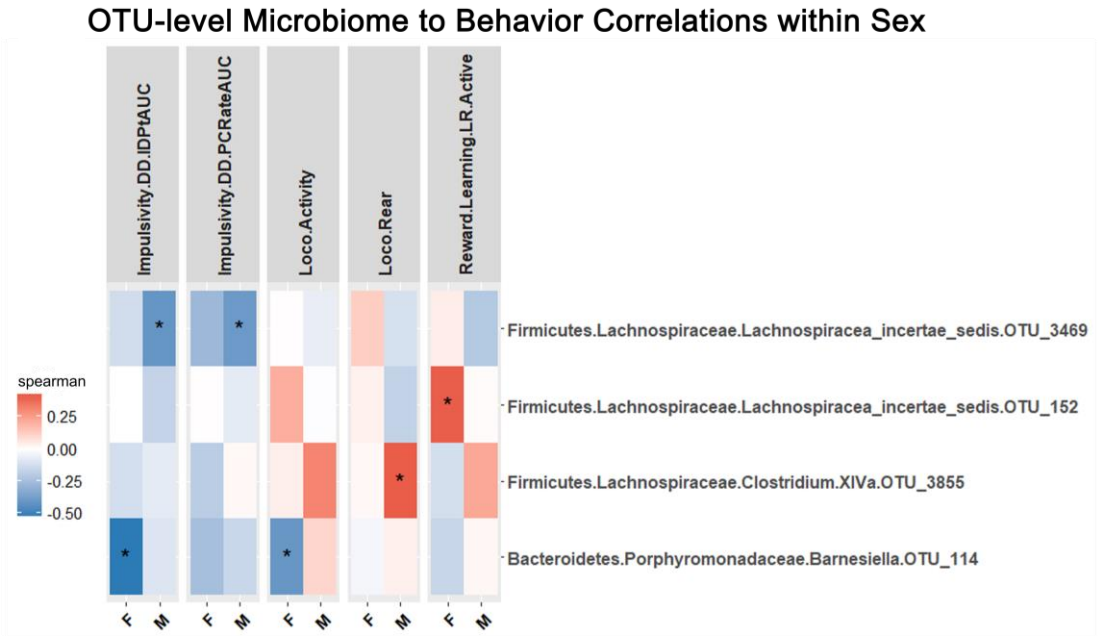


Figure 8 – Correlation Analysis by sex*. Correlations between OTU-level bacteria and behavior in entire female and male populations. Positive correlations indicated in red, negative correlations indicated in blue. Significance that passes FDR indicated by asterisk: '***' q<0.05, '**' q<0.10, '*' q<0.15*

4.5 Discussion

Growing evidence suggests that understanding the complex relationship between addiction-related behaviors and microbiome is important in illuminating factors associated with addiction vulnerability. Overall microbiome composition, as measured by beta-diversity, was not associated to the goal/sign-tracking (GT/ST) phenotype. However, correlations revealed novel sex-dependent links between addiction-associated behavior and microbiome. In females, operational taxonomic units (OTUs) from bacteria *Barnesiella* repeatedly correlated with behavioral measures of impulsivity, measured by indifference point area under the curve in delay discounting task (DD.IDPtAUC) (Fig. 7-8). Moreover, alpha diversity was reduced in female behavioral phenotypes associated to increased addiction vulnerability (Fig. 5). Female PLS-DA cluster phenotype (F.Behav.Clust.1 and F.Behav.Clust.2, Fig. 3A) revealed that behavioral differences followed trends similar to the GT/STphenotype. Most behavioral measures in F.Behav.Clust.1 aligned with sign-trackers (STs) and F.Behav.Clust 2 similar to goal-tracking (GTs) phenotype (Fig. 3B). In males, fewer trends were seen in both microbiome and behavior. All groups had significant correlations between OTU-level bacteria, predominantly belonging to families Ruminococcaceae and Lachnospiraceae, and behavioral measures of impulsivity, attention, reward-learning, and locomotor response to novelty.

Sign-tracking (ST) is defined as the propensity to imbue a conditioned stimulus with incentive salience (Flagel, Watson et al. 2008, Meyer, Lovic et al. 2012). STs are more responsive to both food and drug CSs than GTs and tend to engage in addiction-related behaviors. Increased ST behavior is positively correlated to increased dopamine levels in the nucleus accumbens during amphetamine self-administration (Tomie, Grimes et al. 2008). Additionally, in selectively bred high/low response (HR/LR) rats, both male and females HR rats sign-track while LR counterparts goal-track. HR females have a greater propensity to self-administer cocaine than LR females and

HR & LR male rats (Davis, Clinton et al. 2008). In line with previous work (Pitchers, Flagel et al. 2015, King, Palmer et al. 2016), this study had more females assigned to the addictive-associated ST phenotype, compared to males.

There is contradictory data in humans with regard to gender differences in the incidence of addiction. However, research of both humans and other animal species indicates that females may, in some cases, display a greater propensity for addiction. A human genome-wide-association study (GWAS) showed females having greater impulsive behavior, as measured by delay discounting (Sanchez-Roige, Fontanillas et al. 2018). Furthermore, GWAS studies suggest that opioid dependence is linked to sex specific single nucleotide polymorphisms (Yang, Han et al. 2017). In Cloninger's typology, Type II alcoholism has an early onset, a genetic propensity, and more common in males, while Type I has a late onset and is seen in both females and males (Cloninger, Sigvardsson et al. 1996). Differences in hormones underlie some sex differences in addiction-related behavior. Furthermore, intensity of drug usage and drug withdrawal vary depending on menstrual cycle. In the striatum, dopamine levels are higher during estrus in rats, when estradiol is elevated. Estradiol is known to increase positive affect, locomotor sensitization, and acquisition of self-administration to psychomotor stimulants in female rats (Becker, Lopez et al. 2011).

Measures of impulsivity, attention, reward-learning, and locomotor response to novelty were significantly different by behavioral phenotypes and correlated to microbiome. In delay discounting, the indifference point/area under the curve measure (DD.IDPtAUC) has previously been linked to addiction in humans (Reynolds, Richards et al. 2004). This impulsivity measure correlated to OTUs assigned to family Lachnospiraceae in males and OTUs assigned to genus *Barnesiella* in females. The divergence in gut bacteria correlated to impulsivity indicates that sex-specific commensal bacteria may have similar effects on behavior. In males and females,

measures of Pavlovian Conditioned Approach (PavCA) were significantly correlated with bacterial OTUs in family Ruminococcaceae. In males, reaction time (RT) measures correlated to bacterial OTUs in family Ruminococcaceae. Intriguingly, female STs revealed strong correlations between reaction time attention measure (RT.Omissions) and OTUs in family Ruminococcaceae, including genus *Flavonifrator*, and family Lachnospiraceae, as well as two OTUs in genus *Vampirovibrio*. *Vampirovibrio,* which preys on algae, and is capable of replacing taxa in the microbiome via competition (Baer and Williams 2015, Soo, Woodcroft et al. 2015). Significant correlations were seen in light reinforcement (LR) and locomotor response (Loco) measures and bacterial OTUs in family Ruminococcaceae, including genus *Flavonifrator*, and Lachnospiraceae in the male GT/ST phenotype. In the present study, taxonomic differences between bacterial composition in GT/IN/ST phenotypes did not reach significance. However, the observed correlations repeatedly associated to Ruminococcaceae align with previous addiction research showing reductions in Ruminococcaceae in opioid users (Acharya, Betrapally et al. 2017), alcohol consumption (Bull-Otterson, Feng et al. 2013, Llopis, Cassard et al. 2016), and alcohol addiction severity (Leclercq, Matamoros et al. 2014). Research investigating alcohol consumption and microbiome has also previously reported changes in Lachnospiraceae in mice receiving oral and vapor alcohol (Bull-Otterson, Feng et al. 2013, Peterson, Jury et al. 2017) and alcohol addiction severity in humans (Leclercq, Matamoros et al. 2014). Our recent work on microbiome and addiction phenotype has illuminated intriguing trends in bacteria from families Ruminococcaceae and Lachnospiraceae correlating to dopamine receptor expression in the striatum and measures of anxiety, novelty induced locomotor activity, impulsivity, and compulsive alcohol seeking in male rats (Jadhav, Peterson et al. 2018).

Sex and/or gender are significant factors to consider when investigating the microbiome-gut-brain axis (Jasarevic, Morrison et al. 2016). Previous research reveals sex-associated microbiota differences are strongly linked to metabolic processes (Davey, O'Mahony et al. 2012, Markle, Frank et al. 2013, Amato, Leigh et al. 2014, Zhernakova, Kurilshikov et al. 2016) and to

influences on brain and behavior (Clarke, Grenham et al. 2013, Hoban, Stilling et al. 2016). Segregation of male and female rats by same sex co-housing may confound and/or inflate sex differences observed, especially in light of coprophagic behavior in rats - an environmental factor that may influence microbiota more than host genetics (Rothschild, Weissbrod et al. 2018). Although, in a study of howler monkeys microbiome continued to be distinct between sex despite shared environment and parent (Amato, Leigh et al. 2014). Clearly, further investigation is necessary to elucidate the connections between sex and microbiome composition as well as factors such as genotype (Palmer and de Wit 2012) and cagemates (Baud, Mulligan et al. 2017).

Females associated to a ST phenotype had decreased alpha diversity compared to GTs (Fig. 5). No difference was seen in alpha diversity between GT/IN/ST phenotypes in males (Fig. 4). Reduced alpha diversity is commonly associated to poor health, often the result of common factors, with reductions reported in mental health conditions including stress and depression (Bailey, Dowd et al. 2011, Kelly, Borre et al. 2016). Reduced alpha diversity has also been shown in chronic-intermittent vapor ethanol exposure (Peterson, Jury et al. 2017) and antibiotic depletion is linked to increased locomotor response to cocaine (Kiraly, Walker et al. 2016). Alterations in Ruminococcaceae and Lachnospiraceae have been characterized in many health conditions. Importantly, reduced intestinal permeability, liver damage, and inflammation (Bailey, Dowd et al. 2011, Bajaj, Hylemon et al. 2012, Bull-Otterson, Feng et al. 2013, Leclercq, De Saeger et al. 2014, Llopis, Cassard et al. 2016, Acharya, Betrapally et al. 2017, Leclercq, de Timary et al. 2017, Petrov, Saltykova et al. 2017) have been implicated to alterations in these two family-level bacteria in conditions of psychological stress (Bangsgaard Bendtsen, Krych et al. 2012, Dunphy-Doherty, O'Mahony et al. 2018), depression (Naseribafrouei, Hestad et al. 2014, Jiang, Ling et al. 2015, Chen, Zheng et al. 2018), autism (Hsiao, McBride et al. 2013, Golubeva, Joyce et al. 2017), and dopaminergic-mediated disorders (Leclercq, Matamoros et al. 2014, Petrov, Saltykova et

al. 2017). Many novel bacteria were identified in this study in relation to addictive phenotype and microbiome. OTUs associated to genus *Flavonifractor* were correlated to locomotor activity in males and RT omissions in females. Increased abundance of *Flavonifractor* is linked to major depressive disorder (Jiang, Ling et al. 2015) and bipolar disease (Coello, Hansen et al. 2018); the mechanism of how *Flavonifractor* effects brain is not well known, though it is believed to cause oxidative stress and inflammation. Furthermore, OTUs associated to *Barnesiella* were repeatedly correlated to behaviors in females, particularly impulsivity measures. In a human study comparing microbiota composition of alcohol drinkers and non-drinkers, a top differentiated OTU increased with alcohol consumption belonged to an uncharacterized Barnesiella (Kosnicki, Penprase et al. 2018). Genus *Barnesiella* has also previously been characterized as preventing colonization of infection bacteria (Ubeda, Bucci et al. 2013, Steinway, Biggs et al. 2015), thus reducing inflammatory profile in the gastrointestinal tract. In this study, we see two novel taxa which have been characterized as replacing taxa in the microbiome, *Barnesiella* and *Vampirovibrio.* These novel taxa were also related to measures of attention and impulsivity. Previous research in humans reported a negative relationship between *Clostridium XIVa* and depression in females (Chen, Zheng et al. 2018). Further investigation is necessary to determine if these identified bacteria preferentially colonize certain sexes, the mechanism for the preference, in addition to how these certain bacteria are influencing brain and behavior. Furthermore, the impact of immune function must be investigated in these behavioral phenotypes associated with specific bacteria (Rea, Dinan et al. 2016). Research has shown that gut bacteria impact neuroimmunology (Braniste, Al-Asmakh et al. 2014, Scott, Ida et al. 2017), and neuroimmunology has been linked to addiction (Hofford, Russo et al. 2018). Future studies should also examine how the neurobiological substrates of the behavioral changes observed are regulated by the microbiome. Indeed, the gut microbiome has been shown to regulate cortical morphology and neurotransmitter expression, notably in the prefrontal cortex which is involved in impulsivity behaviors (Hoban, Stilling et al. 2016, Luczynski, Whelan et al. 2016, Hoban, Stilling et al. 2017). Moreover, investigations into

the role of the vagus nerve as a conduit of signals from the gut to the brain is also warranted (Bravo et al., 2011).

4.6 Conclusion

This is the first time, to our knowledge, that extensive characterization of within sex addiction-phenotype and behavioral measures have been associated to microbiome. Overall microbiome composition was not capable of predicting addiction phenotype. The most robust findings in this study indicate that microbiome is associated to locomotor response, reward-stimulus learning, impulsivity and attention. Notably, impulsivity measures were repeatedly correlated to certain bacteria in males and females. This novel work impresses facts that sex as a factor must be considered in both behavior and microbiome research. Further investigation is necessary to elucidate factors that contribute to sex differences in microbiome, and how these differences influence other addiction-related measures like drug-self administration and relapse.

4.7 Supplementary documentation

4.7.1 4.7.1 Preliminary results of Sex Differences (Male vs Female)

4.7.1.1 Behavioral cluster analysis

To assess the ability for the goal/sign-traking phenotype to predict differences in other behavioural measures associated to addiction, an unsupervised ordination plot was generated. A bray-curtis dissimilarity matrix was created from a total of 48 behavioral measures, with the 6 behavioral measures related to PavCA removed. Points were colored and shaped by Behav.Cluster and Tracker group by sex, respectively. Results indicated that differences in non-PavCA behavioural measures separated by this grouping designation on the x-axis (Fig. S1), while females and males separated on the y-axis (Fig. S1A).

Partial least squares differential analysis (PLS-DA) of all 54 behavioral measures constrained by sex and goal/sign tracking (GT/ST) phenotype resulted in a separation between males and females (Fig. S2A). 81% of males are present in the left cluster (L.Behav.Clust = Behav.Clust.1), while only 26% of females are present in the L.Behav.Clust. In the right behavioral phenotype cluster (R.Behav.Clust = Behav.Clust.2), 74% of females are present while only 19% of males are present. Interestingly, the majority of female and male sign-trackers (ST) congregated in the lower right quadrant of this cluster.

4.7.1.2 Behavioral differences between sex and behavioral cluster

Factor analysis (PERMANOVA) of 54 behavioral measures revealed that sex was the only significant contributing factor (R2=0.301, p=0.0002). To ensure this observation was not due to sex differences in weight, factor analysis was also performed for animal weights at experiment endpoint. Weight was not a significant factor contributing to behavior (R2=0.006, p=0.173). Additionally,

sign/goal tracker phenotype was not a significant factor for all behavioral measures (R2=0.007, p=0.371). Of 54 behaviors tested, 32 were significantly different when compared by sex, 34 between left and right behavioral cluster, 17 when females only where compared by behavioral cluster, and 20 when males only were compared by behavioral cluster. Z-score was used to represent behavioral differences by sex, behavioral cluster (R.Behav.Clust and L.Behav.Clust), and behavioral cluster by sex (Fig S2B).

4.7.1.3 Sex differences in Pavlovian Conditioned Approach (PavCA)

Females had significantly increased sign-tracking behavior compared to males (Fig. S2A-B). This was evident by significant increases in Pavlovian Index score on day 4 (PavCA.D4.Index, $p<0.0001$) and day 5 (PavCA.D5.Index, $p<0.01$) in females compared to males. Pavlovian Index score was also significant between behavior cluster (PavCA.D4.Index and PavCA.D5.Index, $p<0.001$) and when males and females were compared individually by behavioral cluster (PavCA.D4.Index and PavCA.D5.Index, $p<0.001$). Sign-tracking behavior, as measured by lever-cue associations, was significantly increased in females, R.Behav.Clust, and R.Behav,Clust.F on day 1, 4, and 5 (PavCA.D1.Lever_CS, $p<0.001$; PavCA.D4.Lever_CS, $p<0.001$; PavCA.D5.Lever_CS, $p<0.001$). Male right behavioral cluster (R.Behav.Clust) was also significantly increased on day 4 and 5 (PavCA.D4.Lever_CS, $p<0.001$; PavCA.D5.Lever_CS, $p<0.001$) compared to left behavioral male cluster (L.Behav.Clust). These finding show that sign-tracking behavior is increased in all females compared to males, as well as right and left behavioral cluster, either compared with all samples or within sex.

4.7.1.4 Sex differences in Pavlovian Conditioned Reinforcement (CRF)

Females had significant increases in reinforcement behavior compared to males (Fig. S2B). Number of CS lever presentations was significantly different between sex, behavioral cluster, and behavioral cluster by sex (CRF.Number.Reinforcers, $p<0.001$), and total number of lever presses

(CRF.LeverPresses, p<0.001). These measures were significantly increased in females vs. males. L.Behav.Clust vs. R.Behav.Clust, and left behavioral clusters within both sexes (Fig. S2B).

4.7.1.5 Sex differences in Cocaine Cue Preference (CCP)

Females had an overall significant increase in locomotor distance travelled compared to males following cocaine administration at both trial 1 and 4 (CCP.T1.Cocaine.Dist and CCP.T4.Cocaine.Dist, p< 0.001) (Fig. S2B). Significant differences in cocaine-induces locomotor travel was also observed between behavioral clusters, however when behavioral clusters were compared within sex, only significant differences were seen within females at trial 1 (CCP.T1.Cocaine.Dist, p< 0.01). Behavioral cluster comparisons in all samples (L.Behav.Clust vs. R.Behav.Clust) showed significant differences in locomotor behavior between cocaine administration and saline (CCP.dtLoco, p<0.01).

There were no significant sex differences in the primary measures of change in time (s) in cocaine-paired (CS+) floor between the post-test and pre-test (CCP.dtCS, p>0.05).

4.7.1.6 Sex differences in Choice Reaction Time Task (RT)

Reaction time (RT) measures of impulsivity total number of per opportunity false alarms was not significant (RT.POFA, p>0.05). A significant increase in total number of per opportunity premature initiations (RT.PerOPInit, p<0.001) was observed in females compared to males and left behavioral cluster compared to right (Fig. S2B).

4.7.1.7 Sex differences in Locomotor Response to Novelty (Loco)

Similar to measures from CCP, females had significant increases in distance travelled (Loco.Distance, p<0.001). This was also seen in comparisons between behavioral cluster and within the male behavioral cluster (Loco.Distance, p<0.05). Additionally, there was significant increases in

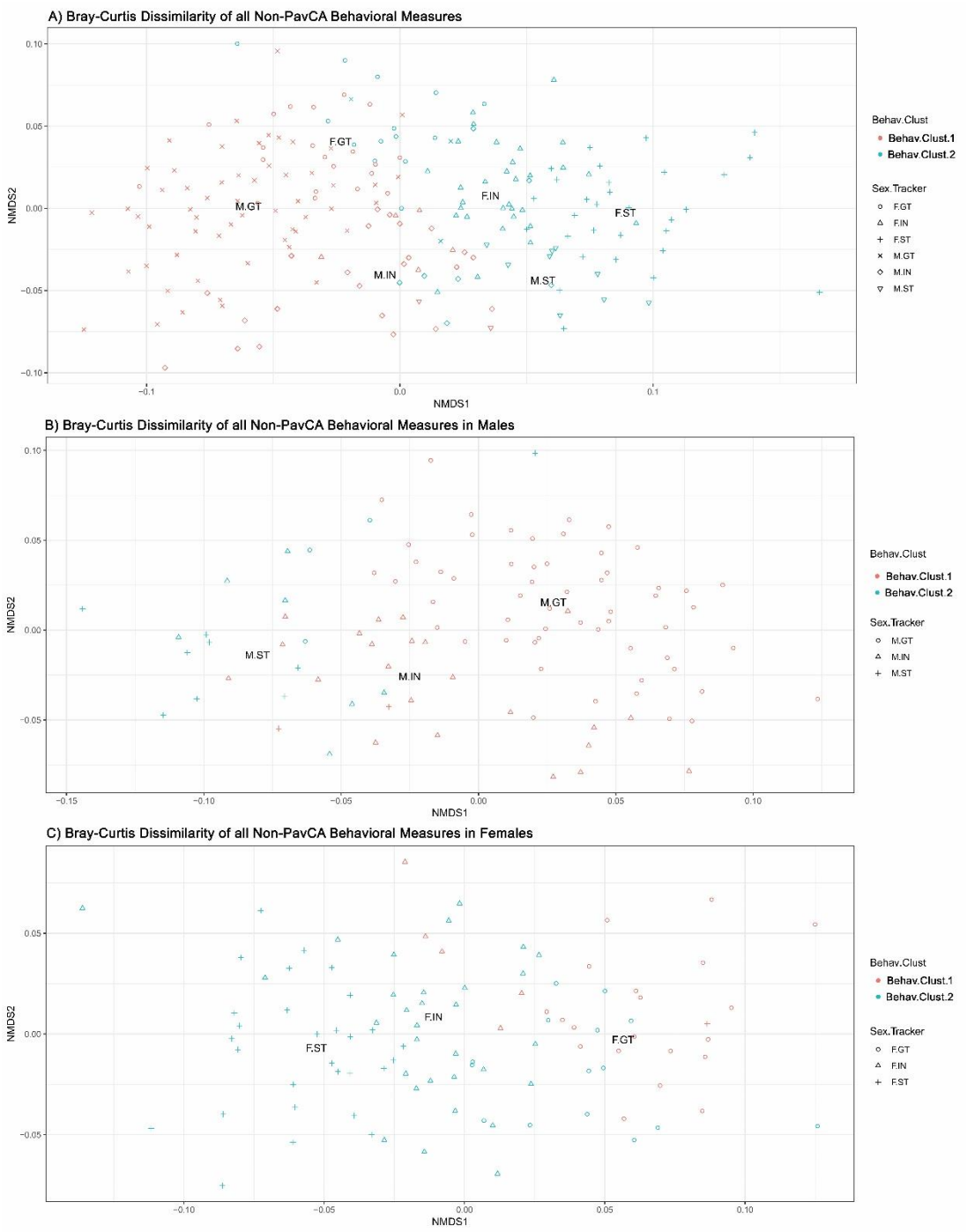
maximum exploratory rearing measures in females, R.Behav.Clust, R.Behav.Clust.F and R.Behav.Clust.M (Loco.MaxRear, $p<0.01$). Significant decreases in total locomotor activity (Loco.Activity, $p<0.001$) in females compared to males.

4.7.1.8 Sex differences in Light Reinforcement (LR)

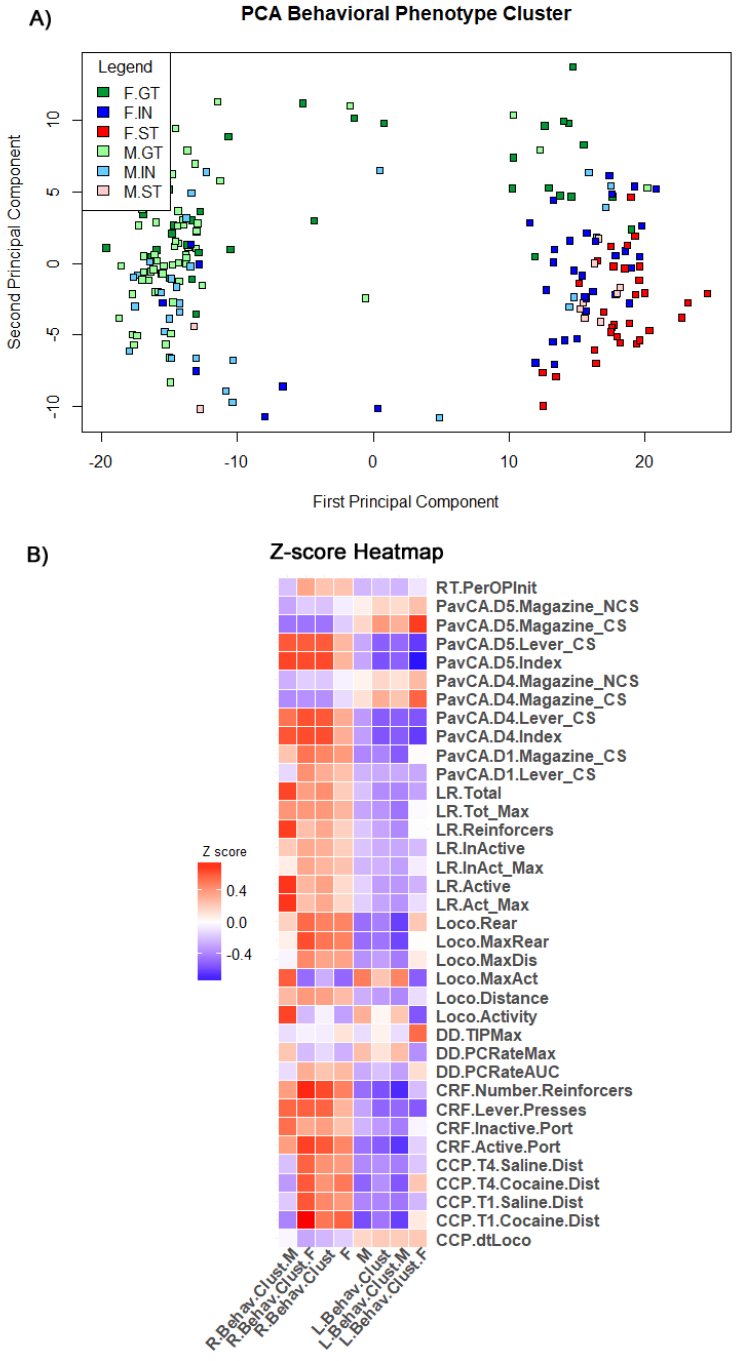
Total Light Reinforcement LR.Reinforcers, $p<0.01$) and total inactive responses (LR.InActive,$p<0.05$) were significantly different by sex, behavioral cluster, and within the male behavioral cluster. Significant increases in measures total active and inactive responses in females, R.Behav.Clust, R.Behav.Clust.F and R.Behav.Clust.M (LR.Total , $p<0.01$) were observed (Fig. S2B).

4.7.1.9 Sex differences in Delay Discounting (DD)

Females had significant increases in impulsivity measure of patch change rate area under the curve (DD.PCRateAUC, $p<0.01$). This was also seen in comparisons between behavioral cluster ($p<0.05$). Primary measures of indifference point area under the curve was not significantly (DD.IDPtAUC, $p>0.05$).



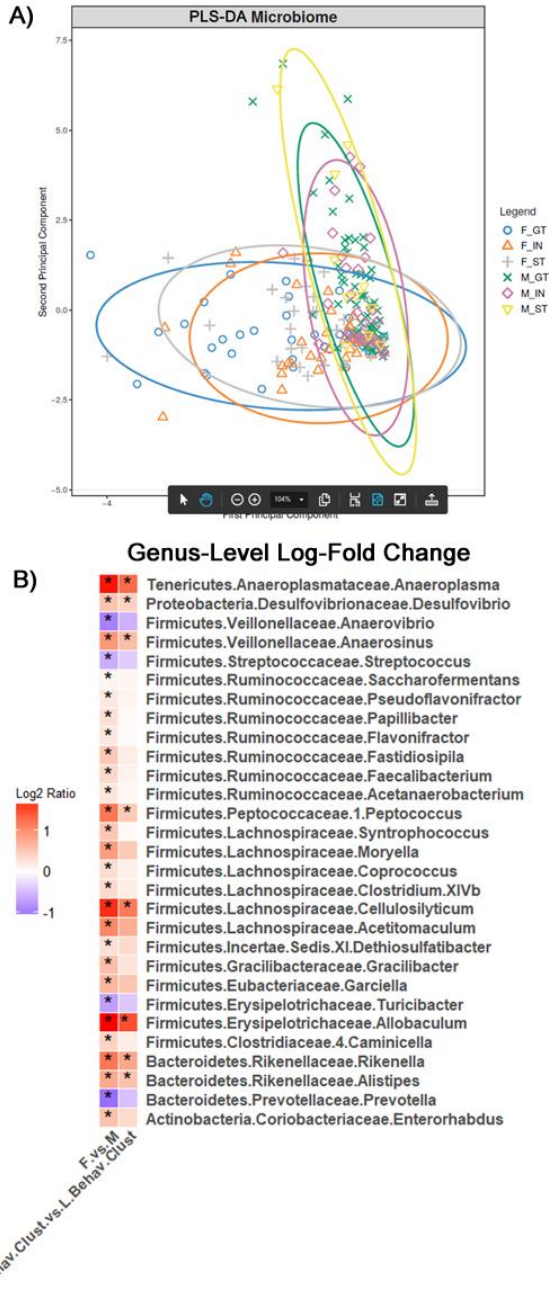
Supplementary Figure 1 – Behavioral dissimilarity in Non-PavCA Measures - *A) in all samples, B) in male subjects, C) in female subject. Shapes on plot indicate tracker and sex designation, red color indicated Behav.Clust.1 (L.Behav.Clust) and blue color Behav.Clust.1 (R.Behav.Clust).*



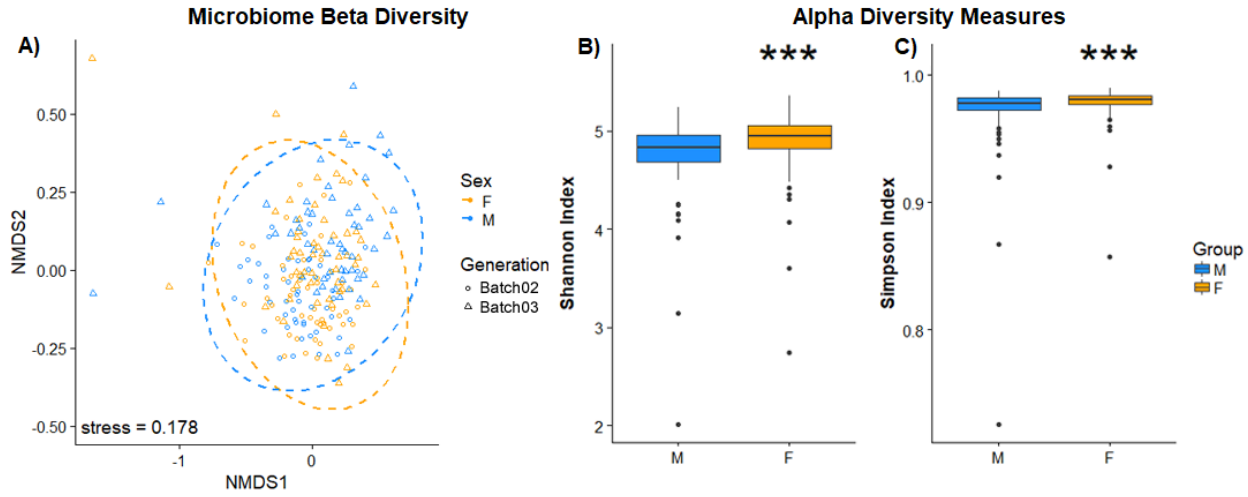
Supplementary Figure 2– Behavioral differences *– A) Behavioral Phenotype Cluster visualized in principal coordinate analysis (PCA) of PLS-DA clustered behavioral measures. Goal-trackers (GT) colored green, intermediate (IN) blue, sign-trackers (ST) red; females in dark colors, and males light colors. B) Z score indicate increases (red) or decreases (blue) in behavioral measures compared to population.*

4.7.1.10 Microbiome diversity

Microbiome beta diversity was significantly different by sex (R2=0.016, p=0.002) and generation (R2=0.032, p=0.0002) (Fig. S3A). Age was also a significant factor contributing to microbiome variance (R2=0.017, p=0.001). No significant differences were found for weight (R2=0.005, p=0.312) or sign/goal-tracker phenotype (R2=0.011, p=0.285). OTU assignments of sign/goal-tracker phenotype only clustered by sex in PLS-DA (Fig 3A). Alpha diversity analysis by sex revealed significant differences in Shannon (p=0.0001) and Simpson (p=0.001) index measures (Fig. S3B). There were no significant differences in alpha diversity measures between behavioral cluster within sex (p>0.05).



Supplementary Figure 3 – Genus Differences in Microbiome by Sex and Behavioral Phenotype *– A) PCA beta diversity plot of PLS-DA clustered microbiome by sign/goal-tracker phenotype and sex (blue circle = female goal-tracker (F_GT), orange triangle = female intermediate (F_IN), grey plus = female sign-tracker (F_ST), green x = male goal-tracker (M_GT),purple diamond = male intermediate (M_IN), yellow triangle = male sign-tracker (M_ST). B) Log-fold change differences at genus level between females vs males and behavioral phenotype clusters (R.Behav.Clust vs. L.Behav.Clust); asterisks indicates significance after FDR corrections (p>0.05)*



Supplementary Figure 4 – Microbiome Diversity *– A) NMDS plot of beta diversity by sex (Female = yellow, Male = blue) and by generation (circles = Batch02, and triangles = Batch03); stress value in lower left corner indicates how well the data is representation in reduced the dimensions. B & C) Alpha diversity of B) Shannon and C) Simpson index measures between males (blue) and females (yellow); asterisks indicate significance p>0.001.*

4.7.1.11 Taxonomic differences between sex

At the phylum level, Tenericutes was significantly greater in females compared to males and ($p<0.001$) (Fig. S3B). Among females and R.Behav.Clust, significantly greater proportions of Peptococcaceae ($p<0.001$) and the corresponding genus *Peptococcus* ($p<0.001$), Rikenellaceae ($p<0.001$) and the genera *Alistipes* ($p=0.01$) and *Rikenella* ($p<0.001$), Desulfovibrionaceae ($p<0.01$) and the genus *Desulfovibrio* ($p<0.01$), and in family Erysipelotrichaceae ($p>0.05$) genus *Allobaculum* ($p<0.01$). In females compared to males, greater abundance of *Gracilibacteraceae* ($p=1.24e-4$) and the genus *Gracilibacter* ($p=1.24e-4$), as well as Enterobacteriaceae ($p=0.002$). Significantly lower *Streptococcaceae* ($p=0.002$) and the genus *Streptococcus* ($p=0.004$) were observed (Fig. S3B). Other significant differences were found at the genus level, including several genera from the families *Lachnospiracea*, *Ruminococcaceae*, *Veillonellaceae*, and *Anaeroplasmataceae* (Fig. S3B).

4.7.1.12 Microbiome correlations to Behavior

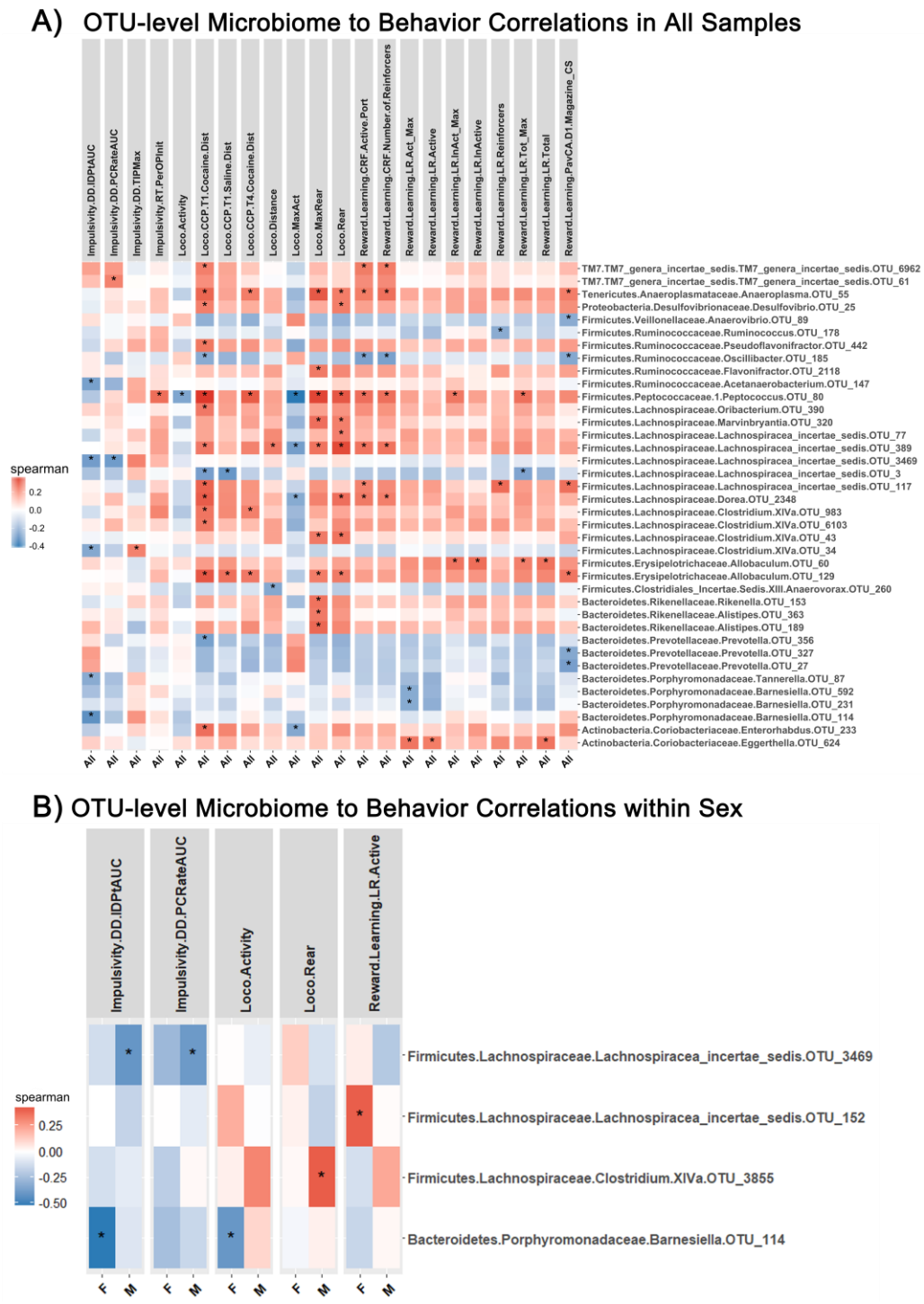
Spearman correlations were performed on all 54 behavioral measures and OTU-level bacterial abundance filtered at >0.01% median abundance, presence greater >50% all samples. For all samples qvalue=0.05, within sex qvalue=0.15.

4.7.1.12.1 Correlations to Behavioral Measures in All Samples

Spearman correlations were performed between behaviors and OTU-level bacteria for all samples (N=202) (Fig. S4A). In all samples, significantly correlations were positively correlated to *Anaeroplasma* OTU_55*, Peptococcus* OTU_80*, Allobaculum* OTU_60 and OTU_129, and *Lachnospiraceae_incertae_sedis* OTU_117, *Allobaculum* OTU_129 were positively correlated to goal tracking behavior on day 1 (Reward.Learning.PavCA.D1.Magazine_CS, rho>0.262, p<0.001); *Anaerovirbrio* OTU_89, *Oscillibacter* OTU_185, and *Prevotella* OTU_327 and OTU_27 were negatively correlated to in goal tracking behavior on day 1 (Reward.Learning.PavCA.D1.Magazine_CS, rho<-0.254, p<0.001). Distance travelled following cocaine administration during trial 1 and 4 was positively correlated to *Allobaculum* OTU_129, *Anaeroplasma* OTU_55, *Peptococcus* OTU_80, *Clostridium XIVa* OTU_983, among others (Loco.CCP.T[1or4]. CocaineDist, rho>0.2.55, p<0.001). Total number of presentations of lever (Reward.Learning.CRF.Number.of.Reinforcers, rho>0.280, p<0.001), and maximum exploratory rears per epoch (Loco.MaxRear, rho>0.282, p<0.0001) were positively correlated to *Anaeroplasma* OTU_55, *Peptococcus* OTU_80*,* and *Lachnospiracea_incertae_sedis* OTU_389. Significant negative correlations to impulsivity measures of indifference point area under the curve and patch change rate area under the curve (Impulsivity.DD.IDPtAUC and Impulsivity.DD.PCRateAUC, rho<-0.303, p<0.001) was observed with *Lachnospiracea_incertae_sedis* OTU_3469. Correlations to locomotor activity (Loco.Activity) and other locomotor response to novelty measures were significantly (p<0.05) associated to *Peptococcus* OTU_80, *Lachnospiracaea_incertae_sedis* OTU_389, and *Allobaculum* OTU_129.

4.7.1.12.2 Correlations to Behavioral Measures within Sex

Spearman correlations were performed between behaviors and OTU-level bacteria within sex (N=101, Males and Females) (Fig. S5B). Impulsivity measures of indifference point area under the curve and patch change rate area under the curve was significantly correlated to *Lachnospiracaea_incertae_sedis* OTU_3469 (Impulsivity.DD.IDPtAUC and Impulsivity.DD.PCRateAUC, rho<-0.393, p<0.001) in males. In females, indifference point area under the curve and locomotor activity were negatively correlated to *Barnesiella* OTU_114 (Impulsivity.DD.IDPtAUC and Loco.Activity, rho<-0.407, p<0.001). In males, exploratory rearing was positively correlated to *Clostridium* XlVa OTU_3855; additionally, males had many OTU correlations to sensory preference for CPP floor before testing A positive correlation between reward learning and *Lachnospiracea incertae sedis* OTU_152 was seen in females (Reward.Learning.LR.Active, rho=0.407, p=2.44e-05) (Fig. S5B).



Supplementary Figure 5 – Correlation Analysis. *A) Correlations between OUT-level bacteria and behavior in all subjects. Significance that passes FDR indicates by asterisk (q<0.05) B) Correlation between OTU-level bacteria within sex and behavior. Significance that passes FDR indicates by asterisk (q<0.15). Positive correlations indicated in red, negative correlations indicated in blue.*

4.7.1.12.3 Correlations to Behavioral Phenotype Cluster X-axis and OTUs

Behavioral Phenotype Cluster (Fig. S2A) separated into two clusters along the X-axis. These X-axis values were correlated to OTU abundance. For all samples qvalue=0.05, within sex qvalue=0.15.

4.7.1.12.4 Correlations to Behavioral Phenotype Cluster X-axis and OTUs in All samples

In all samples, X-axis behavioral phenotype cluster values positively correlated to *Peptococcus* OTU_80, *Anaeroplasma* OTU_55, *Allobaculum* OTU_33 and OTU_129, *Lachnospiracaeae_incertae_sedis* OTU_389, *Dorea* OTU_2348, and *Acetivibrio* OTU_586 (rho>0.241, p<0.001) (Table S1). X-axis phenotype cluster was negatively correlated to *Anaerostipes* OTU_5419, *Tannerella* OTU_1263, *Barnesiella* OTU_871 and OTU_726, and *Lachnospiracea incertae sedis* OTU_3 (rho<-0.240, p<0.001) (Table S1).

4.7.1.12.5 Correlations to Behavioral Phenotype Cluster X-axis and OTUs within Sex

In males, X-axis behavioral phenotype cluster values positively correlated to alpha diversity measures (Observed and Chao1, rho=0.236, p<0.5). In females, X-axis values behavioral phenotype cluster (Fig. S2A) negatively correlated to Shannon index alpha diversity measure and *Barnesiella* OTU_253 (rho<-0.231, p<0.05).

OTU	rho	Pvalue
Firmicutes.Peptococcaceae 1.Peptococcus.OTU_80	0.34596	4.58E-07
Tenericutes.Anaeroplasmataceae.Anaeroplasma.OTU_55	0.324795	2.40E-06
Firmicutes.Erysipelotrichaceae.Allobaculum.OTU_33	0.308244	8.08E-06
Firmicutes.Lachnospiraceae.Lachnospiracea_incertae_sedis.OTU_389	0.276757	6.69E-05
Firmicutes.Erysipelotrichaceae.Allobaculum.OTU_129	0.265397	0.000135
Firmicutes.Lachnospiraceae.Anaerostipes.OTU_5419	-0.25981	0.000188
Bacteroidetes.Porphyromonadaceae.Tannerella.OTU_1263	-0.25524	0.000246
Bacteroidetes.Porphyromonadaceae.Barnesiella.OTU_871	-0.2534	0.000274
Firmicutes.Lachnospiraceae.Dorea.OTU_2348	0.247933	0.000374
Firmicutes.Lachnospiraceae.Lachnospiracea_incertae_sedis.OTU_3	-0.24325	0.000486
Firmicutes.Ruminococcaceae.Acetivibrio.OTU_586	0.241062	0.000548
Bacteroidetes.Porphyromonadaceae.Barnesiella.OTU_726	-0.24049	0.000566

Supplementary Table 1: OTU Correlations to Behavioral Cluster x-axis in all samples *– OTU correlations to x-axis correlations in behavioral cluster that pass FDR corrections (Q<0.05). OUT (first column), rho value (Spearman, second column), Pvalue (raw, third column)*

5 Chapter:

Stress Gone Viral: Chronic social stress induces marked changes in the gut virome in mice

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5.1 Abstract

Current research indicates that the microbiome-gut-brain axis (MGBA) plays a role in stress response. The number of viruses in the gut, notably bacteriophage, vastly outnumber bacteria in the microbiome. Changes in gut bacteria have previously been seen during chronic stress and hypothalamic-pituitary-adrenal (HPA) axis dysfunction. The present study sought to investigate if viruses is the gut microbiome were altered during psychological stress, alongside changes in gut bacteria. Stressed animals were exposed to 3 weeks of chronic social stress. Control and Stress samples (N =20) were sequenced for bacteria (16S) and viruses (metagenomic). Stressed animals had significant increases in depression measures, basal corticosterone, and cytokine IL-6 compared to controls. Bioinformatic analysis revealed marked differences in bacteriome and virome between control and stressed animals. Viral species richness increased in the stress group alongside reductions in bacterial richness. Increased trends of viral and bacterial evenness alpha diversity measures, Shannon and Simpson, were observed following chronic social stress. Significant correlations were observed between viral sequences and Bacteroidetes bacteria belonging to families S24-7 and Rikenellaceae. This is the first study, to our knowledge, to investigate changes in virome in relation to MGBA. Findings from this research further elucidates the impact of psychological stress on the microbiome and suggests that viruses may play a role in HPA axis function.

5.2 Introduction

Every gut contains billions of organisms that contribute to digestion, metabolism, immune function, and even the stress response (Bastiannsen et al 2018). Research into the role of microbiota in health focuses almost exclusively on bacteria yet the number of commensal viruses, most notably bacteriophage (viruses that target bacteria), outnumber bacteria in the gut. Indeed, we are slowly appreciating the significant role that gut bacteriophage

play a role in host immunity, intestinal permeability, and maintaining a healthy microbiome (Dalmasso, Hill et al. 2014, De Paepe, Leclerc et al. 2014). Alterations in bacteriophage have been associated with disease states such as Crohn's disease (Perez-Brocal, Garcia-Lopez et al. 2015), immunodeficiency syndrome (Monaco, Gootenberg et al. 2016, Monaco and Kwon 2017), and *Clostridium difficile* infection (Broecker, Klumpp et al. 2016). Bacteriophage contribute to the stability and dynamics of bacterial populations and may represent a targeted approach to influencing specific populations of bacteria in the gut (Forde and Hill 2018).

A growing body of evidence suggests that the microbiome-gut-brain axis plays a critical role in mood and behavior. Changes in gut bacteria composition are seen during chronic stress, hypothalamic-pituitary-adrenal (HPA) axis dysfunction, and psychiatric disorders such as anxiety and depression (Dinan and Cryan 2012). Previous research has shown that chronic social stress alters microbiome composition (Bailey, Dowd et al. 2011, Bharwani, Mian et al. 2016, Burokas, Arboleya et al. 2017) Furthermore, intervention of probiotic bacteria can produce anxiolytic activity in rodent models (Bravo, Forsythe et al. 2011). Further research is necessary to understand the bi-directional communication between the microbiota-gut-brain axis and how these alterations may, potentially, affect stress response.

To date, there are no published studies investigating the role of bacteriophage composition in association withbrain and behavior. This study sought to investigate changes in viral and bacterial gut composition following chronic social stress.

5.3 Methods

5.3.1 Animals

Male C57BL/6J mice (n=20; Harlan, Cambridgeshire, UK; 8 weeks of age on arrival) were housed in SPF, temperature and humidity-controlled environment

on a 12 hour light/dark cycle. All procedures were performed during the light phase. Experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork.

5.3.2 Chronic Social Stress

Weight-matched animals were randomly assigned to either Stress or Control group. Stress group (N=10) were singly housed and exposed to 3 consecutive weeks of chronic unpredictable social stress and overcrowding, as outlined in Figure 1. Control group (N=10) were group housed (3-4 per cage) and left undisturbed. Procedure for chronic social stress was carried out as previously described (Finger, Dinan et al. 2011, Burokas, Arboleya et al. 2017). Briefly, social defeat was performed by placing test mouse into the homecage of a larger CD1 mouse strain. Once submissive posturing (exposing stomach) was displayed animals were separated by a plexiglass divider for 2 hours. Following the 2-hour period, the divider was removed and following a second defeat the test animal was returned to its homecage. For overcrowding procedure, all ten mice in the stress group were placed in a single cage for 24 hours.

5.3.3 Social interaction test

Following chronic social stress, mice underwent social interaction test to validate stress protocol. This procedure was carried out as previously described (Finger, Dinan et al. 2011, Burokas, Arboleya et al. 2017). In short, test subjects were placed in an open container with a novel CD1 mouse under a metal, mesh cage. Interaction ratio was calculated by the total time spent near the CD1 mouse divided by total time spent by the mesh cage with no CD1 mouse.

5.3.4 Forced Swim test

To assess despair behavior, Forced Swim Test (FST) was used to measure immobility time. This procedure was carried out as previously described (Finger, Dinan et al. 2011, Burokas, Arboleya et al. 2017). In short, animals were placed into a water filled (15-cm depth) clear glass cylinder (24 × 21 cm diameter) for 6 minutes. Water was changed in between individual trials and each trial was video-recorded for later analysis. Immobility time (s) was assessed for the last 4 minutes of the test, with immobility being defined as a total absence of movement except slight motions to maintain the head above the water.

5.3.5 Splenocyte stimulation and Cytokine analysis

Spleens were collected immediately following sacrifice and cultured by homogenizing in media [RPMI (with l-glutamine and sodium bicarbonate, R8758 Sigma) + 10% FBS (F7524, Sigma) + 1% Penicillin/Streptomycin (P4333, Sigma)]. The homogenate was then filtered over a 70um strainer and centrifuged at 200 g for 5 min. Red blood cells were lysed with buffer (R7757, Sigma), cell culture was then centrifuged and resuspended in media. Cells were counted and seeded (4,000,000/mL media). After 2.5 h of adaptation, cells were stimulated with lipopolysaccharide (LPS-2 µg/ml), concanavalin A (ConA-2.5 µg/ml) or vehicle (RPMI media) for 24 h. Following stimulation, the supernatants were harvested to assess the cytokine release using Proinflammatory Panel 1 (mouse) V-PLEX Kit (Meso Scale Discovery, Maryland, USA) for TNFα, IL-10, IL-1β and IL-6. The analyses were performed using MESO QuickPlex SQ 120, SECTOR Imager 2400, SECTOR Imager 6000, SECTOR S 600.

5.3.6 Corticosterone analysis

Tail blood samples were taken one-week prior culling and truck blood collected 45 minutes following forced swim test at the time of cull. Serum corticosterone levels were assayed in duplicate using a Corticosterone Immunoassay Kit

according to the manufacturer's instructions (Enzo Life Sciences, UK) in a single assay using 20 µL plasma per sample with the threshold detection > 32 pg/mL (coefficient of variation limit=20%). Light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm. Concentrations are expressed in ng/mL.

5.3.7 Feacal bacteriome collection and sequencing

The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA using the manufacturer's handbook (Second Edition 2012). Samples were prepared for 16S sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), as described in the Illumina 16S library preparation workflow. 16S bacterial rRNA gene was amplified using primers targeting the V3-V4 hypervariable region (Forward: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; Reverse: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Sigma Aldrich Ireland ltd., Wicklow, Ireland). 16S rRNA amplicons were sequenced on the Illumina MiSeq platform (Teagasc, Morrepark, Ireland).

5.3.8 Feacal virome collection and sequencing

Faecal processed using a previously described protocol (Reyes, Wu et al. 2013, Shkoporov, Ryan et al. 2018). Briefly, samples from control and stress groups were collected before (T0) and after (T1) experiment , virome faecal samples were pooled by cage and timepoint (N=3-4 per pool) to create a 1-2g faecal samples. Pooled samples at each timepoint were resuspended in 10 ml saline magnesium buffer, vortexed for 5 minutes, cooled for 5 minutes on ice, and centrifuged twice at 4,7000 rpm for 10 min at 4^{o}C to remove large particles and bacterial cells. Supernatant was filtered twice through a 0.45 µm pore ceramic filter. Following further refinement and treatment with proteinase K and Phage Lysis Buffer, sample DNA was extracted using the

phenol-chloroform method. After DNA extraction, samples were processed for shot-gun metagenomic sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). Virome metagenomic sequencing was performed on the Illumina MiSeq platform (Teagasc, Morrepark, Ireland).

5.3.9 Bacteriome and Virome Sequence Pipeline

5.3.9.1 Bacteriome Sequence Pipeline

Sequence reads in FASTQ files were filtered using PRINSEQ (Schmieder and Edwards 2011). Sequences with length less than 150 nucleotides or with low quality at the 3' end were removed. Paired-end reads with a minimum overlap of 20 base-pairs were joined using FASTQ-join and analyzed with QIIME (Quantitative Insights Into Microbial Ecology, v1.9.1). Sequence quality was checked, filtered the dereplicates and chimeras removed against gold database, remaining sequences were clustered into Operational Taxonomic Units (OTUs; 97% identity level) using Usearch v7.0 algorithm (Edgar 2010). The average number of high-quality sequences generated per sample was 150,707 ± 69,666 SD. Taxonomy was assigned to OTUs using Ribosomal Database Project (RDP) (Cole, Wang et al. 2014). Alpha and Beta diversity indices were calculated with vegan (v2.5-1).

5.3.9.2 Virome Sequence Pipeline

Shot-gun metagenomic viral sequences were processed using a previously published pipeline (Draper, Ryan et al. 2018, Guerin, Shkoporov et al. 2018, Shkoporov, Ryan et al. 2018). Briefly, sequences underwent quality filtering and trimming using Trimmomatic v0.36. High-quality sequences were then assembled into contigs using metaSPAdes v3.10.1. Contigs were then filtered to only include sequences from viruses using Virsorter.

5.3.10 Statistical Analysis

Data was analyzed in R (v3.5.2) and RStudio (v1.1.463). Plots were generated in R using ggplot2 package (v2.2.1). Beta Diversity between groups (Control and Stress) and factor (NoStress and Stress) was calculated using Adonis (PERMANOVA) vegan function. Sample clustering was visualized with a Non-Metric Multidimentional Scaling (NMDS) plot. Wilcoxson test was used in the analysis of alpha-diversity index comparisons by factor. Spearman correlations were performed on bacteriome and virome count tables filtered to >0.01% relative abundance. In the virome count table, viral contigs were also filtered so that only contigs present in at least 3 were retained. FDR was performed to correct for multiple comparisons using the qvalue package (v2.14.1) with a threshold $q<0.05$ reported as significant.

5.4 Results

5.4.1 Behaviour Measures

5.4.1.1 Social interaction test (SIT)

Social interaction behavioral test for novel CD1 mouse, as measured by percentage of time spend in interaction zone with CD1 divided by time spend in interaction zone without CD1. A significant reduction was observed in Stress group ($t(18)=3.317$, $p=0.004$) (Figure 1C).

5.4.1.2 Forced Swim test (FST*)*

Forced Swim test behavioral measure of despair behavior, measured by immobility time. A significant increase in immobility time was observed in Stress group ($t(18)=-4.946$, $p=0.0001$) (Figure 1A).

5.4.2 Splenocyte stimulation and Cytokine analysis

Inflammatory cytokine measures revealed significant increases in IL-6 Concanavalin A (ConA) and IL-10 in Stress group compared to Control ($t((18)=-2.316$, $p=0.032$;$t(18)=-2.257$, $p=0.037$, respectively) (Figure 1F-G). No significant differences were seen in ConA stimulated splenocytes between Stress and Control group in TNF-alpha ($t(18)=-1.036$, $p=0.314$) and IL-1beta ($t(18)=-1.885$, $p=0.076$). Vehicle stimulated splenocytes (baseline) showed no significant differences in Stress vs. Control groups in TNF-alpha ($U=42$, $p=0.579$), IL-1beta ($t(18)=-0.597$, $p=0.155$), IL-6 ($t(18)=-1.800$, $p=0.089$) (Figure 1D), and IL-10 ($t(18)=-0.916$, $p=0.372$) (Figure 1E). Lipopolysaccharide (LPS) stimulated splenocytes between groups showed no significance difference: TNF-alpha ($U=34$, $p=0.248$), IL-1beta ($t(18)=-1.256$, $p=0.225$), IL-6 ($t(18)=-1.752$, $p=0.097$), IL-10 ($t(18)=-0.916$, $p=0.372$).

5.4.2.1 Corticosterone Analysis

Corticosterone analysis was at performed one week before (Basal) final timepoint and at final timepoint (after FST). A significant increase in Stress group in basal corticosterone was observed one week before culls compared to Controls ($t(15.68)=-2.224$, $p=0.041$) (Figure 1B). Corticosterone measures 45 minutes after FST was near significant between groups ($t(18)=-2.077$, $p=0.052$).

5.4.3 Microbiome

5.4.3.1 Alpha and Beta Diversity of Bacteriome and Virome

Viriome beta diversity was significantly different by group (ADONIS, $r2= 0.682$, $p=0.001$) and factor (ADONIS, $r2= 0.504$, $p=0.002$) (Figure 2A). Bacteriome beta diversity was significantly different by group (ADONIS, $r2= 0.287$, $p=0.001$) and factor (ADONIS, $r2= 0.172$, $p=0.001$) (Figure 2B). Virome alpha diversity measures of richness and evenness were increase following stress, however did not reach significance (Chao, Wilcoxson, $p=0.710$; Shannon, Wilcoxson, $p=0.282$; Simpson, Wilcoxson, $p=0.145$) (Figure 3A-C).There was a trend towards reduced alpha diversity richness in the bacteriome following stress, which did not reach significance (Chao, Wilcoxson, $p= 0.710$) (Figure 3D). Alpha diversity measures of evenness revealed increases in bacteriome following stress (Shannon, Wilcoxson, $p=0.06$; Simpson, Wilcoxson, $p=0.009$) (Figure 3E-F)

5.4.3.2 Correlation between Virome and Bacteriome

Spearman correlations between viral contigs and bacterial OTUs were performed within factor group (NoStress and Stress). Significant positive correlations to uncultured bacteria belonging to phylum Bacteroidetes, families S24-7 and Rikenellaceae (Spearman, $rho>0.9$, $q<0.05$)(Figure 4).

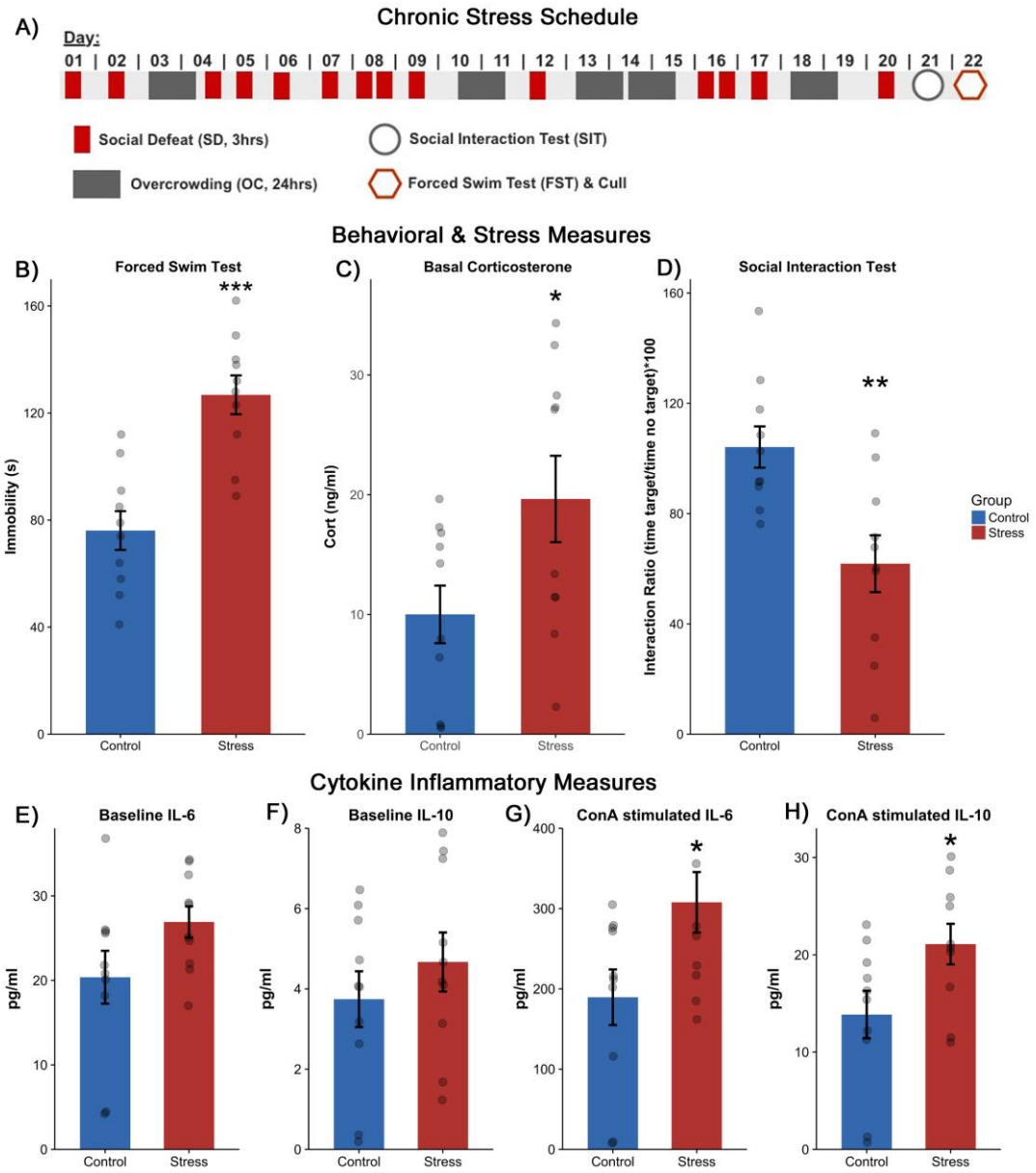


Figure 1 – Effects of Chronic Stress on Behavioural & Immune Parameters *(A), Behavioral (B-D) and Cytokine Measures (E-H) and– A) Stress schedule used in chronic unpredictable social defeat model. Red blocks indicate social defeat (SD) and grey block indicate overcrowding (OC) procedure in stress group only. Circle indicates social interaction test (SIT)and hexagon indicates forced swim test (FST) immediately followed by culling on the same. B) Forced Swim behavioral test C) Basal corticosterone levels 1 week before culls. D) Social Interaction behavioral test E) Unstimulated splenocytes (vehicle) IL-6 levels F) Unstimulated splenocytes (vehicle) IL-10 levels G) Concanavalin A (ConA) stimulated splenocytes IL-6 levels H) Concanavalin A (ConA) stimulated splenocytes IL-10 levels H) day in all subjects. Control group in blue and Stress group in red; significance indicated with asterisks: *** (p<0.001), ** (p<0.01), * (p<0.05).*

5.5 Discussion

This is to our knowledge the first study to investigate changes in the virome in relation to the microbiota-gut-brain axis. Here we show that there are marked differences in both the bacteriome and virome between control and stressed animals. Viral alpha diversity increased following stress, alongside reductions in bacterial richness. Furthermore, we observed a strong correlation between increased alpha diversity in virome and reduced bacterial richness, however this observation did not pass significance after FDR corrections(Supp. Fig. 6A)

These results would indicate a change in dynamic between bacteria and bacteriophage in the microbiome.This is particularly evident in the correlations between viral contig and bacteria belonging to two families, S24-7 and Rikenellaceae. Previous research has shown that these bacteria are altered in chronic social stress (Watanabe, Arase et al. 2016) and inflammation (Scott, Ida et al. 2017). Bacteriophage that do not immediately kill their bacterial host, lysogenic, can make up a substantial amount of bacterial DNA, which are also capable of altering dynamics of their host bacteria (Baugher, Durmaz et al. 2014, Barr, Auro et al. 2015, Knowles, Silveira et al. 2016). In some pathogenic bacteria virulence factors may be carried on lysogenic phage DNA (Mirzaei and Maurice 2017). These can include genes encoding toxins (Joossens, Huys et al. 2011, Melton-Celsa 2014). In some ways it could be said that the bacteria causing infections are, in their own fashion, also infected by their phage (Mills, Shanahan et al. 2013). In addition, stress imposed on bacterial species may potentially lead to the transfer of antibiotic resistance (Baugher, Durmaz et al. 2014, Dalmasso, Hill et al. 2014) and production of toxins that can result in heightened pro-inflammatory state (Van Belleghem, Merabishvili et al. 2017).

There are excessive limitations in the virome database, which may result in unidentified viruses and/or misclassification of viral sequences. Therefore, taxonomy characterization of viruses was not reported in the main manuscript. Further research is necessary to identify changes in specific viruses and bacteria, particularly bacteriophages, in the microbiome affected by chronic social stress. Firstly, it will be important to determine whether a purified virome faecal transfer is capable of conferring stress-susceptibility or stress-resilience in a pre-clinical model. Thereafter, identification of any responsible viruses or bacteria may assist in developing treatments that may potentially reduce stress-susceptibility, thus promoting stress-resilience through reduction of a systemic inflammation.

Exposure to a chronic social stress paradigm significantly increased measures of immobility time during FST, basal corticosterone, and cytokines IL-6 and IL-10 in splenocytes following incubation with Concanavalin A (ConA). The increase of these stress measures in this study aligns with previous studies of chronic social stress (Bailey, Dowd et al. 2011, Finger, Dinan et al. 2011, Finger, Dinan et al. 2012). Findings from this research further elucidates the impact of psychological stress on the microbiome and suggests that virome may play a role in hypothalamic-pituitary-adrenal (HPA) axis function. Future research is needed to elucidate whether this effect is due to the viruses having a direct impact on the host, or if alterations in viruses change bacterial composition which then impacts psychological stress and HPA axis function.

5.6 Supplementary Material

5.6.1 Supplementary Microbiota Methods:

Including Baseline (before stress =T0) and Final timepoint (after stress = T1)

5.6.1.1 Ceacal and feacal microbiome collection and sequencing

Faecal and caecal samples from control and stress animals were collected before stress (T0) and at the end of the experiment (T1) and stored at -80ºC. Bacteria (16S) and viruses (metagenomic) were sequenced and sequence processing and bioinformatic analysis performed.

5.6.1.2 Alternative Virome Sequence Processing Pipeline

To ensure accuracy and reproducibility of virome results, sequences were processed and analysed with two distinct pipelines. The alternative pipeline and results are provided herein. Metagenomic raw sequence reads in FASTQ files were further filtered on the basis of quality (removal all bases at the 3' end with a Phred score < 20 over a sliding window of 10 bp were trimmed) and length (only reads with a minimum length of 150 bases were considered for further analysis) with PRINSEQ. The resulting data were screened against the *Mus musculus* (mouse) reference genome downloaded from Illumina iGenomes (http://support.illumina.com/sequencing/sequencing_software/igeno me.html) to remove host reads using BMTagger (Rotmistrovsky and Agarwala 2011). Read duplicates were removed using the picard v2.7.1 MarkDuplicates tool (Broad Institute 2016) to create fastq files with unique reads only. Afterwards, a quality filtering step of the reads has been implemented, in brief, sequences are trimmed for low quality score using a modified version of the script trimBWAstyle.pl that works directly from BAM files (TrimBWAstyle.usingBam.pl. 2010; https://github.com/genome/genome/blob/master/lib/perl/Genome/Site/TGI/Hmp/HmpSraProcess/trimBWAstyle.usingBam.pl). The script is utilized to trim

bases off the ends of sequences, which show a quality value of two or lower. This threshold is taken to delete all the bases with an uncertain quality as defined by Illumina's EAMMS (End Anchored Max Scoring Segments) filter. Additionally, reads trimmed to less than 160 bp are also removed. The resulting sequences were taxonomically assigned against the complete viral genomes database using BLAST. The taxonomic composition is then determined using either raw number of best hits or number of best hits normalized by genome length using GAAS (Genome relative Abundance and Average Size) (Angly, Willner et al. 2009). The relative abundance of virus among different taxa were estimated by dividing the number of reads that uniquely mapped to that group by the total number of reads from the sample. The average number of high-quality sequences generated per sample was 990,464 ± 153,027 SD. The average number of assigned sequences was 226,374 ± 93,569 SD, approximately 23% of sequences. The average number of contigs per sample was 1842 ± 499 SD.

5.6.2 Supplementary Results

5.6.2.1 Results from Alternative Virome Pipeline

Results from this pipeline are visualized in Supplementary Figures 1-3. Rarefication curves of viral sequences revealed a trend toward increased richness following chronic social stress (Supp. Fig. 1A). Taxonomic assignment of viral sequences also revealed alterations in viral composition between T0 and T1 (Supp. Fig. 1C) and by group (Supp. Fig. 2E), Family-level viral composition showed a trend towards alterations in Podoviridae and Myoviridae following chronic social stress (Supp. Fig. 3).

5.6.2.2 Correlation Results

The following correlations were performed on viral contig data from the primary pipeline outlined main manuscript. Spearman correlation between virome and bacteriome alpha diversity measures at both timepoints (T0 & T1) showed no significant results before or after FDR ($p>0.05$, $q>0.05$). Correlations between virome and bacteriome alpha diversity measures at

final timepoint (T1) revealed a strong negative correlation between Simpson alpha diversity in virome and bacteriome Chao richness (Spearman, rho=-0.886, p=0.03), however this result did not remain significant after FDR (Fig. 6A).

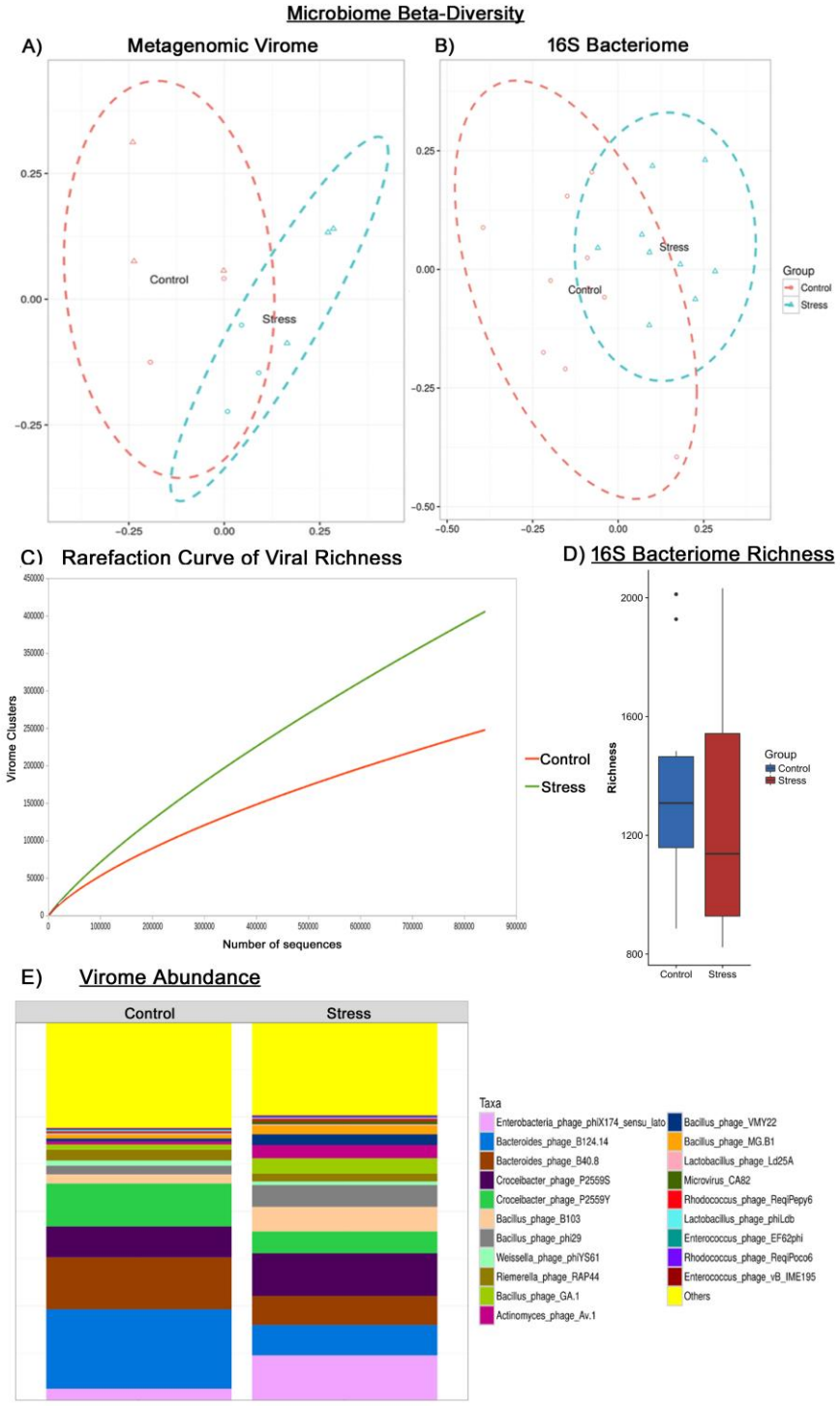
5.6.2.3 Canonical Correspondence Analysis (CCA)

To find major drivers involved in the virome beta diversity separation between NoStress and Stress, CCA performed on virome contigs, virome alpha diversity measures, and virome beta diversity. The adonis vegan function was used to assess viral contigs and virome alpha diversity measures that significantly corresponded to virome beta diversity. Three viral contigs and the virome Shannon Index alpha diversity measure significantly ($p<0.05$) corresponded to variance in virome beta diversity (total explained variance=0.622) These measures were then plotted onto a beta diversity ordination plot with arrows indicating direction of driver, calculated using the vegan ordiArrowMul function (Supp. Fig. 4C).

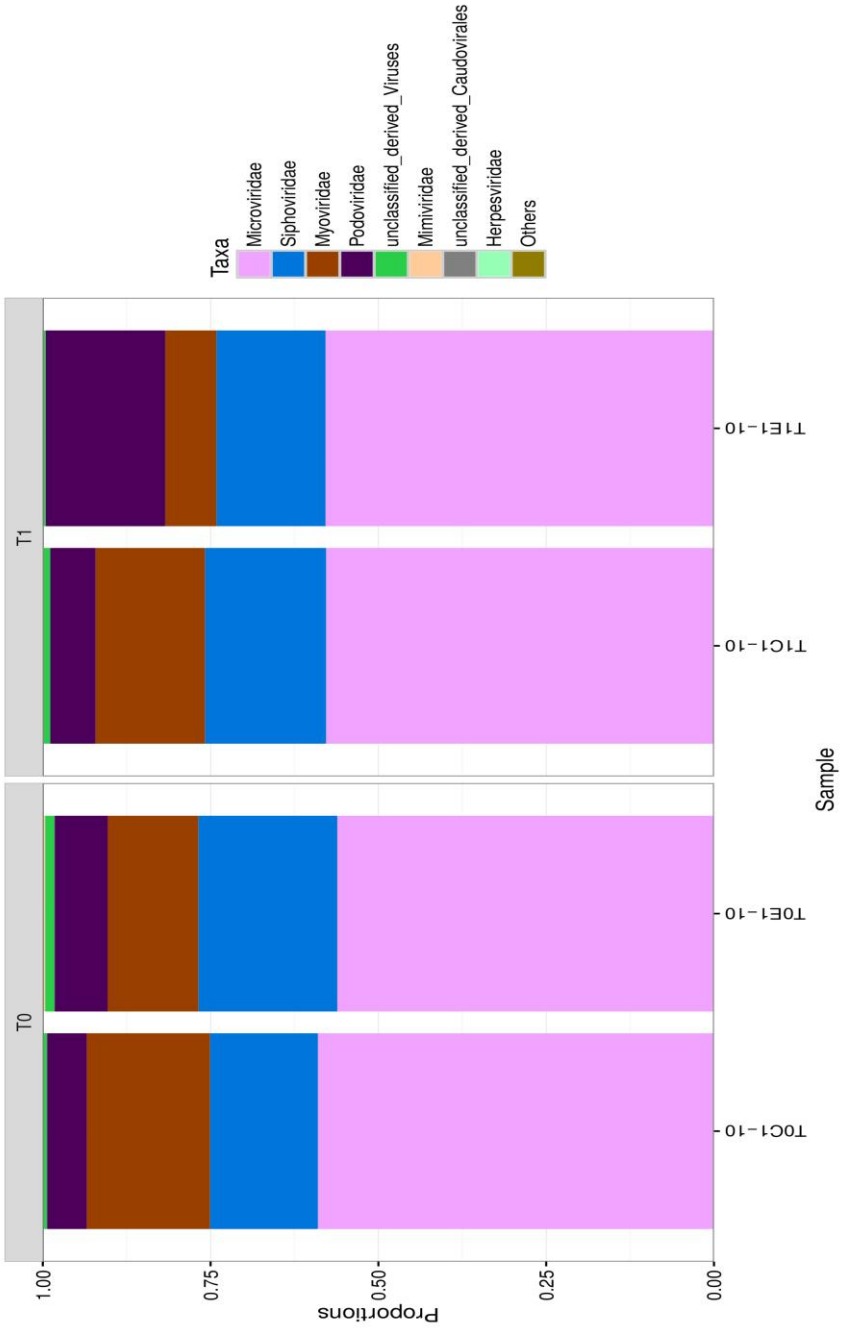
5.6.2.4 Virome Contig Comparisons

Using viral contig data from the primary pipeline from manuscript, comparisons were made between groups at timepoints T0 and T1. Contigs that were altered by chronic social stress and a pvalue<0.05 were visualized, however none passed FDR ($q>0.05$) (Supp. Fig. 5)

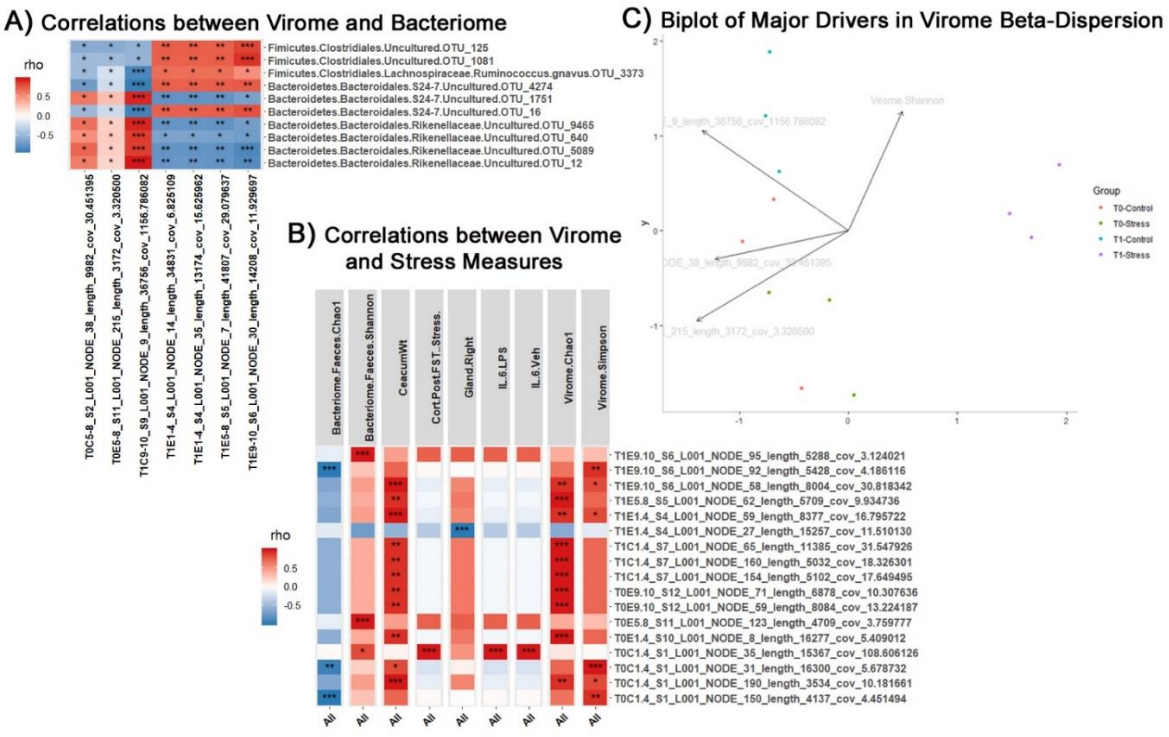
Supplementary Figure 1– Microbiome alpha-diversity and virome abundance*– A) Rarefaction curve of viral sequence; green line indicate stress group at final timepoint (T1E1-10), red indicates control group at final timepoint (T1C1-10); yellow and blue lines indicate stress and control group at beginning timepoint (T0E1-10, T0C1-10, respectively). B) 16S microbiome alpha-diversity richness; red bars indicate feacal Control samples at beginning timepoint (T0-Control); yellow bars indicate Stress feacal samples at beginning timepoint (T0-Stress); green bars indicate 9Control ceacum samples at end timepoint (T1ceacum-Control); teal bars indicate Stress ceacum samples at end timepoint (T1ceacum-Stress); blue bars indicate Control feacal samples at end timepointv (T1- Control); pink bars indicate Stress feacal samples at end timepoint (T1-Stress). C) Virome abundance at beginning (before stress – T0) and end timepoint (after stress – T1) for Stress and Control group. High abundance viruses, colored individually, low abundance viruses colored in yellow (other).*



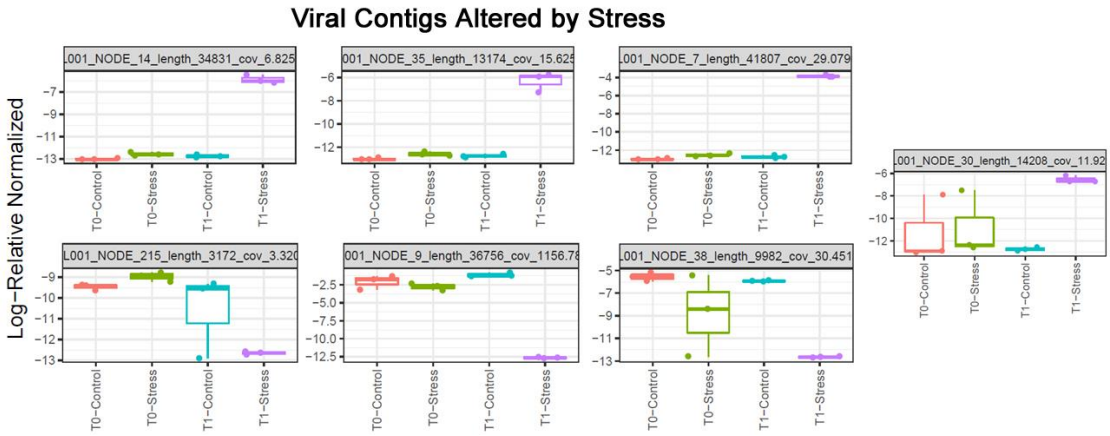
Supplementary Figure 2- Microbiome diversity and virome abundance– *A) Shot-gun metagenomic sequencing of virome faecal samples beta diversity plotted in NMDS; red indicate Control; blue indicate Stress B) 16S microbiome faecal samples beta diversity plotted in NMDS; red indicate Control; blue indicate Stress. C) Rarefaction curve of viral sequence; green line Stress group, red Control. D) 16S microbiome alpha-diversity richness; red bars indicate Stress; blue bars indicate Control. E) Virome abundance for Stress and Control group. High abundance viruses, colored individually, low abundance viruses colored in yellow (other).*



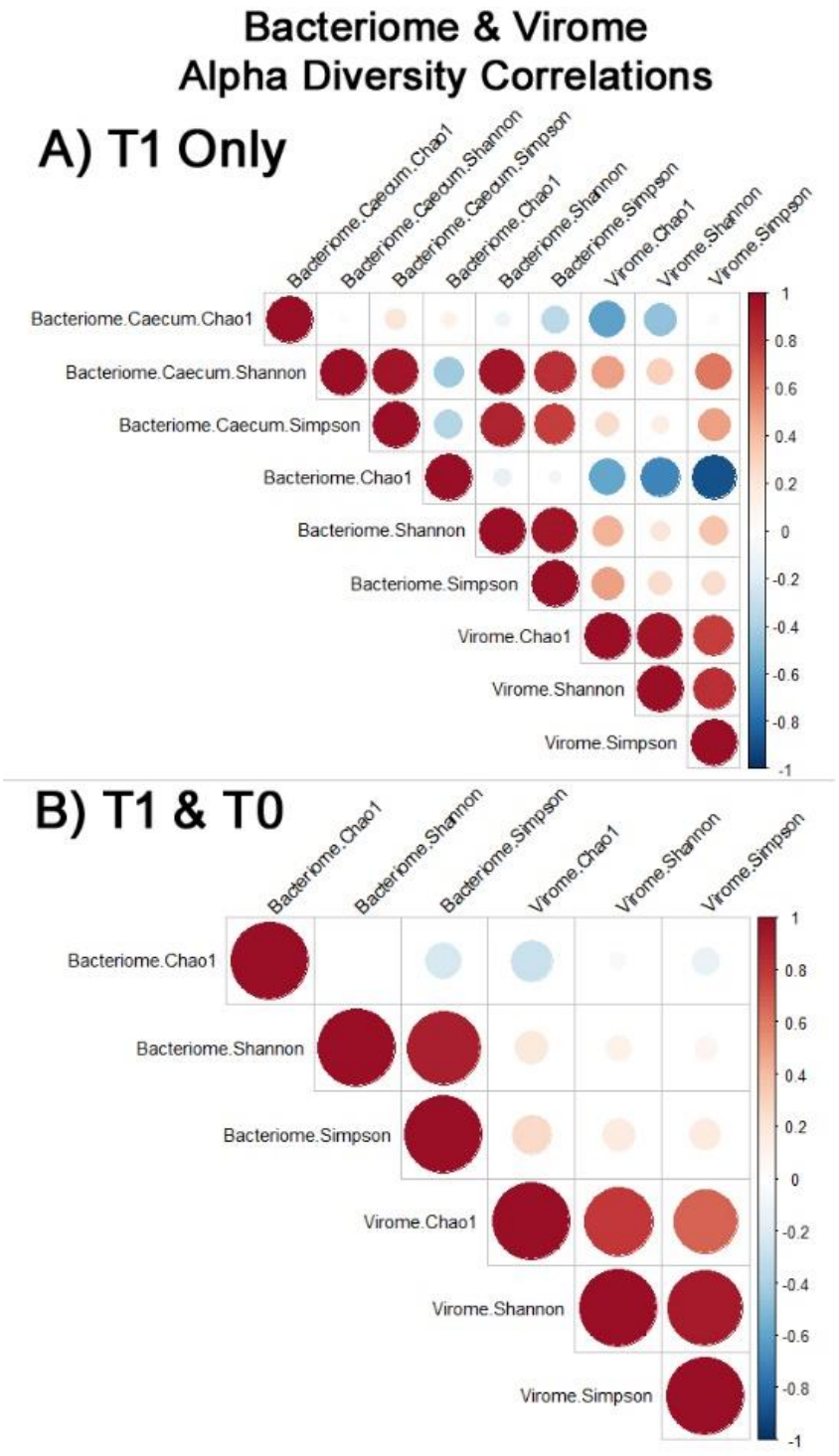
Supplementary Figure 3: Family-Level Virome Composition: *Relative abundance of classified bacteriophage at the family-level. T0C-10 refers to Control group at beginning of study.T0E1-10 refers to Stress group before chronic social stress protocol (beginning of study). T1C1-10 refers to Control group after 3 weeks experiment. T1E1-10 refers to Stress group following 3 weeks chronic social stress.*



Supplementary Figure 4: Virome Correlation Analyses: *A) Spearman correlation heatmap between bacterial OTUs and viral contig. B) Spearman correlation between virome at T1 and measures of stress. C) Biplot of viral contigs and virome alpha diversity that significantly corresponded to beta diversity. Asterisks indicate raw p-value (none passed FDR corrections): *=p<0.05, **=p<0.01, ***=p<0.001*



Supplementary Figure 5: Viral Contig Comparisons by Group: *Viral contigs that were altered by stress exposure. None passed FDR.*



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Supplementary Figure 6: Alpha Diversity Correlations: *A) Spearman correlation between bacteriome and virome alpha diversity measures at T1. B) Spearman correlation between virome at T0 and measures of stress. C) Biplot of viral contigs and virome alpha diversity that significantly correlate to beta diversity. Asterisks indicate raw p-value (none passed FDR corrections): *=p<0.05, **=p<0.01, ***=p<0.001*

6 General Discussion

6.1 Overview and Summary

The findings presented in this thesis show that the microbiome is influenced by alcohol addictionand stress. Bacterial in the gut microbiome have a subtle, yet significant association to addictive measures. Here, the effect of alcohol and chronic social stress has a more profound effect on gut microbiome. Importantly, sex must be considered when interpreting results from pre-clinical studies. The majority of pre-clinical research exclusively uses male animals. Findings in Chapter 4 of sex-specific associations between microbiome and addiction-associated behaviour illuminate the need to consider sex in the context of microbiome and behaviour. Intriguing results from this work suggest that gut microbiome may be a potential therapeutic target for remediation of addictive disorders. Finally, novel research into gut virome and psychosocial stress suggests that future research into bacteriophage, gut virome, and the microbiome-gut-brain axis may be a promising avenue of research.

6.1.1 Findings from Alcohol Studies

Here we see that both oral and vapor administration have effects associated to the microbiome. Notable vapor alcohol administration significantly altered microbiome composition in both alpha and beta diversity, as well as taxonomic bacteria. In our study of oral alcohol self-administration, no significant differences were observed in alpha and beta diversity or at the taxonomic level. This lack of significance may be explained by either the 2-month abstinence period before sample collection or variations in alcohol dosage - as vapor administration reached a BAC higher than most rodents will voluntarily drink. However, in the pre-clinical model of oral alcohol administration significant correlations are observed between microbiome and dopamine-receptor expression in the striatum as well as measures of

addiction severity, congruent with results seen in the addiction phenotype study (Chap. 5).

6.1.2 Findings from the Addiction Phenotype Studies

Addiction is a multifaceted disorder affecting humans worldwide. When considering the microbiome, many factors must be included such as sex, drug, dosage, and psychological comorbidities such as anxiety and depression. In this body of work, we see innate addictive behaviours are significantly correlated to microbiome composition, however are specific to sex.

In the alcohol severity phenotype study in male rats, microbiome correlates to measures of anxiety, impulsivity, dopamine-receptor expression, compulsive alcohol-seeking, and locomotor response to novelty. Cognate results are found in heterogenous bred stock male rats, with bacterial correlations to measures of impulsivity, reward learning, and locomotor response to novelty. Furthermore, in both studies the majority of correlations were to phylum Firmicutes and families Ruminococcoceae and Lachnospiracea.

Females had a more robust microbiota profile between subjects displaying an addictive phenotype compared to others. Significant reductions in alpha diversity were observed in addictive phenotype, a finding that was not seen in males in any of the studies. Importantly, many bacterial OTUs positively correlated to a measure of attention (RT.Omissions) when females were subset by addiction phenotype. Additionally, OTUs assigned to genus *Barnesiella* repeatedly correlated to behavioural measures, mainly a primary measure of impulsivity.

6.1.3 Findings from the Stress Study

Stress has previously been shown to affect the bacteriome (Bailey, Dowd et al. 2011, Burokas, Arboleya et al. 2017). In this novel finding we show that the virome is also affected by chronic psychosocial stress. Stress measures, such as elevated baseline corticosterone, and measures of inflammation were significantly different between Stress and Control groups alongside significant changes in both bacteriome and virome. Intriguingly, virome richness was increased following stress while bacterial richness decreased. This finding during stress may be explained by increased lytic bacteriophage killing bacterial hosts and/or alterations in immunity. Due to limitations in the bacteriophage database, low virome sample sizes due to pooling (N=3 per group), and restrictions on 16S classification at the strain-level, we were unable to find associations between bacteriome and virome. This study is, to our knowledge, the first to characterize gut virome in relation to microbiome-gut-brain axis. These preliminary results suggest that virome may also be changed in other psychiatric conditions linked to gut microbiome. Moreover, bacteriophage may be a significant factor contributing to stress-induced alterations in bacteriome and future microbial interventions may abate the cascading effects of psychological stress. Overall, understanding how both bacteriome and virome contribute to psychological stress is of equal importance.

6.2 Future Directions

6.2.1 Alcohol and Gut Microbiome

Follow-up studies could be performed to further elucidate factors influencing reported results. Since alcohol is a widely consumed product, globally, further understanding of dose-dependant effects on microbiome would illuminate impact on microbiome for all individuals who consume alcohol on the spectrum of acceptable consumption to problematic/severe alcohol abuse. Results of dose-dependant alcohol impact on gut microbiome may further assist in informing regulatory bodies of acceptable levels of alcohol consumption.

6.2.2 Mechanism of Microbiome Influence on Addiction and Dopamine

Results from the addiction phenotype studies indicate there are innate differences in gut microbiome composition between addictive vs. normal subjects, independent of drug administration. Future investigation should use faecal microbiota transplant (FMT) to assess if: 1) an addictive phenotype in rats can be transferred to another rat, and if the phenotype can be transferred between sexes; 2) if clinical samples from addiction patients can confer an addictive phenotype in rodents of the same and alternate sex. If an addictive phenotype can be established via FMT, further intervention studies using psychobiotics could be implemented.

Gut bacteria have previously been shown to influence neurotransmitter receptor expression, resulting in behavioural alterations (Bravo, Forsythe et al. 2011). The novel associations between dopamine receptor expression in striatum and microbiome composition merits further investigation into the mechanisms behind this correlation. Work from Bravo et. al. showed GABA receptors expression are altered by the administration of *Lactobacillus*

rhamnosus GG, resulting in an anxiolytic phenotype. This effect was negated in vagotomised mice, implicating vagal nerve innervations in the mechanistic action. A similar experimental design could be implemented to investigate if the vagus nerve regulates dopamine receptor alterations associated to an addictive microbiota composition. In female rats, addictive phenotype was repeatedly correlated to bacteria belonging to genus *Barnesiella*. In male rats displaying compulsive alcohol seeking behaviour, bacteria from genus *Gemella* correlated to alterations in both D1 and D2 dopamine receptors as well as a majority of the addictive behaviours. Follow-on experiments would administer single cultures of these bacteria, to assess if these identified bacteria increase addiction-associated behavioural measures and dopamine receptor expression.

6.2.3 Stress Faecal Virome Transplant

Investigation into the dynamics of bacteriophage and commensal gut bacteria and their association to the microbiota-gut-brain axis may be harnessed to provide future therapeutic targets to treat mental health disorders. Initial work to establish this field must first prove that a refined faecal phage transplant is capable of conferring a behavioural phenotype. Thus, the first follow-on experiment must administer refined virome from both stressed and non-stressed subjects to a naïve experimental cohort of mice. An additional positive control necessary for this experiment includes inactivated viruses from stressed donor faecal fraction. Importantly, stress hormones and anxiety/depressive behaviour need to be assessed in the virome recipient groups. If transfer of a stressed phenotype is successful, further research would include microbial or dietary interventions to remediate the symptoms of stress by altering gut microbiota.

6.2.4 Sex Matters

Results from Chapter 4 emphasize that sex must always be considered when investigating microbiota associations to brain and behaviour. The relationship between sex hormones, microbiome and behaviour has been

reported in scientific literature (Amato, Leigh et al. 2014, Chaban, Links et al. 2014, Schnorr, Candela et al. 2014, Jasarevic, Morrison et al. 2016). During development, factors that influence addiction, such as genetics, also contribute to sexual dimorphism in microbiome (Jasarevic, Morrison et al. 2016). Clinical and pre-clinical research has shown differences in microbiota composition associated to altered metabolism of essential vitamins and nutrients from the diet, with increased functional richness seen in females compared to males (Amato, Leigh et al. 2014, Bolnick, Snowberg et al. 2014, Zhernakova, Kurilshikov et al. 2016).

In sex-specific addiction worldwide, illicit drug use is significantly lower in females compared to males (World Health Organization 2012). However, research of both humans and other animal species indicates that females may, in some cases, display a propensity for addiction. Females self-administer drugs more readily than males and are more vulnerable to addiction to a variety of licit and illicit drugs (Becker and Hu 2008, Yang, Han et al. 2017). A human genome-wide-association study (GWAS) showed females having greater impulsive behaviour, as measured by delay discounting (Sanchez-Roige, Fontanillas et al. 2018). Furthermore, GWAS studies suggest that opioid dependence is linked to sex specific single nucleotide polymorphisms (Yang, Han et al. 2017). In Cloninger's typology, Type II alcoholism has an early onset, a genetic propensity, and more common in males, while Type I has a late onset and is seen in both females and males (Cloninger, Sigvardsson et al. 1996). Differences in hormones are attributed to some sex differences in addiction-related behaviour. Furthermore, intensity of drug usage and drug withdrawal vary depending on menstrual cycle. In the striatum, dopamine levels are higher during estrus in rats, when estradiol is elevated. Estradiol is known to increase positive affect, locomotor sensitization, and acquisition of self-administration to psychomotor stimulants in female rats (Becker, Lopez et al. 2011). It is important to note that despite extensive literature associating addiction-related behaviours in rodents to drug administration, in humans these only appear to be potential vulnerability factors.

These findings indicate a need for comprehensive understanding of factors associated to addiction propensity, and how different sexes are affected. Females may not necessarily be at greater risk for addiction. Recent findings suggest addiction phenotype is far more complex than just reward-stimulus learning (Singer, Fadanelli et al. 2018). The influence of sex on the microbiome and reward from an evolutionary perspective must also be considered. For enhanced fitness females may have a heightened potential to assign incentive salience to a predictive cue. This would enhance mating with a partner that is healthier, as indicated by association to a natural reward such as food and water to a mate. Importantly, in many mammals rearing of offspring is often exclusively the responsibility of the female, thus heightened sensitivity to cues that predict a natural reward would ensure survival of offspring by enforcing feeding behaviour (Volkow and Wise 2005). This evolutionary advantageous reward pathway circuitry evolved to predict and reinforce behaviour that produced a “reward” that enhanced survival fitness (Newlin 2002). In this paradigm, enhanced metabolic function in the microbiome also has proven to be evolutionarily advantageous for females and in this context may be linked to reward-stimulus learning to enhance learned associations to naturally rewarding stimuli.

6.2.5 Sex Differences in Alcohol, Stress and Virome

Sex differences were not explored in the chronic social stress paradigm, vapor alcohol study, nor the pre-clinical model of alcohol abuse. Given that this is a significant factor in the addiction phenotype study, further research in necessary to investigate how the microbiome of females, compared to males, are affected by drugs of abuse. According to the National Institute of Health, the standard volume of alcohol consumption for females is lower than males. In follow-up research of dose-dependent effects of alcohol consumption on microbiome, sex must be included in the experimental design. Furthermore, research investigating sex differences in psychological stress related to bacteriome and virome should be investigated.

6.2.6 Can the Microbiome Treat Addiction and Stress?

While FMT is a valuable method for conducting pre-clinical research and treating life-threating *Clostridium difficile* infection, this method is not a targeted psychobiotic treatment in humans (Bojanova and Bordenstein 2016). A faecal sample contains a host of microbes (bacteria, viruses, fungi, etc.) along with variety of molecules (bacterial toxins, hormones, and bile salts) that may have undesired effects for the FMT recipient. More targeted therapies to treat psychological disorders via the gut microbiota include prebiotics, probiotics, phageome therapy, and dietary intervention.

Pre-clinical work from our lab has shown that prebiotic treatment can reduce symptoms of stress (Burokas, Arboleya et al. 2017). Prebiotic intervention is suggested to affect brain and behaviour through increasing communities of beneficial bacteria via direct metabolization of prebiotic or secondary metabolites, these metabolic by-products from bacterial include short-chain fatty acid. This also reduces the pro-inflammatory profile associated with stress (Bailey, Dowd et al. 2011). Stress is a significant factor in addiction and previous research investigating gut microbiome and substance abuse has found connections to a pro-inflammatory state (Leclercq, Cani et al. 2012, Bull-Otterson, Feng et al. 2013, Allais, Kerckhof et al. 2016, Lowe, Gyongyosi et al. 2017, Plein and Rittner 2018). These data would suggest that prebiotic treatment may also be beneficial for addiction and substance abuse disorders.

Probiotic administration in animal studies of *Lactobacillus rhamnosus* has been shown to be effective for reducing anxiety and improving intestinal barrier function during alcohol exposure (Bravo, Forsythe et al. 2011, Bull-Otterson, Feng et al. 2013). Actions of this and other probiotics include: altering vagus nerve signalling and brain mRNA expression (Bravo, Forsythe et al. 2011), improving barrier function which reduces inflammation by

preventing bacterial endotoxins to cross into the circulatory system (Bull-Otterson, Feng et al. 2013), direct immune modulation (Hsiao, McBride et al. 2013), and by altering gut microbiome community composition (Bastiaanssen, Cowan et al. 2018).

Other potential therapeutic interventions may include diet intervention and targeting gut bacteriophage. A heathy diet is an age-old treatment for many health conditions. Diet is known to be one of the most significant factors influencing microbiome composition (Zhernakova, Kurilshikov et al. 2016). Research has shown that depression is associated with low fibre intake (Kelly, Borre et al. 2016), indicating that a diet high in fibre may benefit the brain. Diets rich in healthy fats and phytonutrients are shown to have an anti-inflammatory effect (Ricker and Haas 2017).

Phage therapy is an intriguing possibility for removing pathogenic bacteria that may contribute to a pro-inflammatory phenotype. However, in our current study we found that bacteriophage diversity is linked to bacterial diversity which indicates that therapeutic intervention requires altering community dynamics. An intriguing intervention to pursue, which targets phage populations, would be administration of polyphenols which has been shown to interact with bacteriophage (Catel-Ferreira, Tnani et al. 2015). Phage administration has also been shown to influence intestinal permeability of host (Tetz and Tetz 2016).

Route of Action:	Prebiotic	Probiotic	Phageome	Diet
Altering Gut Microbiome Composition	XX	XXX	XX	XXX
Anti-Inflammatory/Immune Modulatory	X	XXX	X	XX
BBB and Intestinal Barrier Integrity	X	XXX	X	XX
Vagus Nerve Signalling/Brain mRNA Expression		XX		X

Table 6.2: Microbiome Therapies to Treat Stress and Addiction*: x's indicate robustness of the literature supporting purposed the route of action. Prebiotic references: (Schmidt, Cowen et al. 2015, Burokas, Arboleya et al. 2017); Probiotic references: (Whorwell, Altringer et al. 2006, Rousseaux, Thuru et al. 2007, Kirpich, Solovieva et al. 2008, Forsyth, Farhadi et al. 2009, Bravo, Forsythe et al. 2011, Messaoudi, Lalonde et al. 2011, Wang, Liu et al. 2012, Dinan, Stanton et al. 2013, Korpela, Salonen et al. 2018); Phageome references: (Angly, Willner et al. 2009, Reyes, Semenkovich et al. 2012, Barr, Auro et al. 2013, Mills, Shanahan et al. 2013, Reyes, Wu et al. 2013, Dalmasso, Hill et al. 2014, Barr, Auro et al. 2015, Lim, Zhou et al. 2015, Knowles, Silveira et al. 2016, Silveira and Rohwer 2016, Tetz and Tetz 2016, Forde and Hill 2018); Diet references: (Claesson, Jeffery et al. 2012, Tuohy, Conterno et al. 2012, Bull-Otterson, Feng et al. 2013, Jacka, Gama et al. 2014, Schnorr, Candela et al. 2014, Jacka, Cherbuin et al. 2015, Kelly, Borre et al. 2016, Lin, An et al. 2016, Zhernakova, Kurilshikov et al. 2016, Ryan, Patterson et al. 2017, Hassan, Mancano et al. 2018, Ma, Wang et al. 2018, Mulders, de Git et al. 2018, Olson, Vuong et al. 2018, Rothschild, Weissbrod et al. 2018)*

6.3 Limitations of the reported studies

6.3.1 16S Sequencing

16S sequencing is limited to the genus level (Zhernakova, Kurilshikov et al. 2016, Cronin, Barton et al. 2018). Metabolomic sequencing would provide better resolution of functional profile and lower-level taxonomic classifications, such as bacterial species and strain. Functional profiling in individual studies may help further elucidate microbiome interactions in separate studies.

6.3.2 Database Limitations

The databases are a continual limitation of microbiome studies. More so for virome as reference databases for taxonomic identification are very limited, due to a lack of whole-genome characterization. Additionally, updates to bacterial databases temporally affect bacterial assignments, potentially influencing results and data interpretations.

6.3.3 Limitations of Virome Analysis

The field of virome in relation to the microbiome and host health is currently in a state of development and refinement. Many of the limitations of the present study reflects broad limits in the field. As previously mentioned, virome reference databases for taxonomic identification are incomplete due to lack of research characterizing/classifying viral genomes. Virome sequencing was performed at two timepoints alongside 16S sequencing. A scarcity in fresh faecal samples compounded by the need to acquire 2-3 grammes of faeces for virome analysis forced a decision to collect fresh faeces for 16S and dried faecal pellets, found in mouse bedding, for virome sequencing. Thus for virome samples, prolonged exposure of faecal pellets to the environment may be a confounding factor when associated with bacteriome at the same timepoint. Furthermore, due to housing shortages in

the animal facility, control mice had to be group housed (2x cages of3 mice, 1x cage of 4 mice). Due to coprophagia, co-housed control subjects (N=10) only provided 3 unique faecal virome samples for analysis. Finally, bacteriophage viruses are species/strain specific to their bacterial hosts. Thus, metagenomic analysis of bacteriome and virome would be preferable as bacterial metagenomic sequencing is capable of assigning bacteria to these lower levels of taxonomic identification.

6.4 Conclusions

Together this data indicates that addiction and stress measures are associated to microbiome composition. Moreover, this affects linked factors including, drug dosage, sex, and inflammation. In this current work we sought to characterize microbiome changes in alcohol addiction, sex dependent addiction-related behaviours, and stress. The main aim of this exploratory work assessed is 16S rRNA gene and virome shot-gun metagenomic sequences by comparing alpha, beta, and taxonomic abundance between groups as well as correlating composition to biological and behavioural metadata.

These studies show that there is both innate and acute variations in gut microbiota in models of drug addiction. Furthermore, microbiome composition and correlations to behavioural metadata is sex-specific. Abundance of specific bacteria, predominantly in the phylum Firmicutes and families Ruminococcaceae and Lachnospiracea, correlate to behavioural measures of impulsivity, reward learning, compulsive alcohol seeking, dopamine-mediated locomotor response to novelty, and striatal dopamine receptor expression.

This is the first time, to our knowledge, that gut virome has been characterized in the context of the microbiome-gut-brain axis. Investigation of gut microbiome in a mouse model of chronic psychosocial stress revealed that both bacteriome and virome composition and alpha diversity are significantly affected in stressed mice compared to non-stressed controls. Here, we observe increases in virome richness and decreases in bacterial richness. This finding, alongside alterations in bacteriophage abundance between stressed and non-stressed mice, suggests that lytic bacteriophage may be implicated in bacteriome alterations during stress. Further research is necessary to elucidate if: 1) refined virome from stress mice transplanted into naïve mice can confer a stress phenotype; 2) if targeting virome alpha diversity is capable of inducing or remediating stress severity; 3) if targeting particular viruses are capable of altering the stress phenotype. Our preliminary results presented here suggest the gut virome as an intriguing new field to investigate for novel microbiome-gut-brain axis therapies.

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8 Appendix I:

Abstracts of Co-Authored Manuscripts

First page of published co-authored manuscripts presented herein. Contribution to publications consisted of: i) biostatistical analysis and interpretation of microbiome (Golubeva, Joyce et al. 2017, Scott, Ida et al. 2017, Cussotto, Strain et al. 2018, Dunphy-Doherty, O'Mahony et al. 2018), ii)participation in animal study (Burokas, Arboleya et al. 2017), and iii) splenocyte stimulation for measurement of cytokines (Ryan, Patterson et al. 2017).

Order:

- Golubeva et. al. (2017). Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism
- Scott et. al. (2017). Revisiting Metchnikoff: Age-related Alterations in Microbiota-Gut-Brain Axis in the Mouse
- Burokas et. al. (2017). Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice
- Doherty et. al. (2017). Post-Weaning Social Isolation of Rats Leads to Long-term Disruption of the Gut Microbiota-Immune-Brain Axis
- Ryan et. al. (2017). Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice
- Cussotto et. al. (2018). Psychotropic Drugs and the Microbiome-Gut-Brain Axis

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Research Paper

Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism

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ABSTRACT

Autism spectrum disorder (ASD) is one of the most prevalent neurodevelopmental conditions worldwide. There is growing awareness that ASD is highly comorbid with gastrointestinal distress and altered intestinal microbiome, and that host-microbiome interactions may contribute to the disease symptoms. However, the paucity of knowledge on gut-brain axis signaling in autism constitutes an obstacle to the development of precision microbiota-based therapeutics in ASD. To this end, we explored the interactions between intestinal microbiota, gut physiology and social behavior in a BTBR T^{+} *Itpr3*^{tf}/J mouse model of ASD. Here we show that a reduction in the relative abundance of very particular bacterial taxa in the BTBR gut – namely, bile-metabolizing *Bifidobacterium* and *Blautia* species, - is associated with deficient bile acid and tryptophan metabolism in the intestine, marked gastrointestinal dysfunction, as well as impaired social interactions in BTBR mice. Together these data support the concept of targeted manipulation of the gut microbiota for reversing gastrointestinal and behavioral symptomatology in ASD, and offer specific plausible targets in this endeavor.

1. Introduction

Autism spectrum disorder (ASD) is one of the most serious neurodevelopmental conditions worldwide, affecting on average 1 in 150 children (Lyall et al., 2017). The hallmarks of ASD include impaired social communication skills and repetitive behaviors, frequently co-occurring with intellectual disability (Association, 2013). There are limited treatment options for ASD symptoms, and thus there is a great need to develop strategies that would improve the quality of life in ASD subjects. More recently, the gut microbiome has emerged as a novel important player and therapeutic target in autism (Vuong and Hsiao, 2017). Intestinal bacteria can orchestrate a range of gastrointestinal (GI) functions (Sommer and Backhed, 2013), and therefore are poised to affect gut-brain axis signaling in ASD. Animal studies have demonstrated that the gut microbiota plays a crucial role in anxiety (Neufeld et al., 2011), cognitive performance (Desbonnet et al., 2015), repetitive behaviors (Desbonnet et al., 2014) and social communications (Buffington et al., 2016; Sherwin et al., 2017). There is a growing body of evidence that ASD subjects display substantial alterations in microbiota composition, along with marked symptoms of GI distress, including changes to bowel habits, increased intestinal permeability, bloating and abdominal pain (Angelis et al., 2013; D'Eufemia et al., 1996; Kang et al., 2013; Luna et al., 2017; McElhanon et al., 2014; Tomova et al., 2015). In a recent small open-label clinical study, transplantation of standardized human gut microbiota to ASD-diagnosed children induced an improvement of GI function, as well as a reduction of behavioral ASD scoring (Kang et al., 2017). However, we do not yet know whether alterations

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Named Series: BBI and the Microbiome

Revisiting Metchnikoff: Age-related alterations in microbiota-gut-brain axis in the mouse

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ABSTRACT

Over the last decade, there has been increased interest in the role of the gut microbiome in health including brain health. This is by no means a new theory; Elie Metchnikoff proposed over a century ago that targeting the gut by consuming lactic acid bacteria such as those in yogurt, could improve or delay the onset of cognitive decline associated with ageing. However, there is limited information characterising the relationship between the behavioural and physiological sequelae of ageing and alterations in the gut microbiome. To this end, we assessed the behavioural, physiological and caecal microbiota profile of aged male mice. Older mice (20–21 months old) exhibited deficits in spatial memory and increases in anxiety-like behaviours compared to younger mice (2–3 months old). They also exhibited increased gut permeability, which was directly correlated with elevations in peripheral pro-inflammatory cytokines. Furthermore, stress exacerbated the gut permeability of aged mice. Examination of the caecal microbiota revealed significant increases in phylum TM7, family Porphyromonadaceae and genus *Odoribacter* of aged mice. This represents a shift of aged microbiota towards a profile previously associated with inflammatory disease, particularly gastrointestinal and liver disorders. Furthermore, Porphyromonadaceae, which has also been associated with cognitive decline and affective disorders, was directly correlated with anxiety-like behaviour in aged mice. These changes suggest that changes in the gut microbiota and associated increases in gut permeability and peripheral inflammation may be important mediators of the impairments in behavioural, affective and cognitive functions seen in ageing.

1. Introduction

Over 100 years ago, Nobel Prize winner Elie Metchnikoff hypothesised that beneficial bacteria contained in fermented foods could influence health and delay cognitive decline by manipulating the intestinal environment (Mackowiak, 2013; Cryan and Dinan, 2015). Although popular at the time, the theory was largely ignored by the medical community until a recent resurgence in interest over the past 20 years (Mackowiak, 2013). The gut microbiome is now recognised to play a critical role in health and disease, and this is especially true at the extremes of life (Brussow, 2013; Borre et al., 2014; Candela et al., 2014; Diaz Heijtz, 2016; Dinan and Cryan, 2016). Indeed, changes in microbial composition have been shown to have a distinct impact on health outcomes in infants and in the elderly and are also thought to play a key role in modulating gut-brain axis function, influencing brain and behaviour (Jeffery and O'Toole, 2013; Borre et al., 2014). However, there is limited information on how ageing regulates this axis. With advances in healthcare, longevity has markedly increased; currently it is estimated that within 50 years, approximately 20% of the world population will be classified as elderly (Ellison et al., 2015). Ageing is associated with a number of behavioural and

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Biological Psychiatry

Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice

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ABSTRACT

BACKGROUND: The realization that the microbiota-gut-brain axis plays a critical role in health and disease, including neuropsychiatric disorders, is rapidly advancing. Nurturing a beneficial gut microbiome with prebiotics, such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), is an appealing but underinvestigated microbiota manipulation. Here we tested whether chronic prebiotic treatment modifies behavior across domains relevant to anxiety, depression, cognition, stress response, and social behavior.

METHODS: C57BL/6J male mice were administered FOS, GOS, or a combination of FOS+GOS for 3 weeks prior to testing. Plasma corticosterone, microbiota composition, and cecal short-chain fatty acids were measured. In addition, FOS+GOS- or water-treated mice were also exposed to chronic psychosocial stress, and behavior, immune, and microbiota parameters were assessed.

RESULTS: Chronic prebiotic FOS+GOS treatment exhibited both antidepressant and anxiolytic effects. Moreover, the administration of GOS and the FOS+GOS combination reduced stress-induced corticosterone release. Prebiotics modified specific gene expression in the hippocampus and hypothalamus. Regarding short-chain fatty acid concentrations, prebiotic administration increased cecal acetate and propionate and reduced isobutyrate concentrations, changes that correlated significantly with the positive effects seen on behavior. Moreover, FOS+GOS reduced chronic stress-induced elevations in corticosterone and proinflammatory cytokine levels and depression-like and anxiety-like behavior in addition to normalizing the effects of stress on the microbiota.

CONCLUSIONS: Taken together, these data strongly suggest a beneficial role of prebiotic treatment for stress-related behaviors. These findings strengthen the evidence base supporting therapeutic targeting of the gut microbiota for brain-gut axis disorders, opening new avenues in the field of nutritional neuropsychopharmacology.

Keywords: Animal behavior, Anxiety, Microbiota-gut-brain axis, Prebiotics, SCFAs, Stress

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Increasing evidence suggests that the microbiota-gut-brain axis plays a key role in regulating brain functions, particularly emotional processing and behavior (1,2). Indeed, the microbiota plays an important role in neurodevelopment, leading to alterations in gene expression in critical brain regions and resulting in perturbation to the programming of normal social and cognitive behaviors in mice (3–6). The gut microbiota has principally been exploited to yield positive effects on brain health via probiotics, with various bifidobacteria and lactobacilli strains shown to have anxiolytic and procognitive effects in both rodents (7–10) and humans (11–14). Although single- or multistrain probiotics have shown potential to modify behavior, they also are limited by their ability to have relatively narrow spectrum effects on the microbiome. Moreover, given that they are live biotherapeutics, there are formulation and storage issues to consider.

An alternative but underinvestigated strategy to target the microbiome is via dietary prebiotics. These are defined as selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thereby conferring benefits on host health (15). Unabsorbed/undigested carbohydrates in the small intestine are fermented by the gut microbiota in the large bowel, producing their main end products, short-chain fatty acids (SCFAs) and lactic acid (16), which may have multiple effects, including the modulation of enteroendocrine serotonin secretion (17).

Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are soluble fibers extensively used as prebiotics that

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Full-length Article

Post-weaning social isolation of rats leads to long-term disruption of the gut microbiota-immune-brain axis

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ABSTRACT

Early-life stress is an established risk for the development of psychiatric disorders. Post-weaning isolation rearing of rats produces lasting developmental changes in behavior and brain function that may have translational pathophysiological relevance to alterations seen in schizophrenia, but the underlying mechanisms are unclear. Accumulating evidence supports the premise that gut microbiota influence brain development and function by affecting inflammatory mediators, the hypothalamic–pituitaryadrenal axis and neurotransmission, but there is little knowledge of whether the microbiota-gut-brain axis might contribute to the development of schizophrenia-related behaviors. To this end the effects of social isolation (SI; a well-validated animal model for schizophrenia)-induced changes in rat behavior were correlated with alterations in gut microbiota, hippocampal neurogenesis and brain cytokine levels. Twenty-four male Lister hooded rats were housed in social groups (group-housed, GH, 3 littermates per cage) or alone (SI) from weaning (post-natal day 24) for four weeks before recording open field exploration, locomotor activity/novel object discrimination (NOD), elevated plus maze, conditioned freezing response (CFR) and restraint stress at one week intervals. Post-mortem caecal microbiota composition, cortical and hippocampal cytokines and neurogenesis were correlated to indices of behavioral changes. SI rats were hyperactive in the open field and locomotor activity chambers traveling further than GH controls in the less aversive peripheral zone. While SI rats showed few alterations in plus maze or NOD they froze for significantly less time than GH following conditioning in the CFR paradigm, consistent with impaired associative learning and memory. SI rats had significantly fewer BrdU/NeuN positive cells in the dentate gyrus than GH controls. SI rats had altered microbiota composition with increases in Actinobacteria and decreases in the class Clostridia compared to GH controls. Differences were also noted at genus level. Positive correlations were seen between microbiota, hippocampal IL-6 and IL-10, conditioned freezing and open field exploration. Adverse early-life stress resulting from continuous SI increased several indices of 'anxiety-like' behavior and impaired associative learning and memory accompanied by changes to gut microbiota, reduced hippocampal IL-6, IL-10 and neurogenesis. This study suggests that early-life stress may produce long-lasting changes in gut microbiota contributing to development of abnormal neuronal and endocrine function and behavior which could play a pivotal role in the aetiology of psychiatric illness.

1. Introduction

Exposure to early-life stress is an established risk factor for the development of several common psychiatric conditions; including anxiety-related disorders, schizophrenia, post-traumatic stress disorder and depression (Heim et al., 1997). Furthermore, stress is the strongest predictor of developing depression in clinical

Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; CFR, conditioned freezing response; GH, Group-housed; HPA, hypothalamic pituitary-adrenal axis; NeuN, neuronal nuclei; NOD, novel object discrimination; PFC, prefrontal cortex; SI, social isolation; SEM, standard error of the mean.

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Recombinant Incretin-Secreting Microbe Improves Metabolic Dysfunction in High-Fat Diet Fed Rodents

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The gut hormone glucagon-like peptide (GLP)-1 and its analogues represent a new generation of anti-diabetic drugs, which have also demonstrated propensity to modulate host lipid metabolism. Despite this, drugs of this nature are currently limited to intramuscular administration routes due to intestinal degradation. The aim of this study was to design a recombinant microbial delivery vector for a GLP-1 analogue and assess the efficacy of the therapeutic in improving host glucose, lipid and cholesterol metabolism in diet induced obese rodents. Diet-induced obese animals received either Lactobacillus paracasei NFBC 338 transformed to express a long-acting analogue of GLP-1 or the isogenic control microbe which solely harbored the pNZ44 plasmid. Short-term GLP-1 microbe intervention in rats reduced serum low-density lipoprotein cholesterol, triglycerides and triglyceride-rich lipoprotein cholesterol substantially. Conversely, extended GLP-1 microbe intervention improved glucose-dependent insulin secretion, glucose metabolism and cholesterol metabolism, compared to the high-fat control group. Interestingly, the microbe significantly attenuated the adiposity associated with the model and altered the serum lipidome, independently of GLP-1 secretion. These data indicate that recombinant incretin-secreting microbes may offer a novel and safe means of managing cholesterol metabolism and diet induced dyslipidaemia, as well as insulin sensitivity in metabolic dysfunction.

GLP-1 is a short conserved mammalian peptide produced by enteroendocrine L-cells that can stimulate insulin production to lower postprandial glucose levels in patients with type-2 diabetes (T2D)[1,2]. While GLP-1 has been known to interact with an array of additional systems – including the brain[3], liver[4] and stomach[5] – its role in lipid metabolism and cardiovascular health has only recently emerged[6]. However, the incretin is rapidly cleaved and rendered inactive by circulating dipeptidyl peptidase (DPP)-4. As such, numerous drugs have explored extending the effects of GLP-1, either by synthetic stimulation of the GLP-1 receptor (GLP-1R) by DPP-4-resistant analogues (*e.g.* exendatide and lirglutitide) or direct inhibition of DPP-4 activity (*e.g.* gliptins).

GLP-1, GLP-1R agonists and DPP-4 inhibitors have all shown varying abilities to suppress enteric cell chylomicron production, made phenotypically evident by a reduction in circulating triglycerides and apolipoprotein B-48 (apoB-48)[7–9]. Chylomicrons are triglycerides-rich lipoprotein particles, which are formed in the endoplasmic reticulum of enterocytes and transport triglycerides to adipose, skeletal and cardiac tissues. These structures have now been identified as contributors to arterial cholesterol accumulation and consequently, are at the core of dyslipidaemia-induced atherosclerosis in obese and insulin resistant individuals[10]. While it is acknowledged that GLP-1 impacts upon host satiety and weight, factors which could credibly alter lipid metabolism, these studies

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ORIGINAL INVESTIGATION

Differential effects of psychotropic drugs on microbiome composition and gastrointestinal function

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Abstract

Rationale Growing evidence supports a role for the microbiota in regulating gut-brain interactions and, thus, psychiatric disorders. Despite substantial scientific efforts to delineate the mechanism of action of psychotropic medications at a central nervous system (CNS) level, there remains a critical lack of understanding on how these drugs might affect the microbiota and gut physiology.

Objectives We investigated the antimicrobial activity of psychotropics against two bacterial strain residents in the human gut, *Lactobacillus rhamnosus* and *Escherichia coli*. In addition, we examined the impact of chronic treatment with these drugs on microbiota and intestinal parameters in the rat.

Results In vitro fluoxetine and escitalopram showed differential antimicrobial effects. Lithium, valproate and aripiprazole administration significantly increased microbial species richness and diversity, while the other treatments were not significantly different from controls. At the genus level, several species belonging to *Clostridium*, *Peptoclostridium*, *Intestinibacter* and *Christenellaceae* were increased following treatment with lithium, valproate and aripiprazole when compared to the control group. Animals treated with escitalopram, venlafaxine, fluoxetine and aripiprazole exhibited an increased permeability in the ileum.

Conclusions These data show that psychotropic medications differentially influence the composition of gut microbiota in vivo and that fluoxetine and escitalopram have specific antimicrobial activity in vitro. Interestingly, drugs that significantly altered gut microbial composition did not increase intestinal permeability, suggesting that the two factors are not causally linked. Overall, unravelling the impact of psychotropics on gastrointestinal and microbiota measures offers the potential to provide critical insight into the mechanism of action and side effects of these medications.

Keywords Psychotropics · Intestinal permeability · Gut microbiota · Diversity · Richness · Short-chain fatty acids · Antimicrobial

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Introduction

The burden of psychiatric disorders on society continues to grow, with estimates from the World Health Organization (WHO) suggesting that worldwide, 322 million and 60 million people are affected by depression and bipolar disorder, respectively (World Health Organization 2017). Treatment options used in the management of psychiatric disorders are often associated with metabolic side effects (Beyazyüz et al. 2013; Correll et al. 2015; Olguner Eker et al. 2017; Reynolds and Kirk 2010; Tschoner et al. 2007) and high non-response rates (Ackenheil and Weber 2004; Al-Harbi 2012; Hatta and Ito 2014; Nelson 1998). Over the last decade, a growing body of evidence has highlighted a significant role for the gut microbiota in interactions between the gut and the brain (Bercik et al. 2012; Collins et al. 2012; Cryan and Dinan 2012b; Dinan et al. 2013; Mayer et al. 2014; Mayer et al. 2015). Moreover,

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