PROBIOTIC EFFECTS ON ADULT ANXIETY AND SYSTEMIC INFLAMMATION AFTER EXPOSURE TO WESTERN DIET

by

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DEDICATION PAGE

This thesis is wholeheartedly dedicated to my partner, Matthew, for all his love, motivation, and support during the completion of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Research shows that the microbiome-gut-brain axis affects physiological systems related to food intake, obesity, anxiety, and inflammation. These disorders are related to stress exposure and stress responding is influenced by programming events during early-life. In this study, we investigated the impact of early-life probiotic supplementation and lifelong Western diet exposure on metabolic consequences, systemic inflammation, and anxiety-like behaviours in adulthood. Overall, it was found that probiotic treatment: 1) led to a greater inflammatory response following acute stress perhaps due to the lack of inhibition from enhanced stress hormone release; 2) may lead to fewer escape behaviours during anxiety testing; 3) mitigated the effects of weight gain after Western diet exposure in males; and 4) prevented weight gain in response to increased food intake in females. These results have implications for the treatment of anxiety and metabolic disorders along with highlighting the importance of diet and sex differences on health outcomes.

LIST OF ABBREVIATIONS USED

UNITS

%	Percentage
°C	Degrees Centigrade
Cq	Quantification cycle value
cm	Centimetre
mm	Millimeter
M	Molar
mM	Millimolar
µM	Micromolar
g	Gram
mg	Milligram
µg	Microgram
ng	Nanogram
pg	Picogram
L	Litre
mL	Millilitre
µL	Microlitre
CFU	Colony Forming Unit
W	Watt
RPM	Revolutions Per Minute
g	Standard gravity
m/s	Meters per second
s	Second

DISCIPLINE SPECIFIC ACRONYMS

GD	Gestational Day x
Px	Postnatal Day X
HPA	Hypothalamic-Pituitary-Adrenal
MGB	Microbiome-Gut-Brain Axis
ARC	Arcuate Nucleus
PVN	Paraventricular Nucleus
GRE	Glucocorticoid Responsive Element
CORT	Corticosterone
CRF	Corticotropic-Releasing Factor
ACTH	Adrenocorticotropic Hormone
GR	Glucocorticoid Receptor
MR	Mineralocorticoid Receptor
PTSD	Post-Traumatic Stress Disorder
LDB	Light-Dark Box
OFT	Open-Field Test
IBD	Irritable Bowel Disease
SH	Standard Housing
SNH	Semi-Naturalistic Housing

MOLECULAR TERMINOLOGY

bp	Base Pairs
qPCR	quantitative Polymerase Chain Reaction
RT-qPCR	Reverse Transcription qPCR
NPY	Neuropeptide Y
PYY	Peptide YY
PP	Pancreatic Polypeptide
RPL13a	Rat Ribosomal protein L13a
DNA	Deoxyribonucleic Acid
cDNA	complementary DNA
RNA	Ribonucleic Acid
mRNA	messenger RNA
DAMPs	Damage-Associated Molecular Patterns
PBS	Phosphate Buffered Saline

INFLAMMATORY MARKERS

IL-x	Interleukin-x
NF-κβ	Nuclear Factor kappa beta
IFN-γ	Interferon gamma
TNF-α	Tumor Necrosis Factor alpha
CRP	C-Reactive Protein
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte/Macrophage-CSF
M-CSF	Macrophage-CSF
GRO/KC	Growth Related Oncogene/ Keratinocyte Chemoattractant
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
MIP-1α	Macrophage Inflammatory Protein-1 alpha

PMSF	Phenylmethylsulfonyl Fluoride	MIP-3α	MIP-3 alpha
DMS	Dimethyl Sulfide	VEGF	Vascular Endothelial Growth Factor
		MCP-1	Monocyte Chemoattractant Protein-1

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CHAPTER 1 INTRODUCTION

1.1 THE MICROBIOME-GUT-BRAIN AXIS

1.1.1 Overview of the Microbiome

Comprising an estimated 100 trillion cells in the human body (i.e., 10-fold greater than the number of human cells; Qin et al., 2010), microbes are abundant and have a profound impact on our biology and behaviour. Analyses of human fecal DNA reveal a catalog of 3.3 million microbial genes (99.1 % bacterial); 150-fold more than the human genes that are present (Qin et al., 2010). Due to acid, bile, and pancreatic secretions, most bacteria have difficulty colonizing the stomach and proximal small intestine but are found in great abundance in the distal small intestine and large intestine (O'Hara & Shanahan, 2006).

In the gut, recent research has shown that the microbiome (i.e., the gut bacterial composition, the gut microflora) plays a role in many physiological systems related to food intake and obesity, even being implicated in the development of neuropsychiatric disorders such as anxiety and depression (Kao, Harty, & Burnet, 2016). Described as an "essential organ" in the human body (Eckburg et al., 2005, p. 1635), two major bacterial phyla make up around 90% of the human microflora: the Bacteroidetes and the Firmicutes (Eckburg et al., 2005).

1.1.2 Overview of Probiotics

The International Scientific Association for Probiotics and Prebiotics (ISAPP) came to a consensus that a probiotic is a regulated compound that, when administered in specific amounts to a host species, confers a health benefit. Included in this classification are probiotic drugs, probiotic medical foods, probiotic foods, non-oral probiotics, probiotic animal feed, defined microbial consortia, probiotic dietary supplements, and probiotic infant formulas. Products not included are dead microbes, fermented foods with undefined microbial content, and undefined consortia including fecal microbiota transplants (Hill et al., 2014). In contrast, the term 'prebiotic' has changed slightly over

recent years, but an agreed upon definition is any ingredient that is fermentable by digestive bacteria (e.g., inulin fiber) that affects the microbiota (its composition or its activity) and benefits host health (Roberfroid et al., 2010). For instance, many prebiotics target the Lactobacilli and Bifidobacteria genera to stimulate the growth or activity of these beneficial microbes (Slavin, 2013).

In Canada, there are certain bacterial species that, when delivered in food or as a supplement at a dose of 1×10^9 colony forming units (CFU), are recognized to provide general health benefits. The list comprises *Bifidobacterium* species (i.e., *adolescentis, animalis, bifidum, breve, longum*) and *Lactobacillus* species (i.e., *acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus, salivarius*; Health Canada, 2009). Important to consider in the definition of probiotic is the commensal microorganisms that inhabit the human gut from which specific strains of probiotics are derived (Hill et al., 2014). Furthermore, according to the ISAPP, "the current state of science does not allow the clear definition of a healthy gut microbiota based on microbial composition" (Hill et al., 2014, p. 508).

In the literature, probiotics are most often discussed as promoting a healthy digestive tract and a healthy immune system (Hill et al., 2014). In their review, Hill and colleagues (2014) conclude that many probiotics do promote a healthy digestive tract due to their benefits related to diarrhea, gut transit time, irritable bowel syndrome, abdominal pain and bloating, ulcerative colitis, and necrotizing enterocolitis. Specifically, there seems to be broad mechanisms underlying the health benefits of probiotics (e.g., inhibition of potential pathogens, production of specific metabolites and enzymes) that are beginning to be characterized in research. The promotion a healthy immune system (e.g., preventing allergies, infection) by probiotics seems to be less widespread and more strain-specific. Other more recent claims that probiotics aid with reproductive function, oral health, lung health, skin health, and the gut–brain axis are under-researched and also strain (not species) specific (Hill et al., 2014).

1.1.3 The Importance of the Microbiome-Gut-Brain Axis for Health

The vast number of bacterial species that inhabit the gut are susceptible to various environmental factors such as antibiotics, diet, immune dysfunction, and infection which can alter the microbial composition toward a harmful or disease-inducing state (i.e., dysbiosis; Stecher, Maier, & Hardt, 2013). To maintain homeostasis, bidirectional communication between the gastrointestinal system and the brain (i.e., the gut-brain axis) is required (Cryan & O'Mahony, 2011). This communication is regulated peripherally and centrally at neural, hormonal and immunological levels (Cryan & O'Mahony, 2011). Grenham, Clarke, Cryan, and Dinan (2011) and Moloney, Desbonnet, Clarke, Dinan, and Cryan (2014) describe the microbiome as a crucial part of the gut-brain axis that contributes to health and disease (e.g., by affecting digestion, immune function, brain development) and proposed updating the term "gut-brain" axis to "Microbiome-Gut-Brain" (MGB) axis. Furthermore, the authors describe the therapeutic possibilities of targeting the microbiome to treat specific disorders that can result from dysbiosis (e.g., stress related disorders, gastrointestinal disorders). It is also emphasized that research has yet to elucidate what differentiates a normal from abnormal microbiome as well as the mechanisms by which this bacterial composition leads to changes in disease progression (Grenham et al., 2011; Moloney et al., 2014).

Barker's early programming of adult disease hypothesis states that prenatal, postnatal, and host genetics play a role in brain health and adult disease onset (Barker, 2004). Recently, Codagnone et al. (2018) proposed an addition that accounts for the fact that the host microbiota is both influenced by, and can in turn influence, the previously stated factors that affect adult disease outcomes; thus, it should be considered a fourth programming agent. In their detailed review on the consequences of the early-life microbiome on later health outcomes, Stiemsma and Michels (2018) conclude that pre and postnatal factors can alter a newborn's microbiome composition and that environmental exposures leading to dysbiosis (e.g., antibiotic use, poor diet, infection) can have dire consequences for health and disease development later in life. They stress that multifaceted approaches to analyse causal data that combine epigenetic,

transcriptome, and microbiome analyses are required to gain a clearer picture of the ways in which the human microflora interacts with the host to affect disease susceptibility and overall health.

In their review, Rieder, Wisniewski, Alderman, and Campbell (2017) further describe that, although studying the MGB axis is at its early stages, the extant literature suggests that the microbial environment has critical impacts on immune function, adaptive stress responding, neural functioning, and behaviour. Moreover, Rieder et al. (2017) conclude that important communication routes within the MGB axis include the vagus nerve, the neuroendocrine system, neurotransmitters within the central nervous system, and growth factors. Hill and colleagues (2014) describe that determining which strains are depleted or eliminated from dysbiosis and effectively replacing them is an important area for the prevention and treatment of obesity and inflammatory disease caused by dysbiosis. As it stands, no causal relationship between microbial composition and disease has been shown, but there are associations between dysbiosis from antibiotics and later development of obesity (Trasande et al., 2013) and Irritable Bowel Disease (IBD; Hviid, Svanstrom, & Frisch, 2011). Overall, although research is gaining a clearer picture of which diseases are associated with dysbiosis (e.g., diabetes, celiac disease, metabolic syndrome) by comparing microbial colonization patterns to healthy controls, the idea of what comprises a 'healthy microbiome' is yet to be determined (Bäckhed et al., 2012).

1.2 The Stress Response and Mental Health

1.2.1 Overview of Stress Responding Behaviours

Stress refers to a set of responses to an adverse environmental or physiological condition to regain homeostasis in the body (Reichmann & Holzer, 2016). In all vertebrate animals, including humans, a perceived stressor activates noradrenergic neurons in the brain stem, both sympathetic and parasympathetic systems, and the Hypothalamo-Pituitary-Adrenal (HPA) axis, which leads to the release of hormones that have many effects on bodily functioning in response to that stressor (Björntorp, 2001, Smith & Vale, 2006).

Specifically, the HPA axis is comprised of the Paraventricular Nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary, and the adrenal glands (Smith & Vale, 2006). Within the PVN, specialized neurons synthesize and release Corticotropin-Releasing Factor (CRF) in order to regulate the HPA axis (Smith & Vale, 2006). CRF binds to the anterior pituitary gland which induces the release of Adrenocorticotropic Hormone (ACTH) to the circulatory system (Smith & Vale, 2006). ACTH acts mainly on the adrenal cortex to stimulate the synthesis and release of glucocorticoids and catecholamines (Smith & Vale, 2006).

Corticosteroids include mineralocorticoids (e.g., aldosterone) and glucocorticoids (e.g., predominantly cortisol in humans, corticosterone in rats; de Kloet, Joëls, & Holsboer, 2005). Glucocorticoids bind to mineralocorticoid and glucocorticoid receptors (MRs and GRs, respectively) and receptor complexes (MR and GR homodimers or heterodimers; de Kloet et al., 2005) in the brain and periphery (Smith & Vale, 2006). The binding is crucial for the regulation of the HPA axis through negative feedback mechanisms (i.e., to control the magnitude and duration of the stress response; Smith & Vale, 2006). Broadly, MR-specific binding occurs early in the stress response, whereas GRs are bound when glucocorticoid concentrations are high as a way of terminating the stress response and facilitating storage of the event as a memory for future use (de Kloet et al., 2005). More specifically, MR/GR homodimers and heterodimers can interact with Glucocorticoid Responsive Elements (GREs) and lead to repression or continuation of the stress response (de Kloet et al., 2005; Smith & Vale, 2006). Conversely, when glucocorticoids bind to GR monomers, there is a conformational change in the GR causing it to dissociate and translocate from the cytosol into the nucleus of the cell where it can interfere with the stress response by stopping the formation of stress hormones (e.g., CRF, vasopressin) via interaction with specific transcription factors (e.g., nuclear factor-κβ; NF-κβ; de Kloet et al., 2005; Smith & Vale, 2006).

There are important individual differences in the stress response where an individual can be more prone to a vulnerable stress phenotype as a result of genetic and/or environmental factors, especially during early-life (de Kloet et al., 2005). Specifically,

genetics can modify stress responding in the face of environmental factors and these environmental stressors can also impact genetics through epigenetic mechanisms (i.e., heritable or non-heritable changes in the structure of genetic material, not the specific sequence, as a result of the environment; Ebner & Singewald, 2017). For example, in rodents, chronic separation from their mother early in life enhances emotional and stress responsiveness to acute stressors later in life (Ladd, Huot, Thrivikraman, Nemeroff, & Plotsky, 2004). This chronic stress also increases CRF mRNA in the amygdala and PVN and decreases GR mRNA in response to adult acute stress, interfering with the termination of the stress response (Ladd et al., 2004).

Upon cessation of the stressor, termination of the stress response in a timely manner is critical. If the body either does not terminate the stress response or releases too many glucocorticoids, there can be detrimental physiological consequences as a result (Ebner & Singewald, 2017). At a molecular level, glucocorticoids increase blood glucose indirectly by inducing glycogen breakdown by epinephrine and norepinephrine and prevent the uptake of glucose into tissues not involved with the stress response (e.g., muscles would be allotted more glucose in response to a stressor to aid with the fight vs. flight response and digestive processes would slow). Glucocorticoids also block the secretion of growth hormones, which is helpful during acute stress, but the presence of chronic stress can inhibit overall growth and long-term survival. Specifically, Brown, Varghese, and McEwen (2004) have linked the release of too many glucocorticoids to diseases such as depression, obesity, osteoporosis, and cognitive impairment.

1.2.2 Stress, Anxiety, and Behavioural Testing

Increased activation of the HPA axis is seen with psychiatric patients (e.g., with major depressive disorder, panic disorder; Flandreau et al., 2012). A basic definition of anxiety would be a state of increased physiological and behavioural activation as a result of a potential threat to survival whereas a fear response occurs when there is an immediate and real danger (Steimer, 2011). The anxiety response is designed to aid with stressful or threatening situations, but anxiety can become pathological if over-activated or activated

in the wrong situations (Steimer, 2011). To measure coping and anxiety responses in animals, researchers expose a subject to a potentially anxiety-inducing situational stressor and measure the behavioural and physiological responses that result (Steimer, 2011). Beneficial coping strategies (e.g., active coping such as fight or flight), if appropriately matched to the situation, reduce the likelihood of detrimental psychological and physiological consequences that may arise from the activation of stress mechanisms (Steimer, 2011). Importantly, an organism can be more prone to pathological anxiety or having a stress system that does not respond appropriately to its surroundings due to predisposing genetic risk or environmental factors that occur during development or during life (Steimer, 2011).

Animal studies show that exposure to situations designed to induce stress result in anxiety-like behaviours that can be measured during later behavioural testing (Steimer, 2011). For example, to demonstrate the effectiveness of an antidepressant drug, Bondi, Rodriguez, Gould, Frazer, and Morilak (2008) used a behavioural test (i.e., the elevated plus-maze) to show that after exposure to a chronic unpredictable stress paradigm, there was a decrease in observed anxiety-like behaviours with the treatment. More broadly, the anxiety-like behaviours that are measured in an experiment may vary by both the type of behaviours observed and which behavioural test is being conducted (e.g., the Open-Field Test vs. the Light-Dark Box). Specifically, the Open-Field Test (OFT; Hall, 1934) is a conflict test that demonstrates that less anxious rats will spend their time in the open centre area (due to their innate desire to explore open areas) and more anxious rats will spend their time in the perimeter (due to an innate fear of open spaces; Prut & Belzung, 2003). Similar to the OFT, the traditional Light-Dark Box (LDB) test (Crawley & Goodwin, 1980) is also used to measure the conflict between spending time in the anxiety-inducing lit area to explore and staying in the less anxiety-inducing dark area (Arrant, Schramm-Saptya, & Kuhn, 2013).

Some common behaviours measured in both the OFT and the LDB include distance travelled, rearing behaviours, time spent in anxiety inducing areas, and number of entries into the higher anxiety areas. More time spent in and more entries into the more

anxious areas, are consistently indicative of a subject that is showing low anxiety-like behaviours (Kalueff & Tuohimaa, 2004). However, while increased distance travelled (e.g., line crosses) in behavioural tests such as the OFT is regarded by some as a measure of decreased anxiety (Kalueff & Tuohimaa, 2004), locomotor behaviours such as this should be interpreted with caution when body weight is not consistent among treatment groups as a higher weight can lead to less locomotion regardless of level of anxiety (Barrow & Leconte, 1996). Rearing is another behaviour that is commonly examined, but often measured inconsistently by different research groups. Rearing may depend partly on exploration (i.e., a low anxiety behaviour) but can also vary due to overall locomotor ability which is influenced by more than just anxiety (e.g., by weight, sex; Tanaka, Young, Halberstadt, Masten, & Geyer, 2012). It has recently been recommended that the types of rearing (e.g., unsupported vs. supported) are included in studies as separate variables with unsupported rearing as an indicator of exploration and supported rearing as a locomotor measure to be interpreted with caution (Sturman et al., 2018).

1.2.3 Sex Differences in Anxiety-Like Behaviours

In animal models of anxiety, the majority of extant literature has focused on the behavioural patterns of males only (Kokras & Dalla, 2014) and does not consider the higher prevalence of anxiety in women compared to men (i.e., 4.6% vs. 2.6% globally and 7.7% vs. 3.6% in the Americas, respectively; WHO, 2017). Indeed, important differences in rodent behaviour are evident by sex (e.g., due to the presence and levels of various steroid hormones; Palanza, 2001). Furthermore, the type of anxiety test given seems to influence which sex is deemed more anxious which again highlights the important of behavioural research using both sexes (Palanza, 2001; Kokras & Dalla, 2014). For example, it has been shown that females are more anxious than males in the presence of a predator odour (Blanchard, Shepherd, de Padua Carobrez, & Blanchard, 1991) and males have more aversion to light compared to females in the LDB (Kokras & Dalla, 2014). In the OFT, males are usually less active than females when it comes to rearing behaviour and overall locomotion (Blizard, Lippman, & Chen, 1975) which

highlights a potential confound for studies interpreting increased locomotion as decreased anxiety (Kokras & Dalla, 2014).

1.2.4 Probiotics as Therapeutics for Anxiety and Stress Disorders

Stress exposure is associated with changes in the microbial environment whereby animals exposed to stressors show a depletion in beneficial bacteria from their microbiome (Fourie et al., 2017). In the literature, there has been a recent focus on how we can use probiotics to modulate both the behaviour and physiology of a host. There seems to be a critical period after birth where bacteria will colonize the gut and remain stable throughout the life of the animal (Sudo et al., 2004). Exposure to microbes deemed to be beneficial early in life has been shown to regulate and normalize the development of the HPA axis, leading to a more stable and adaptive stress response (Sudo et al., 2004).

Using live *Lactobacillus rhamnosus* (strain R0011; 95% composition) and *Lactobacillus helveticus* (strain R0052; 5% composition; Lacidofil®), Gareau, Jury, MacQueen, Sherman, and Perdue (2007) demonstrated that probiotic administration to maternally separated rats of both sexes decreased the HPA response as measured by decreased serum corticosterone (CORT) at 20 days old. Based on the premise that there is a relationship between gut health and stress/mood disorders, another study demonstrated that a formulation of Probio'Stick^{TM} (i.e., *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) improved stress-induced gastrointestinal symptomatology in healthy adults (Diop, Guillou, & Durand, 2008). Also using Probio'Stick^{TM}, Messaoudi et al. (2011) demonstrated decreased anxiety-like behaviours in rats and improved levels of psychological distress in human participants. Another *Lactobacillus* strain, *L. farciminis*, has been shown to lead to a decreased HPA axis response following acute stress exposure in adult female rats (Ait-Belgnaoui et al., 2012). In another study, Probio'Stick^{TM} was also successful in reducing the stress response by regulating glucocorticoid negative feedback in the HPA axis of adult male mice (Ait-Belgnaoui et al., 2018).

1.3 Obesity and Feeding Behaviours

1.3.1 Gut Microbes and Obesity

Obesity rates worldwide have almost tripled in the last 40 years (World Health Organization [WHO], 2018). As of 2016, approximately 25% of the planet was overweight (i.e., body mass index greater than 25) of which almost 10% were considered obese (i.e., body mass index greater than 30; WHO, 2018). What is of more concern is that there has been a jump in rates of childhood obesity, which increases the onset of both childhood and adult disease. Notably, the prevalence of children and adolescents (aged 5-19) who are overweight or obese has risen from 4% (in 1975) to 18% (in 2016; WHO, 2018). Although its causes are multifaceted, at its core, obesity develops due to an imbalance of energy taken up and used by the body; whereby if energy intake is greater than the energy used by the body, then excess energy is stored as fat and increases over time (Kolida et al., 2017; Turnbaugh et al., 2007).

Previously described as an "essential" (Eckburg et al., 2005, p. 1635) or a "microbial" (Jia et al., 2008, p. 123) organ, recent literature has updated the definition of the human gut microbiota to describe a "metabolic" organ as its vital role in metabolic dysfunction and obesity is now a focus (Guinane & Cotter, 2013, p. 295). In one study, researchers found an association that bacteria of the Bacteroidetes phyla are decreased by 50% in obese compared to lean people, whereas bacteria of the Firmicutes phyla are increased by 50% comparatively in the obese participants (Ley et al., 2005). Nevertheless, a more recent meta-analysis (Walters, Xu, & Knight, 2014) cautions against the robustness of using the Firmicutes:Bacteroidetes ratio as it does not seem to be a consistent tool to compare lean and obese human microbiota and that microbiome sequencing technology is still in its infancy. One review also cautions use of the Firmicutes:Bacteroidetes ratio and has further linked obesity to increased presence of members of the phylum Actinobacteria, genus *Bacteroides* (inconsistent among studies), and family *Prevotellaceae* along with decreased overall bacterial diversity, species *Ruminococcus*

flavefaciens, genus *Bifidobacterium,* and genus *Methanobrevibacter* (Guinane & Cotter, 2013).

The literature has yet to elucidate if diseases such as obesity and type 2 diabetes are directly caused by changes to the composition of the microbiome or if the progressive development of these diseases (e.g., by unhealthy diet) changes the microbiome in measurable ways (Guinane & Cotter, 2013). Although limited, research has begun characterizing the mechanisms behind why certain bacteria may be beneficial for metabolic diseases such as obesity. An association has been shown between decreased presence of species *Faecalibacterium prauznitzii* and genus Roseburia (butyrate producing bacteria) and type 2 diabetes (Karlsson et al., 2013; Qin et al., 2012). Butyrate (along with acetate and propionate) is a short-chain fatty acid molecule found in the intestines of all mammals that is produced when dietary fiber is fermented (Cummings, 1981). A recent review by Hartstra, Bouter, Bäckhed, and Nieuwdorp (2015) hypothesizes that decreased butyrate from microbial imbalance affects satiety, hepatic glucose, and lipid production, contributing to obesity development.

1.3.2 How Stress alters Feeding Behaviours

The hypothalamus has been described as a "hub that controls appetite and energy balance" (Loh, Herzog, & Shi, 2015, p. 125). Referred to as non-homeostatic eating, the consumption of palatable foods in response to stress may decrease HPA axis activity and thus, diminish the stress response (Maniam & Morris, 2012). Grissom, George, and Reyes (2017) found that offspring mice of both sexes whose mother received a poor-quality diet during pregnancy and lactation (i.e., both low-protein and high-fat diets) showed elevated glucocorticoid release in response to stressor exposures (i.e., physiological lipopolysaccharide stressor and psychological restraint stressor).

As diet directly affects the composition of the microflora (Zinöcker & Lindseth, 2018), the microbiome has been hypothesized to affect the relationship between food intake and mental health disorders. In fact, the HPA axis has been shown to influence the balance

between energy use and storage as fat (Cagampang, Poore, & Hanson, 2011). If the HPA axis becomes maladaptive, it can affect an organism's response to stressors and increase their risk of developing metabolic syndrome, which is defined as the presence of three metabolic symptoms in an individual (e.g., overweight/obesity, insulin resistance, increased plasma triglycerides, low high-density lipoprotein cholesterol, and increased blood pressure; Cagampang et al., 2011). Pecoraro, Reyes, Gomez, Bhargavea, and Dallman (2004) report that CRF mRNA is reduced in rats given palatable foods to consume, indicative of less HPA activity. Furthermore, there is evidence that the combination of increased glucocorticoids and insulin release drive the consumption of palatable foods and lead to an increased likelihood of energy storage as abdominal fat (Dallman, Pecoraro, & la Fleur, 2005).

1.3.3 Unhealthy Diet affects the Microbiome and Metabolic Syndrome

The Western diet comprises highly processed and fried foods, refined grains, and sugary products (Jacka et al., 2010). Jia et al. (2008) state that the Western diet combined with "antibiotic abuse" (p. 124) directly affects the composition of the gut microbiome and can lead to alterations in energy use and storage. Indeed, in their study, Turnbaugh et al. (2007) conclude that obese mice have important changes in how efficiently they can absorb and use nutrients from food based on their gut microbial compositions. Another study found that mother mice who were administered Western diet (i.e., high-fat, high-carbohydrate diet) perinatally had offspring with an increased Firmicutes:Bacteroidetes ratio (compared to a low-fat, high-carbohydrate control diet; Steegenga et al., 2017) which, as previously described, has been associated with an obese phenotype (Ley et al., 2005).

Studies have also examined the effects of high-fat diet and stress on obesity development. Tamashiro, Terrillion, Hyun, Koenig, and Moran (2009) found that pups from dams who were fed high-fat diet, exposed to stressors, or exposed to both high-fat diet and stress were heavier starting at seven days old and demonstrated symptoms of insulin resistance after weaning and in adulthood. Bridgewater and colleagues (2017) found important

differences based on sex in mice that were fed a high-fat diet and exposed to chronic unpredictable stress. Specifically, male mice in the high-fat diet group exhibited more anxiety-like behaviours in the OFT (i.e., decreased distance travelled in centre area) and more anxiety-like behaviours in the elevated-plus maze (i.e., decreased time spent in and less entries into the open arms of the maze). Furthermore, when examining the composition of microbiota, female mice exposed to chronic stress had a microbial composition similar to that of female mice fed a high-fat diet throughout life with no changes in weight gain (Bridgewater et al., 2017). These results highlight the disparity in how males and females respond to high-fat diet administration and chronic stress.

1.3.4 Probiotics as a Potential Treatment for Obesity

The composition of the intestinal microbiome is established early in life and remains relatively stable over one's lifespan (Thompson, 2012). The microbiome also seems to affect obesity development (Walker & Parkhill, 2013). Extant literature focuses on *Lactobacillus* and *Bifidobacterium* strains as agents for obesity reduction (Kobyliak et al., 2016). Karlsson et al. (2011) demonstrated that supplementation with *Lactobacillus plantarum* decreased weight gain, adipose tissue around abdominal organs, and plasma leptin compared with rats supplemented with *E. coli*.

1.4 INFLAMMATION

1.4.1 Inflammation, Stress, and Mental Health

There is a strong relationship between mood disorders and overall presence of inflammation in the body (Bauer & Teixeira, 2018). Psychological stress has been associated with an increase in molecules that induce inflammation (i.e. pathogen- and damage-associated molecular patterns such as circulating uric acid; Bauer & Teixeira, 2018). In particular, Damage-Associated Molecular Patterns (DAMPs) can induce an inflammatory response centrally and peripherally in response to psychosocial stress (Fleshner, Frank, & Maier, 2017). A recent review suggests that systemic inflammation

impairs mood in both males and females, with greater impairments in females (Bekhbat & Neigh, 2017).

Bauer and Teixeira (2018) describe mood disorders as chronic stress disorders that result from a chronically impaired stress response system. This impairment likely involves the immune system as glucocorticoids regulate the HPA axis and the immune system through an anti-inflammatory, or suppressing, effect on inflammatory pathways (e.g., inhibition of pro-inflammatory transcription factor, NF-κβ; Bauer & Teixeira, 2018). Due to chronic stress, cells can become resistant to glucocorticoids by downregulation of GRs (to deal with the constant release of cortisol) and, therefore, NF-κβ is not suppressed as it should be, activating pro-inflammatory pathways. In the brain, cytokines that were released in the periphery can modulate brain regions related to mood disorders by 1) impairing plasticity; 2) altering neurochemistry; and 3) affecting neuroendocrine axes by activating microglia and astrocytes (Bauer & Teixeira, 2018).

In fact, in a review of studies on neuroendocrine and neuroinflammatory dysfunction in major depressive disorder, glucocorticoid resistance and increased levels of pro-inflammatory cytokines in plasma were found in 85% of the studies reviewed (Horowitz & Sunszain, 2015). In a meta-analysis on inflammatory markers associated with Post-Traumatic Stress Disorder (PTSD), Interferon gamma (IFN-γ), IL-1β, and IL-6 were found to be increased in patients with PTSD (Passos et al., 2015). Moreover, levels of Tumor Necrosis Factor alpha (TNF-α), IL-1β, IL-2, IL-6, and C-Reactive Protein (CRP) have been associated with anxiety disorders in the literature (Michopoulos, Powers, Gillespie, Ressler, & Jovanovic, 2017). A meta-analysis, with nearly 15,000 participants, revealed that human participants exposed to significant stress (i.e., a traumatic event) had higher levels of CRP, IL-1β, IL-6, and TNF-α (Tursich et al., 2014). Carpenter et al. (2009) found that children that were maltreated released more IL-6 in response to acute stress and had more circulating levels of plasma IL-6 compared to control participants. In contrast it has been shown that social isolation stress after weaning in rats decreased levels of both IL-6 (a pro-inflammatory marker) and IL-10 (an anti-inflammatory marker) in the hippocampus (Doherty et al., 2018). In addition, O'Mahony and colleagues (2009)

found increases in plasma CORT and TNF-α in response to maternal separation stress in newborn rats.

1.4.2 Inflammation and Obesity

As previously discussed, improper and inadequate diet choices affect the microbiome and inflammation. Pro-inflammatory cytokines are found to be increased in rats fed a high-fat diet for 12 weeks (e.g., IL-6, TNF-α, IL-1β; De Souza et al., 2005). De la Serre et al. (2010) found increased intestinal inflammation and increased intestinal permeability (i.e., decreased epithelial barrier integrity) in rats with an obesity-prone phenotype (i.e., rats that developed obesity after being fed a high-fat diet) compared to rats with an obesity-resistant phenotype (rats who did not develop obesity on the same diet previously mentioned). Interestingly, regardless of phenotype, rats fed the high-fat diet had an increase in a specific order of the Bacteroidetes, the Bacteroidales, and of the Firmicutes, order Clostridiales (de la Serre et al., 2010).

In their review, Dandona, Aljada, and Bandyopadhyay (2004) concluded that chronic overeating leading to obesity in humans may lead to oxidative stress and induce inflammation. They also found that obesity and type 2 diabetes were associated with increased IL-6, TNF-α, and CRP, and that fat cells might actually produce IL-6 and TNF-α into plasma (Dandona et al., 2004). Verdam et al. (2013) determined through cluster analysis that participants who were obese (i.e., BMI greater than 30) had a significantly decreased Bacteroidetes to Firmicutes ratio. While not detectable in all participants, fecal calprotectin (a marker of the inflammatory response) was observed in about 40% of the obese cluster and 0% of the non-obese cluster (Verdam et al., 2013).

1.4.3 Probiotics and Inflammation

As the intestinal mucosa creates the barrier between what is ingested from the external environment and the immune system (Jia et al., 2008), it is no wonder the bacteria that inhabit this mucosa have effects on immune and nervous system function. Most probiotics have anti-inflammatory properties and could be therapeutic options in diseases

arising from inflammation such as IBD and psychiatric disorders (Bambury et al., 2017). However, in order for probiotics to be used as therapeutics for these diseases, there is a crucial need for studies that can elucidate specific mechanisms of action in how probiotics affect the gut microbiome and, in turn, influence the inflammatory response. Furthermore, it remains unknown how these bacteria interact with non-neuronal cells (e.g., microglia, astrocytes) which can affect neural development, neurotransmission, immune activation in the central nervous system, and the integrity of the blood brain barrier (Fung, Olson, & Hsiao, 2017).

A probiotic combination, administered to colitis-induced rats, including a combination of Bifidobacteria, Lactobacilli, and one *Streptococcus* species, induced production of the anti-inflammatory marker, IL-10, and decreased production of pro-inflammatory analytes such as TNF-α and IL-6 in both colonic tissue and serum (Dai et al., 2013). Furthermore, Bifidobacteria have been shown to inhibit toll-like receptors (i.e., receptors that induce a pro-inflammatory response by recognizing structural components of bacteria; Bambury et al., 2017) which decrease the inflammatory response (Zhou et al., 2015). After being exposed to maternal separation stress, rats fed a specific probiotic (i.e., *Bifidobacterium infantis* 35624) were reported to have normalized noradrenaline levels, decreased IL-6, and decreased CRF mRNA in the amygdala (Desbonnet et al., 2010). In response to a combination of eight strains of probiotic bacteria, Syrian golden hamsters exhibited decreased stress behaviours in behavioural tests and decreased levels of pro-inflammatory factors (e.g., IL-1β; NF-κβ; Avolio et al., 2019).

1.5 The Neuropeptide Y System

1.5.1 NPY Background

Neuropeptide Y (NPY) is a 36 amino acid peptide and belongs to the Neuropeptide Y family of proteins along with Peptide YY (PYY) and Pancreatic Polypeptide (PP; Holzer, Reichmann, & Farzi, 2012). NPY has been shown to be active at all levels of the MGB

axis, unlike PYY and PP that exert their effects mainly on the digestive system (Holzer et al., 2012). Furthermore, NPY is the most abundant neuropeptide in the brain and found from the brainstem to the cerebral cortex (Holzer et al., 2012) and is highly conserved between animal species (e.g., shark, mammal; Dimitrijević and Stanojević, 2013).

While high levels of NPY can be found in the Arcuate Nucleus (ARC) region of the hypothalamus (Lin, Boey, & Herzog, 2004), NPY is also found in high levels in the adrenal glands where it functions with noradrenaline in the sympathetic nervous system response (Schütz, Schäfer, Eiden, & Weihe, 1998). As part of the enteric nervous system, NPY is released as a regulator of digestion (Holzer et al., 2012). Both central and peripheral NPY exert their effects via G-coupled protein receptors (namely Y1, Y2, Y4, Y5, Y6; Lin et al., 2004). The Y6 receptor is absent in rats and is considered a pseudogene in humans (i.e., non-functional; Holzer et al., 2012).

1.5.2 NPY, Stress, and Mental Health

NPY has an anti-stress effect that is seen in a variety of animal models that display varying emotional responses (Heilig, 2004). A more pronounced stress response in rats has been related to downregulation of NPY and can be significantly reduced in animals administered an NPY receptor agonist (Cohen et al., 2012). Importantly, increased NPY appears to be related to successful coping and resilience to stress in animal models (Hawley et al., 2010), and is described as promoting stress resiliency, or the ability to cope with stress, by Reichmann and Holzer (2016). In humans, levels of NPY are lower in patients with PTSD (Rasmusson et al., 2000) and intranasally delivered NPY in rats has been shown to decrease behavioural symptoms associated with single prolonged stress (i.e., an animal model of PTSD; Serova et al., 2013).

NPY has an anxiolytic effect in a variety of animal models, primarily due to effects mediated by the Y1 receptor (Reichmann & Holzer, 2016). Specifically, it has been shown to have anti-anxiety properties similar to medications designed to reduce anxiety

(Heilig, 2004). NPY seems to counteract the effects of CRF (Heilig, Koob, Ekman, & Britton, 1994), which is anxiogenic in animal models (Bakshi & Kalin, 2000).

1.5.3 NPY and Obesogenic Outcomes

Stress can contribute to the development of obesity and metabolic problems, but what remains unclear are the mechanisms behind this observed effect (Aschbacher et al., 2014). It is reported that NPY acts on the Arcuate Nucleus–Paraventricular Nucleus (ARC–PVN) of the hypothalamus as a strong appetite stimulant (Kalra & Kalra, 2004). The NPY family of peptides has also been described as one of the most important systems in the regulation of appetite and energy expenditure (Loh, Herzog, & Shi, 2015). Specifically, NPY stimulates food intake and increases energy storage as fat, which likely has important effects on obesity development and metabolic disease (Loh et al., 2015). In mice, chronic stress has been shown to increase visceral fat formation and symptoms of metabolic syndrome in response to Western diet compared to non-stressed animals (Kuo et al., 2007). In particular, sympathetic NPY stimulates the growth of these fat cells and contributes to the detrimental effects of chronic stress exposure on obesogenic outcomes (Kuo et al., 2007). NPY is increased in offspring male and female rats after mothers and offspring are exposed to a 43% high-fat diet. Increased abdominal fat and metabolic syndrome have also been observed in women that reported high levels of chronic stress and palatable food consumption (Aschbacher et al., 2014). Levels of plasma NPY were elevated in those women reporting chronic stress and the correlations of increased highly palatable food intake and levels of abdominal fat were stronger in those women who had higher levels of plasma NPY (Aschbacher et al., 2014).

1.5.4 The Immune Consequences of NPY

Immune stimulation has been shown to decrease NPY expression in the hypothalamus of rats (Kim et al., 2007). In both rats and humans, NPY receptor subtype Y1 (Bedoui et al., 2002; Bedoui et al., 2008; Petitto et al., 1994; Rethnam et al., 2010), subtype Y2 (Bedoui et al., 2002; Bedoui et al., 2008; Dimitrijević et al., 2010; Mitić, Stanojević, Kuštrimović, Vujić, & Dimitrijević, 2010; Nave et al., 2004) and subtype Y5 (Bedoui et al., 2008;

Dimitrijević et al., 2010; Mitić et al., 2010) have been found on immune cells, with subtype Y4 receptors further being identified on human immune cells (Bedoui et al., 2008). In the brain, NPY has been suggested to be an important signalling molecule between neuronal and immune cells (Malva et al., 2012). In fact, microglia cells, which protect the brain after pathogen attack or injury, have been shown to express NPY (Alvaro et al., 2007; Ferreira et al., 2010).

Overall, NPY and PYY have been reported to slow gastric emptying time, inhibit gastric and pancreatic secretions, and stimulate the absorption of water and electrolytes (El-Salhy & Hausken, 2016). PP has an almost opposite effect to that of PYY and NPY in that it stimulates gastric motility and gastric acid secretion along with relaxing the gallbladder, inhibiting pancreatic secretions, and increasing transit time through the small intestine (El-Salhy & Hausken, 2016). Multiple reviews on this topic suggest that NPY has pro-inflammatory activity through the gastrointestinal system (El-Salhy & Hausken, 2016; Farzi, Reichmann, & Holzer, 2015; Holzer et al., 2012). Indeed, NPY levels have been shown to be increased in the brain and colon of mice with chemically-induced colitis (Baticic et al., 2011; Pang et al., 2010). Of all the members of the NPY family, NPY seems to be the most important in inflammation and the development of IBD (El-Salhy & Hausken, 2016).

1.5.5 How does NPY relate to the Microbiome-Gut-Brain Axis?

The NPY family likely plays an important role throughout the MGB axis. Holzer et al. (2012) suggest that the abundance of evidence demonstrating NPY involvement in pain, mood, and stress disorders, makes studying the effects of NPY as they relate to gut-brain axis functioning critical. Within the gut mucosa, the microbiome is in direct communication with endocrine cells (Holzer et al., 2012) and NPY is reported to be an antimicrobial agent against certain gut bacteria (e.g., *E. coli, L. acidophilus*; El Karim et al., 2008).

1.6 Objectives and Preface to Thesis

1.6.1 Current Research Gap

Current research focuses on how we can manipulate the microbiome to confer health benefits with respect to the gut and brain of a host using probiotics. The *Bifidobacterium* and *Lactobacillus* genera are frequently studied as they are not comprised of lipopolysaccharide chains that induce an inflammatory response when they enter the gut. While the study of psychobiotics (i.e., prebiotics and probiotics that affect the MGB axis) is still a relatively new field of neuroscience research, the literature consistently demonstrates that these agents have effects on both psychological and physiological outcomes in humans and animals (Sarkar et al., 2016).

A crucial conclusion from many studies on probiotic administration is that the effects of these agents are dependent on the bacterial species used in the study; certain species have different effects and groups of species can either work together to lead to beneficial outcomes or cancel each other out via competition (Bruce-Keller, Salbaum, & Berthoud, 2017; Cryan & O'Mahony, 2011; Luna & Foster, 2015). Given this finding, studies on the effects of different strains of probiotics and how these probiotics communicate with the MGB axis are imperative for researchers to elucidate which bacteria may be beneficial for which specific diseases and dysfunctions (Bruce-Keller et al., 2017; Cryan & Dinan, 2012).

1.6.2 Probio'Stick™ and Mental Health

The probiotic (Probio'Stick™; Lallemand Health Solutions Inc., Montreal, Canada) used in the present experiment is comprised of *Lactobacillus helveticus* R0052, and *Bifidobacterium longum* R0175. This probiotic has been demonstrated to improve gastrointestinal function in response to stress in rats (Diop et al., 2009), improve anxiety symptoms in both rats and human participants (Messaoudi et al., 2011), decrease the stress response by affecting glucocorticoids in the HPA system (i.e., by decreasing

plasma CORT levels and normalizing GR expression in response to chronic stress; Ait-Belgnaoui et al., 2018), and prevent gut barrier impairment and corticosterone increase in response to chronic stress (Ait-Belgnaoui et al., 2014). Furthermore, in a systematic review and meta-analysis on double-blind, placebo-controlled trials testing the effects of probiotic supplementation on psychological symptoms in humans, Probio'Stick™ was effective at reducing symptoms of stress, anxiety, and depression (McKean et al., 2017).

1.6.3 Probio'Stick™ and Metabolic Consequences

Overall, the bacterial strains present in Probio'Stick™, while more established at reducing the negative symptoms associated with stress and other psychological disorders, may have an impact on food intake patterns and obesity, which warrants further investigation. As summarized above, recent research suggests that there is an important relationship between psychological distress and obesity in that psychological dysregulation may promote factors that lead to obesity such as increased weight gain, increased food intake, and chronic stress (Hemmingsson, 2014**)**. Limited research exists on the effects of Probio'Stick® on obesity and related metabolic consequences; however, in response to a high-fat diet, rats fed two Bifidobacteria strains isolated from healthy human participants showed less weight gain compared to rats not given this intervention (Yin, Yu, Fu, Liu, & Lu, 2010). Another group demonstrated that rats fed a high-fat diet in combination with a probiotic treatment that included *Bifidobacterium longum* species had lower lipid and obesity levels compared to rats that were not given the probiotic (An et al., 2011). Shin et al. (2017) demonstrated that a 6-strain probiotic that contained *B. longum* BG7 was successful in decreasing weight after a 60% fat diet administration. In another study examining the modulation effect of diet and the probiotic *Lactobacillus helveticus* R0052 on the gut microbiota, the effect of the probiotic on physiological and psychological functioning was shown to be dependent on diet (Ohland et al., 2013).

1.6.4 Probio'Stick™ and Immune Functioning

Bacteria that inhabit the intestinal mucosa have effects on immune, mood, and nervous system function (Jia et al., 2008). Some probiotics have anti-inflammatory properties and

have the potential to be used as therapeutic options in diseases arising from inflammation such as IBD and psychiatric disorders (Bambury et al., 2017). In saying this, depending on the specific strains used, research shows that probiotics can regulate, stimulate, or modulate immune responses and can have an immunoprotective effect (Azad, Sarker, & Wan, 2018). A probiotic containing *Lactobacillus helveticus* R0052, *Lactobacillus plantarum* R1012, and *Bifidobacterium longum* R0175 (i.e., two of the three strains present in Probio'Stick^{TM}) was successful in reversing an increase in the pro-inflammatory marker, TNF-α, after a chronic mild stress protocol (Li et al., 2018). There still remains a crucial need for studies that can elucidate these probiotic-strain specific effects as the area remains largely unexplored (Fung, Olson, & Hsiao, 2017).

1.6.5 The Microbiota and NPY

To summarize prior research relating to NPY, high levels of NPY both stimulate appetite and lead to a more adaptive response to stress; yet, high levels of stress, which are associated with lower levels of NPY (Cohen et al., 2012), can induce food intake and increase levels of abdominal fat (Maniam & Morris, 2012). Thus, the relationship between stress and food intake is not clear, especially with respect to the role of NPY. As NPY is expressed throughout the MGB axis by enteric neurons, primary afferent neurons, and a large number of neurons in the brain, microglia, and immune cells (Holzer et al., 2012), its effects on the MGB axis necessitate investigation. While there are no specific studies examining the effects of Probio'Stick^{TM} or its strains on NPY levels, Lesniewska et al. (2006) found that a probiotic formulation with *L. rhamnosus* GG and *B. lactic* Bb12 combined with inulin fiber (a prebiotic) increased plasma NPY levels in adult (i.e., 6-month-old) rats compared to before treatment. However, a study in zebrafish found that although *L. plantarum* decreased anxiety-like behaviours, they did not find a difference in NPY levels between the probiotic and control groups (Davis et al., 2016). In another study on tilapia larvae, *Lactobacillus rhamnosus* IMC 501® treatment increased NPY mRNA levels (Giorgia et al., 2018).

1.6.6 Goals and Hypotheses

In their recent review on the effects of psychobiotics on health and disease, Sarkar and colleagues (2016) detail important gaps in the literature that should be addressed and suggest that probiotics and prebiotics be studied separately to effectively tease out their differential effects. First, to address the knowledge gap of "ecosystem and structural change" (Sarkar et al., 2016), we will examine if probiotic supplementation could lead to changes in the composition of the microbiome (e.g., taxonomic differences, diversity) after administration across the subjects' lifespan, including mothers during pregnancy and lactation. Second, we will tackle the question of the "role of moderators" (Sarkar et al., 2016) in that we will examine what other factors may alter the effects of our probiotic, including sex and diet.

As previously described, the literature has consistently shown that the MGB axis is related to a variety of behavioural and physiological outcomes including anxiety (e.g., Luna & Foster, 2015), diet-related behaviours and obesity (e.g., food intake; Hussain & Bloom, 2013), immune function (Higgins, Frankel, Douce, Dougan, & MacDonald, 1999) and specific neuropeptide levels such as Neuropeptide-Y (NPY; Holzer, Reichmann, & Farzi, 2012), which will be the focus of this thesis. With this present study, we aim to increase knowledge on how Probio'Stick^{TM} affects these outcome measures. The overall goal of this experiment is to examine if supplementation with Probio'Stick^{TM} (across the lifespan, including prenatally) has a protective effect on weight and food intake changes as a result of Western diet (i.e., high-fat and high-carbohydrate) exposure. To add, our aim with this experiment is to examine whether other health consequences such as anxiety-like behaviours in response to stress, systemic inflammation, and NPY levels vary based on our experimental manipulations.

We hypothesize that probiotic treatment with Probio'Stick^{TM} will improve health outcomes in our subjects compared to a placebo group. Based on previous literature, we expect probiotic treatment will lead to decreased weight gain, food intake, systemic inflammation (i.e., lower pro-inflammatory markers, higher regulatory and anti-

inflammatory markers), and anxiety-like behaviours after acute stress exposure. We also expect that inflammation in plasma will be complementary to inflammation in the brain. While NPY is an appetite-stimulating hormone (Loh et al., 2015) that is related to increased inflammation (Farzi, Reichmann, & Holzer, 2015), it is also related to successful stress coping (Hawley et al., 2010). Previous, although limited, research has suggested that probiotic treatment may increase NPY (Giorgia et al., 2018; Lesniewska et al., 2006). Thus, we hypothesize that probiotic treatment in this experiment will also increase NPY.

We further hypothesize that Western diet administration will worsen these previously stated health outcomes compared to a control diet in that this unhealthy diet will increase weight, food intake, levels of systemic inflammation, and anxiety-like behaviours. Overall, we hypothesize that NPY will be increased in the group administered a Western diet (Choi, 2018). We also expect that probiotic administration could counteract some of the negative impacts of unhealthy diet in subjects administered both a probiotic and Western diet.

With respect to sex differences, we predict that males will have increased food intake and adulthood weight compared to females (Bridgewater et al., 2017). Based on previous literature, we also anticipate that males will demonstrate more anxiety-like behaviours than females in the LDB control trial, the LDB vanilla trial, and the OFT. Nonetheless, we hypothesize that females will have more of an aversion to the cat urine stimulus in that trial of the LDB. We further hypothesize that there will be no difference by sex in levels of NPY (Choi, 2018). Due to the lack of research examining sex differences in relation to systemic inflammation with probiotic and diet exposure, we have no specific hypotheses related to overall levels of inflammatory markers by sex.

CHAPTER 2 METHODOLOGY

2.1 ANIMALS AND BREEDING

All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the Dalhousie University Committee on Laboratory Animals (protocol #18-023). All rats were housed in a colony room maintained at 21 °C ± 2 °C under a 12h:12h reversed light cycle (lights off at 1000h). Timeline for experimental procedures is summarized in Figure 1.

2.1.1 Pre-Breeding

A total of 16 Long-Evans hooded rats (8 females, 8 males) were ordered from the supplier (Charles River Laboratories, Quebec) to be used for breeding. All male breeders were born on 02/20/2018 and arrived with an approximate weight of 275 – 300g from colony room "R06" at the supplier. All female breeders were born on 02/19/2018 and arrived with an approximate weight of 200-225g from colony room "R06" at the supplier.

Upon arrival, breeders were given two weeks to acclimate to our facilities before any experimental manipulation. They were housed in same-sex pairs, in standard housing polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids, with Shaw Resources "Fresh Bed" softwood bedding (Rona, Canada), and with a black PVC tube (12 cm length, 8 cm diameter). Rat chow (Rodent Laboratory Chow, St. Louis, MO, USA) and tap water were supplied *ad libitum*.

2.1.2 Breeding Protocol

A total of four naïve breeding pairs were placed in a colony room designated 'probiotic' (PR) and the remaining four breeding pairs were placed in a similarly sized colony room designated 'placebo' (PL). Breeding for all pairs was achieved by placing one male and one female together in a standard home cage for seven days in identical housing conditions as the acclimatization period, after which time the female was assumed to be

pregnant and the male was removed. Male breeders were housed alone after breeding due to aggressive behaviour between male rodents being reported post-breeding (Taylor, 1980). Along with a PVC tube (12 cm length, 8 cm diameter), males were given Froot Loops (Costco Wholesale Canada, Halifax, NS) and wooden blocks (Home Depot, Halifax, NS) as a form of environmental enrichment to help to counteract the stress of housing in isolation. Female breeders were housed in pairs until estimated gestational day 17, after which they were housed singly until giving birth with a PVC tube and food and water provided *ad libitum*. Weight of the female breeders was monitored bi-weekly (Figure 2). In total, seven of the eight female breeders produced a litter; female "H" in the placebo group did not produce pups at the end of 21-days after breeding.

2.1.3 Sacrifice of Breeders

Once the female dams had given birth, male breeders in the probiotic room were sacrificed and various tissues and plasma were collected to be used as practice during molecular optimization. To reduce the number of animals used, male breeders in the placebo room were used as part of a second round of breeding in another master's student's thesis project as male breeders did not receive probiotic or placebo solutions. Female breeders were kept with their pups until weaning (i.e., postnatal day 21), after which time they were sacrificed, and tissue was collected for practice and for future analyses.

2.2 Offspring Groups

Offspring birth and wean weights are summarized in Table 1. At weaning, there was a total of 42 animals from probiotic litters and 30 animals from placebo litters. Due to the large variation in sample sizes, three of the larger probiotic litters were randomly culled from twelve offspring to eight, ensuring equal sample sizes remained in each of the four probiotic experimental groups. This left final sample sizes of 32 probiotic animals and 30 placebo animals (Table 2). Overall there was a total of eight experimental offspring groups: 1) PL-CD-M - placebo males exposed to control diet (CD; $n = 7$); 2) PL-WD-M - placebo males exposed to Western diet (WD; $n = 7$); 3) PL-CD-F - placebo females

exposed to CD (*n* = 8); 4) PL-WD-F - placebo females exposed to WD (*n* = 8); 5) PR-CD-M - probiotic males exposed to CD (*n* = 8); 6) PR-WD-M - probiotic males exposed to WD (*n* = 8); 7) PR-CD-F - probiotic females exposed to CD (*n* = 8); 8) PR-WD-F - probiotic females exposed to WD (*n* = 8). Attempts were made to maximize litter distribution in each of the eight experimental group, but due to sex ratios and our pair-housing protocols, all litters could not always be represented in each experimental group (Table 3).

2.3 CONTROL ANIMALS

A total of 4 control animals were ordered (Charles River Laboratories, Quebec) around age P40 for future qPCR spiking experiments to test for strain presence in caecum of our probiotic and placebo animals. Upon arrival, animals were given two weeks to acclimate to our facilities and housed in same-sex pairs, in standard housing polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids. Identical to the breeders for the experiment, rats were given Shaw Resources "Fresh Bed" softwood bedding (Rona, Canada), a black PVC tube (12 cm length, 8 cm diameter), along with rat chow (Rodent Laboratory Chow, St. Louis, MO, USA), and tap water *ad libitum.*

First, "C1" and "C2" were sacrificed at P65 to obtain practice tissue and control caecum that was not exposed to the probiotic or placebo solution. Control animals were sacrificed at days similar to the sacrifices in the two main experiments occurring in the lab that would require practice caecum and/or milk. "C3" and "C4" were mated to produce control milk samples for another study. After breeding, C4 (a male) was sacrificed at P94. C3 gave birth on Aug 24th (P87) and was sacrificed after lactation and offspring weaning at P118. Three control offspring were randomly chosen to grow up to adulthood (i.e., "C5", "C6", and "C7") and were sacrificed at P67. Caecum was stored at -80°C until contents were aliquoted and removed from lining. Contents were also stored at -80°C until processing.

2.4 HOUSING

2.4.1 Semi-Naturalistic Housing

On the day of parturition, PL and PR females and their litters were transferred into large cages with a burrow section (Semi-Naturalistic Housing; SNH; Figure 6). Bedding was comprised of pine shavings Shaw Resources "Fresh Bed" softwood bedding (Rona, Canada). We have previously demonstrated that SNH affects dam and offspring behaviour in particular ways, with dams showing more maternal care during rearing and offspring benefiting by having decreased anxiety behaviours in adulthood (Korgan, O'Leary, King, Weaver, & Perrot, 2018). Thus, so as to not introduce an early-life stressor into our experiment by rearing the rats in standard cages, we chose to rear all rats in the more ecologically relevant SNH housing.

2.4.2 Standard Housing

After weaning at Postnatal Day 21 (P21), same-sex littermates were caged in pairs or in threes (to avoid the stress of housing them in isolation) in Standard Housing (SH) polypropylene cages (Figure 5; 47 cm x 24 cm x 20.5 cm) with wire lids. Bedding was comprised of pine shavings Shaw Resources "Fresh Bed" softwood bedding (Rona, Canada), and a black PVC tube (12 cm length, 9 cm diameter) was also added.

2.5 PROBIOTIC DELIVERY

The probiotic or placebo was administered at a dosage of 1 billion Colony Forming Units (CFUs) per mL, per day. Per rat, this amounted to be 0.0500 g of probiotic (or placebo) per 0.5 mL of reverse osmosis water. The probiotic or placebo were delivered via syringe feeding as per the protocol developed by Tillmann and Wegener (2017) early during the light cycle (i.e., 11 am +/- 1 hour). The probiotic and placebo solutions were made daily and both the raw powder and the mixed solutions were maintained at 4°C until use to ensure bacterial viability. The probiotic powder and the placebo powder both contained a

polysaccharide food additive (i.e., maltodextrin) which was slightly sweet to ensure the probiotic was palatable for the rats, minimizing stress associated with the feeding.

The syringe feeding protocol required a 3 to 4-day training period whereby the rat was lap fed by the experimenter on a towel at 11 am +/- 1 hour. From the third day of training, the rat was held in its cage to facilitate the association of syringe-feeding with the home cage. Eventually, all rats learned to feed voluntarily from the syringe, simply by placing the syringe through the wire bars at the top of the cage and releasing the contents slowly. The training phase of holding the rat while feeding took 20 seconds to 5 minutes per rat but only seconds to deliver once the rats were trained to voluntarily consume the solution.

2.5.1 Maternal Probiotic Delivery

Female dams were randomly assigned to receive a probiotic or a placebo solution. Probiotic and placebo solutions were delivered to the dam daily, throughout pregnancy and during breastfeeding to maximize benefits to the offspring. There is evidence suggesting that probiotics, namely *Lactobacilli* and *Bifidobacteria* spp., are transferred through the act of breastfeeding; however, the mechanisms behind this transmission are yet to be elucidated (Mueller et al., 2015; Rautava, 2018). There is also recent evidence that human infant microbial gut colonization may begin *in utero* as there are shared microbiota profiles between placenta and amniotic fluid with infant meconium (Collado, Rautava, Aakko, Isolauri, & Salminen, 2016).

Females were trained with 0.25 M sucrose solution (i.e., 0.4279 g sucrose 0.5 mL reverse osmosis water) for four days prior to breeding to expedite training with probiotic or placebo solution after breeding. Sucrose was chosen as the sweetener due to the fact that the effects of artificial sweeteners on the microbiome have not been conclusive (Spencer et al., 2016) and may lead to undesired weight gain compared to sucrose (de Matos Feijo et al., 2013). Half of the females voluntarily took the syringe with sucrose solution after the four days. After the seven-day breeding period, on assumed Gestational Day 0 (GD0),

probiotic and placebo solution were delivered without added sucrose. All females voluntarily fed from the cage at that time.

2.5.2 Offspring Probiotic Delivery

As described previously for the mothers, the offspring received daily probiotic or placebo solution identical to their mother and at the same dosage. This treatment began on P22 (after weaning) and continued until sacrifice. All offspring rats learned to voluntarily take the solution after 27 days at the latest. It remains unclear if this delay and difficulty in training compared with the mother rats had to do with young age, the lack of sucrose (even though both solutions were sweetened with maltodextrin), or the physical size of the syringe compared to the size of the young rat's mouths.

2.6 DIET DELIVERY

2.6.1 Diet Composition

From weaning, all rats were fed a specific rat chow (i.e., control diet vs. high-fat + high-carbohydrate diet) and provided water *ad libitum* from P22 until sacrifice. During this time, they were weighed weekly. Diets were supplied by Research Diets (New Brunswick, NJ, USA) and contained an equivalent number of kilocalories matched by the supplier. Offspring rats were randomly assigned to receive control diet (Product #D14042701; see Table 4; Carbohydrate kcal% of 73, fat of kcal% of 10, protein kcal% of 17) or Western Diet (Product #D12079B; see Table 4; Carbohydrate kcal% of 43, fat of kcal% of 40, protein kcal% of 17).

2.6.2 Food Intake Measurements

When offspring were in early adulthood, food intake was measured for five consecutive days (i.e., P66-70 for "B", "D", and "G" litters; P65-69 for "C", "E", and "F" litters; P63-P67 for "A" litters) to determine if there were any differences in the amount of food eaten per cage of animals by treatment, diet, or sex. The protocol consisted of weighing the

starting amount of food in each cage and subtracting the final amount of food 24 h later divided by the number of rats in each cage (i.e., 2 or 3). Over five days, this allowed for four measurements in the change in food levels.

2.7 Offspring Behavioural Testing

Offspring were tested in both the OFT (Figure 3; between P60 and P61), a control trial of the LDB (Figure 4; between P62 and P63), a vanilla odour trial of the LDB (between P64 and P65), and a cat urine odour trial of the LDB (between P71 and P73). Rearing and line crosses were live scored, but all behavioural tests were recorded by video camera for future scoring of remaining measures. OF was performed under 60W red light. LDB testing was conducted under 60W red light, but with a 60W white light mounted 45 cm from the base of the light area of the testing apparatus. All behavioural apparatuses were cleaned with a solution of 70% ethanol and 30% reverse osmosis water between trials and before the first trial of the day. Furthermore, odour stimuli (i.e., vanilla, cat urine; described below) were discarded and replaced between test animals. Behaviours were monitored, and behavioural testing was conducted as per descriptions in Appendix A.

2.7.1 Open-Field Testing

The OFT employs a black plexiglass arena (77.5 cm x 77.5 cm x 34.3 cm) with no lid. The test room is illuminated with red light so as not to disrupt the circadian cycles of our rats housed in a reverse light-dark cycle. Animals were placed individually in the center of the arena at the start of the test which lasted five minutes (as in Bridgewater et al., 2017; Soulis, Papalexi, Kittas, & Kitraki, 2007). Behaviours measured were: time spent in the center, locomotion (i.e., crosses between 16 equal squares marked off by white Fisherbrand™ labelling tape), unsupported rearing in centre of open-field, supported and unsupported rearing in perimeter, and latency to move from centre. See Table 5 for breakdown of behavioural measures and Appendix B for behavioural testing scoring sheet.

2.7.2 Light-Dark Odour Testing

We modified the traditional LDB by adding three odour trials to increase the range of observed behaviours by making the light section of the LDB more or less aversive compared to a control. The trials included: 1) simple light/dark preference; 2) light/dark with vanilla odour in light; 3) light/dark with cat urine odour in light. With respect to the vanilla odour, rats are able to detect vanilla odour (Mosienko, Chang, Alenina, Teschemacher, & Kasparov, 2017; Wallace, Gorny, & Whishaw, 2002) and it has been shown to be non-aversive. Brake (1981) describes that when presented with a novel olfactory stimulus while suckling, rats acquire a conditioned preference for that stimulus. Thus, to ensure that the vanilla odour was desirable, we presented the vanilla odour from P8 to P20 (weaning on P21) in the SNH cage and further reintroduced the odour at P57 in the SH cage for 8 or 9 days prior to testing (depending on when the vanilla trial LDB occurred) in the SH cage. The vanilla extract and predator odor (i.e., the cat urine) were placed in tea strainers and hung from the top of the apparatus (approximately 35 cm above the base of the apparatus) so as not to be interfered with by the rat during testing.

Measurements during each trial included: latency time (i.e., time to enter light area), transitions between light and dark, supported and unsupported rearing in light and dark regions, time spent in light and dark, and locomotion. Locomotion was measured by frequency of line crosses using white Fisherbrand^{TM} labelling tape to divide the apparatus floor into eight equal sections. The vanilla odour consisted of 0.5 mL of pure vanilla extract (Costco Wholesale Canada, Halifax, NS) that was placed on a new cotton pad for each rat. The cat urine odour consistent of 0.5 mL of cat urine that was collected from the floor of cat colony rooms with a syringe and refrigerated during the three months prior to testing. See Table 5 for breakdown of behavioural measures and Appendix C for behavioural testing scoring sheet. During five-minute observations, the rats were placed in the dark region (made of opaque black plexiglass on three sides and clear black plexiglass) of the apparatus facing the door to the light region (made of clear plexiglass that was completely transparent). LDB dimensions were 60 cm x 30 cm x 45 cm with an

8 cm x 8 cm opening between sections in the middle and a 50/50 light-dark split (Miller, Piasecki, & Lonstein, 2011).

2.8 Offspring Sacrifice and Tissue Analysis

2.8.1 Sacrifices and Tissue Collection

All surgical equipment was cleaned and rinsed with 70% ethanol before the first sacrifice of the day and between animals to prevent cross-contamination of tissues. On specific days, sacrifices and dissections from the placebo group were always performed prior to the probiotic group. Sacrifices were conducted over five days when animals were between P71 and P75. To prepare for collection of fresh tissue for 62 rat offspring, rats were deeply anesthetized with Euthanyl (sodium pentobarbital) at a dosage of 0.1 mL/ 100 g body weight. Once it was determined they were sufficiently anesthetized by the lack of the toe pinch reflex, they were decapitated using a guillotine. Brains were removed and gross dissected for hippocampus and hypothalamus on dry ice. Then the abdominal cavity was opened, and the following tissues were collected and quickly stored on dry ice: adrenal glands, colon, caecum, small intestine, stomach, fat, liver, and spleen. All samples remained on dry ice until they could be placed in a freezer of -80^{o}C.

2.8.2 Luminex Rat Cytokine 23-Plex

For detection of immunological analytes, we used the Bio-Plex 200 system with the Bio-Plex Pro™ Rat Cytokine 23-Plex Assay Kit (Bio-Rad, cat. #12005641). This kit included the Bio-Plex Pro™ rat standard (Bio-Rad, cat. #171NZ0001), coupled magnetic beads for rat (Bio-Rad, cat. #10021236), rat detection antibodies (Bio-Rad, cat. #10021239), streptavidin-PE (Bio-Rad, cat. #171304501), assay buffer (Bio-Rad, cat. #10014822), standard diluent (Bio-Rad, cat. #9703888), 10X wash buffer (Bio-Rad, cat. #171304040), and sample diluent (Bio-Rad, cat. #10014641). We further needed the Bio-Plex™ Cell Lysis Kit for brain samples in order to effectively extract our protein. This kit included cell wash buffer (Bio-Rad, cat. #9704158), cell lysis buffer (Bio-Rad, cat. #9704159), cell lysis buffer factor 1 (Bio-Rad, cat. #9704161) and cell lysis buffer factor 2 (Bio-Rad,

cat. #9704162). Protein extraction methods for multiplex analysis in brain and plasma (Hulse, Kunkler, Fedynyshyn, & Kraig, 2004) were adapted accordingly for the type and amount of sample, and for the lab equipment being used. See Table 6 and Table 7 for descriptions and functions of all 23 analytes.

First, all hippocampus samples were weighed, and weights were recorded. Using sterile forceps, each brain sample was placed in a new 2 mL bead beating tube (Sarstedt, cat. #72.693) containing 4-5 sterile 3 mm beads (Thermo Fisher Scientific, cat. #11-312A). Exactly 1 mL of cell lysis buffer cocktail was added to each bead beating tube. To make 10 mL of cell lysis buffer cocktail, the following reagents were used: 40 µL cell lysis buffer factor 1 (Bio-Rad, cat. #9704161), 20 µL cell lysis buffer factor 2 (Bio-Rat, cat. #9704162), 9 mL cell lysis buffer (Bio-Rad, cat. #9704159), and 40 µL 500 mM Phenylmethylsulfonyl Fluoride (PMSF; Sigma-Aldrich, cat. #P7626-250mg) dissolved in Dimethyl Sulfide (DMS; Sigma-Aldrich, cat. #D2650-100 mL). Next, using the mouse brain protocol (6.0 m/s for 40 seconds, quick prep), bead beating was conducted with the MP FastPrep® 24 5G Homogenizer (MP Biomedicals). After bead-beating, supernatants were transferred from bead-beating tubes into 1.5 mL microtubes and centrifuged at 11,000 RPM for 10 minutes at 4°C.

For hippocampus samples, a 1 in 4 dilution (i.e., 25 µL of sample into 75 µL of sample diluent) was performed as previous tests determined that this was the best dilution for yielding analyte readings within the standard curve. Hippocampal samples were analyzed using the Bio-Plex 200 system as per manufacturer's instructions. Since hippocampal samples were acquired via gross dissection, we ran a DC™ protein assay (Bio-Rad, cat. #5000111) with bovine γ-globulin standard included, which is a more stable and quicker reaction than the similar Lowry protein assay. These total protein concentrations were used to calculate a ratio of the concentration of each analyte (in pg) to the total protein concentration (µg) of the sample as per Franklin and Perrot-Sinal (2006).

To prepare plasma samples after trunk blood collection, they were first centrifuged at 1,000 *g* for 15 minutes at 4°C and transferred into a clean microtube. A second

centrifugation at 10,000 *g* for 10 minutes at 4°C was then conducted to completely remove platelets and precipitates and samples were transferred into a clean microtube. Samples were diluted to 1 in 10 using Bio-Plex® sample diluent (Bio-Rad, cat. #10014641; found in the Bio-Plex Pro™ Rat Cytokine 23-Plex Assay Kit) again based on previous tests analyzing different dilutions. Identical to the brain samples, plasma samples were analyzed using the Bio-Plex 200 system following the manufacturer's guidelines.

2.8.3 RT-qPCR Analysis for NPY

Total RNA was isolated from adrenal and hypothalamus samples using a Trizol™-chloroform extraction method. mRNA from whole adrenal glands and gross hypothalamic coronal sections was isolated using Trizol™ reagent (Simms, Cizdziel, & Chomczynski, 1993). Specifically, in a fume hood, cells were lysed by added 1 mL of Trizol™ Reagent to 2 mL bead beating tubes (Sarstedt, cat. #72693) that were previously autoclaved two times and contained 4-5 3 mm glass beads (Thermo Fisher Scientific, cat. #11.312A). Tissues were homogenized by bead beating (FastPrep® 24 5G Homogenizer, MP Biomedicals) using the mouse brain protocol (6.0 m/s for 40 seconds, quick prep setting) 1-2 times depending on how easily specific samples homogenized. Tissue homogenates were added to 2 mL Phase Lock Gel-Heavy tubes (Quantabio, cat. #2302830) that were pre-spun to collect gel at tube bottoms at 1,500 *g* for 30 seconds at room temperature. In the fume hood, 250 µL of chloroform was added to each tube. Tubes were shaken vigorously by hand for 15 seconds, and then incubated at room temperature for 3 minutes. After incubation, tubes were centrifuged at 12,000 *g* for 10 minutes at 4^0C, separating the Trizol™ into a lower phenol-chloroform phase with DNA and an upper aqueous phase containing RNA.

Next, the aqueous phase containing the extracted RNA was transferred to a fresh microtube. We precipitated the RNA using 500 µL of isopropyl alcohol (2-propanol) by mixing samples by repeated inversion, incubating at room temperature for 10 minutes, and centrifuging at 12,000 *g* for 10 minutes at 4^0C. Upon examining the samples, the

adrenal samples were the only samples to produce visible pellets. Supernatant was aspirated and pellet (or cloudy remains at the bottom of the tube in the case of hypothalamic samples), was washed by adding 1 mL of 70% ethanol prepared using RNase-free water from 100% ethanol that was filtered with a 0.22 µmicron filter. Samples were vortexed to dislodge the pellet and centrifuged at 12,000 *g* for 10 minutes at 4^0C. The supernatant was again carefully aspirated, and samples were allowed to air dry open in the vents of the fume hood for 30 minutes to ensure complete evaporation of ethanol. Samples were dissolved in 50 µL of RNase-free water and quick spun. Finally, samples were incubated at 56^0C for 10 minutes while occasionally inverting solutions to allow for complete dissolution. RNA was quantified using the NanoDrop™ (Thermo Fisher Scientific) to determine purity before clean-up step. RNA samples are deemed "pure RNA" if they show a 260/280 ratio of 2.00 – 2.20 after the DNase clean-up step and a 260/230 between 1.8 and 2.00, but ideally greater than 2.00 (Thermo Scientific Technical Bulletin, T042).

To begin the RNA Clean-up step to increase purity, samples with NanoDrop™ values greater than 1500 ng/µL had their volumes increased from ~50 µL to ~100 µL by adding 50 µL of RNase-free water to ensure the RNA sample was not too concentrated as to not plug up the mini spin columns used in this step. Next, 350 µL of RLT buffer (found in the QIAGEN RNeasy Mini Kit 50, cat. #74104) was added to each tube and mixed by pipette. Then, 250 µL of 0.22 µmicron filtered 100% ethanol was added to the tubes and mixed by repeated inversion. Samples were transferred to mini spin columns (found in the QIAGEN RNeasy Mini Kit 50, cat. #74104). Tubes were centrifuged at 8,000 *g* for 1 minute at room temperature. Outflow was discarded, and columns were placed back into collection tubes.

Then, 500 µL of prepared RPE buffer (with ethanol; found in the QIAGEN RNeasy Mini Kit 50, cat. #74104) was added to the tubes and they were again centrifuged at 8,000 *g* at room temperature for 1 minute. Flow was again discarded, and tubes were placed into new collection tubes. Another 500 µL of prepared RPE buffer (with ethanol; found in the QIAGEN RNeasy Mini Kit 50, cat. #74104) was added to the tubes and they were

centrifuged at 8,000 *g* for 2 minutes at room temperature. Outflow was discarded, and columns were placed in new collection tubes and centrifuged at maximum speed (21,000 *g*) for 2 minutes at room temperature. Upper tube columns were placed into a clean RNase-free 2 mL Eppendorf tube and 50 µL of RNase-free water was added. Tubes were incubated at room temperature for 1 minute and then centrifuged at 8,000 *g* for 1 minute. Outflow was discarded and another 50 µL of RNase-free water was added to the tubes. The tubes were again incubated at room temperature for 1 minute and centrifuged at 8,000 *g* for 1 minute. Finally, RNA was quantified again using the NanoDrop™ and recorded. To determine the quality of RNA, samples were run on an Agilent Bioanalyzer RNA 6000 Nano System chip as per the manufacturer's instructions (RNA 6000 Nano Kit, Agilent Technologies, cat. #5067-1511).

To remove genomic DNA contamination, 4 µg of RNA (adrenal samples) or 250 ng of RNA (hippocampus samples) was pipetted into Eppendorf tubes along with 10X TURBO DNase Buffer, TURBO DNase, and RNase-free water. Reagents were mixed together so each tube contained 30 µL total volume. Sample tubes were incubated at 37^0C for 25 minutes after which DNase Inactivation Reagent was added to terminate the reaction. Samples were then incubated at room temperature for 5 minutes while mixing occasionally. Samples were then centrifuged at 10,000 *g* for 2 minutes and RNA was transferred to a fresh tube. Finally, 15-20 µL of supernatant was transferred into another new tube without transferring the stop reaction pellet.

To ensure that the DNase step was successful and there was no genomic DNA contamination of the RNA samples, we performed qPCR of the DNAse treated RNA (i.e., no amplification would infer no DNA was present) using SYBR green chemistry. First, primers were reconstituted to 100 µM using RNase free water (e.g., if a primer was 29.7 nmol, we added 297 µL to make a 100 µM stock concentration). Then, 60 µL of each reconstituted primer was transferred into a clean Eppendorf tube to work from in order to minimize primer contamination. Forward and reverse primers for reference gene Rat Ribosomal Protein L13a (RPL13a; i.e., 5′–GGATCCCTCCACCCTATGACA–3' and 5′CTGGTACTTCCACCCGACCTC–3', respectively; Langnaese, John, Schweizer,

Ebmeyer, & Keilhoff, 2008) and forward and reverse primers for NPY (i.e., 5′GCTAGGTAACAAACGAATGGGG–3′ and 5′–CACATGGAA GGGTCTTCAAGC–3′, respectively; Shi et al., 2009), along with SYBR, template/sample RNA, and RNase-free water comprised the master mix. RPL13a is a gene encoding for the structural component of the 60S (large) ribosomal subunit with an amplicon length of 132 bp (Langnaese et al., 2008).

The reference gene RPL13a has been previously used by Korgan et al. (2018) in a generational experiment exposing sire rats to high-fat diet and examining CRF hnRNA in the PVN of the hypothalamus. We also validated the use of RPL13a including melt curve analysis (compared to GAPDH and HPRT1 in both adrenal and hypothalamus samples) with a small (n = 7) sample of rats of different ages given probiotic or not to ensure the reference gene remains stable with probiotic treatment. The NPY primer was also validated in this optimization experiment and while amplification specificity was achieved, the melt curve analysis was inconclusive with respect to how many products were amplifying. Upon conducting PCR on the cDNA after the RT-qPCR was performed and running the amplified product on a gel, it was determined that there was only one product amplifying and it was of the expected size (i.e., 288 bp)

qPCR was run on the DNase treated RNA using the 7300 Real Time PCR System (Applied Biosystems) under the following cycling conditions: 1) Stage 1- 1 rep of 50°C for 2 minutes; 2) Stage 2- 1 rep of 95°C for 2 minutes; 3) Stage 3- 40 reps each of 95°C for 15s, 60°C for 30s, and 72°C for 30s; and 4) Stage 4 (dissociation stage)- 1 rep of 95°C for 15s, 60°C for 30s, and 95°C for 15s. No genomic DNA contamination was observed.

Next, mRNA, was converted back into cDNA for RT-qPCR. In preparation for reverse transcription, we added oligo dT, dNTP mix, and RNase-free water to our RNA but we did not normalize amount of RNA (e.g., to 1µg) in order to use all RNA available. Samples were incubated at 65^0C for 5 minutes and then incubated on ice for 1 minute. After these incubations, the reverse transcription enzyme mix containing first strand

buffer, DTT, and superscript IV (i.e., the reverse transcriptase enzyme) was added to sample tubes to convert RNA into cDNA. Another incubation was done at 50^0C for 50 minutes, and then we stopped the reaction by heating at 85^0C for 5 minutes**.** Finally, 1 µL of RNase mix was added to the sample tubes and all samples were incubated at 37^0C for 30 minutes.

RT-qPCR was conducted in two 384 well plates with standard curves in a 10 µL reaction volume containing 1 µL of 1 in 5 diluted cDNA template. For accuracy as we were using multiple plates, plates were prepared using the ep*Motion*® *5075t* liquid handling robot (Eppendorf, cat. #5075006022). Cycling conditions were identical to those for qPCR, again with an annealing temperature of 60°C. Plates were analyzed using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, cat. #1855485). Expected product size for RPL13a was 132 base pairs (bp) and 288 bp for NPY. Amplification specificity was assessed by examining melt curves. PCR efficiency for each primer on each of the two plates was calculated manually using standard curve slopes.

2.9 Statistical Analyses

All statistical analyses were performed using the IBM SPSS Statistics (Version 25; IBM Corporation, USA) software. Significance level varied based on how many comparisons were being conducted. A threshold level of 0.05 was used when analyzing differences on only one dependent variable. If cases arose where groups were compared on multiple similar dependent variables (e.g., the multiple behavioural measures observed to indicate level of anxiety), significance level was lowered to 0.01 as a way to minimize type 1 error.

Specifically, plasma inflammatory analytes (23 comparisons), hippocampal (23 comparisons), and behavioural measures (36 comparisons) were analyzed by two-way factorial Analysis of Variance (ANOVA) with treatment, diet, and sex as factors at a significance level of 0.01. Food intake, weight at weaning, weight at sacrifice, and neuropeptide Y levels were analyzed separately again using two-way ANOVA, but at a significance level of 0.05.

RT-qPCR results are presented as an expression fold change (i.e., quantification of relative changes in gene expression) in the probiotic group compared to the placebo group (i.e., the $2^{-\Delta\Delta Cq}$ method; described in Livak & Schmittgen, 2001) with a two-way ANOVA being conducted comparing the ΔCq values from the probiotic group and the placebo group by treatment, diet, and sex. Correlations were analyzed by Spearman's rho (r_s) at a significance level of 0.01 to correct for multiple correlations using the same dependent variables. Outliers were removed from analyses if they were greater or below 3 standard deviations from the mean. Sample size was determined by previous publications from our laboratory that examined similar models of diet and stress (Korgan et al., 2016, Korgan et al., 2018).

Similar to r^2, eta squared (η^2) is a statistic used with categorical independent variables (e.g., in ANOVA) to measure the proportion of total variance accounted for in the dependent variable that is associated with the independent variable (e.g., if $\eta^2 = .250$, then the dependent variable explains 25.0% of the variation in terms of the independent variable; Richardson, 2011). η^2 varies between 0 and 1 where a value of 0 indicates that none of the variance in the dependent variable is explained by the independent variable (Richardson, 2011).

Partial eta squared (η_p^2) is often used to determine the effect size with more than one independent variable (i.e., the effects of multiple independent variables or covariates are "partialled" out and mathematically controlled; Richardson, 2011). In studies with only 1 independent variable, $\eta^2 = \eta_p^2$, but in factorial designs where η_p^2 does not equal η^2, the interpretation is more complex (Richardson, 2011). In such designs, η^2 values for the independent variables and their interactions, can be regarded as additive percentages of variation and will still vary between 0 and 1 (Richardson, 2011). Due to how it is calculated, values for partial eta squared for the different independent variables and their interactions can equal a number greater than 1 when added and can no longer simply be explained as a proportion of total variance (Richardson, 2011). However, in this experiment, effect sizes were reported as partial eta squared (η_p^2) due to its wide use in research studies (Richardson, 2011) and due to having defined numerical guidelines for

the size of the effect (i.e., a small effect above 0.01, a medium effect above 0.06, and a large effect above 0.14; Cohen, 1988, pp. 284-287).

CHAPTER 3 RESULTS

3.1 DO PROBIOTICS AFFECT INFLAMMATION IN RESPONSE TO STRESS AND DIET?

Using multiplex technology, we analyzed plasma and hippocampus samples for inflammatory analytes including growth factors, cytokines, chemokines, and regulatory factors. The following analytes were not consistently measurable by the Bio-Plex Pro™ Rat Cytokine 23-Plex in plasma: IL-2, 6, 13, IFN-γ, and G-CSF. Furthermore, G-CSF was not consistently measurable in hippocampal tissue. In plasma, there were no significant effects of treatment, sex, or diet for IL-12p70, 18, and RANTES. In the hippocampus, there were no significant effects of the independent variables for the following analytes: IL-1β, IL-2, 4, 5, 6, 7, 10, 12p70, 13, 17, 18, GM-CSF, IFN-γ, MIP-1α, MIP-3α, RANTES, TNF-α, VEGF, and MCP-1. All graphs denote trending (i.e., *p*-value < .05) effects with one asterisk, significant at *p*-value < .01 with two, and significant at $p < .001$ with three.

3.1.1 Plasma Analyte Readings

A series of treatment x diet x sex ANOVAs examining differences in inflammatory analyte levels in rat plasma samples revealed that the probiotic group was higher in specific anti-inflammatory, pro-inflammatory, and regulatory analytes (as summarized in Table 8 and Figure 7). Treatment x diet x sex ANOVAs further revealed significant and trending main effects of sex (Table 9), main effects of diet (Table 10), and interactions between sex and diet for: IL-1β, IL-7, GM-CSF, GRO/KC, MIP-1α, and MCP-1 (Table 11). Specifically, Western diet exposed animals were consistently higher in IL-1β, IL-7, GM-CSF, MIP-1α, and MCP-1 than control diet exposed animals. Furthermore, males were consistently higher in IL-1β, IL-7, GM-CSF, GRO/KC, MIP-1α, and MCP-1 compared to females. There was also a significant main effect of diet for the analyte, VEGF ($F(1, 52) = 7.972$, $p = .007$, $\eta_p^2 = .133$) in that Western diet rats ($M = 473.77$; $SD =$

391.46) had significantly greater levels of VEGF compared to control diet rats (M = 233.70; SD = 240.40; see Figure 9).

IL-1β, IL-7, GRO/KC, MIP-1α, and MCP-1 sex and diet interactions were analyzed post hoc by simple effects analyses. Western diet exposed males had significantly higher IL-1β and IL-7 levels compared to control diet exposed males ($p < .001$; Table 11, Figure 8). Furthermore, Western diet males had significantly higher readings for the same analytes compared to Western diet females ($p < .001$; Table 11, Figure 8). Western diet exposed males had significantly higher GRO/KC levels compared to control diet exposed males ($p = .001$; Table 11, Figure 8). Western diet males had significantly higher readings for GRO/KC compared to Western diet females ($p = .023$; Table 11, Figure 8). Western diet exposed males had significantly higher MIP-1α levels compared to control diet exposed males ($p = .001$; Table 11, Figure 8). Next, Western diet males had significantly higher readings for MIP-1α compared to Western diet females ($p = .004$; Table 11, Figure 8). Western diet exposed males had significantly higher MCP-1 levels compared to control diet exposed males ($p = .001$; Table 11, Figure 8). Furthermore, Western diet males had significantly higher readings for MCP-1 compared to Western diet females ($p < .001$; Table 11, Figure 8).

There was a trending 3-way interaction between sex, diet, and treatment for the analyte, GM-CSF ($F(1, 51) = 4.133$, $p = .047$, $\eta_p^2 = .072$). Analyses of this interaction revealed: 1) that placebo control males (M = 57.13; SD = 28.37) had significantly lower analyte readings ($p < .001$) than placebo Western males (M = 886.52; SD = 596.70); 2) that probiotic western males (M = 458.94; SD = 435.02) had significantly higher analyte readings ($p < .001$) than probiotic Western females (M = 248.53; SD = 142.60); and 3) that probiotic Western males (M = 458.94; SD = 435.02) had significantly ($p = .006$) lower GM-CSF levels compared to placebo Western males (M = 886.52; SD = 596.70; Figure 10). Spearman correlations revealed that weight at weaning was significantly and consistently negatively correlated with multiple plasma inflammatory analytes including IL-1α, IL-4, IL-5, IL-10, IL-12p70, IL-17, M-CSF, MIP-3α, RANTES, TNF-α, and VEGF (Table 14).

3.1.2 Hippocampus Analyte Readings

A series of two-way ANOVAs examining differences in inflammatory analytes in hippocampus revealed that there was a trending sex by diet interaction for IL-1α (F(1, 53) = 4.233, p = .045, η_p^2 = .074) with Western males (M = .0228; SD = .0126) having significantly higher (p = .015) levels than Western females (M = .0143; SD = .0055; Figure 8). There was also a significant treatment by sex interaction for TNF-α (F(1, 51) = 9.233, p = .004, η_p^2 = .153). Post hoc analyses revealed: 1) that placebo females (M = 0.6563; SD = 0.2185) had significantly lower (p = .005) levels of this analyte compared to placebo males (M = 1.11; SD = 0.5173); and 2) that there was a trend (p = .026) for placebo males (M = 1.11; SD = 0.5173) having higher levels of TNF-α in the hippocampus compared to probiotic males (M = 0.7590; SD = 0.3692; Figure 11).

3.2 DO PROBIOTICS ALTER STRESS RESPONDING AND ANXIETY IN ADULTHOOD?

3.2.1 Open-Field Test

Since multiple comparisons were being conducted to analyze behavioural data, p-values less than .05 will be denoted as trending, and p-values < .01 will be considered significant. A series of treatment x diet x sex ANOVAs were conducted to analyze data recorded from the OFT. First, there was a main effect of sex in the locomotion measure, line crosses (F(1, 54) = 14.063, p < .001, η_p^2 = .207). Specifically, females (M = 94.69; SD = 24.25) crossed more lines than males (M = 72.73; SD = 21.39). There was a trending main effect of sex for total rearing (F(1, 54) = 4.173; p = .046; η_p^2= .072) whereby females (M = 27.31; SD = 11.62) reared more frequently than males (M = 21.73; SD = 10.21). With specific types of rearing, there was a trending main effect of sex (F(1, 53) = 4.390; p = .041; η_p^2= .076) with supportive rearing (in the perimeter of the open-field) where females (M = 21.77; SD = 9.24) performed more supportive rears than males (M = 17.63; SD = 8.19). There was also a main effect of treatment for total rearing (F(1, 54) = 11.19, p = .002, η_p^2 = .172) in that placebo rats (M = 29.03; SD =

11.85) reared more than probiotic rats (M = 20.22; SD = 8.69; Figure 12). When examining the types of rearing, there was a main effect of treatment for supported rearing ($F(1, 53) = 17.21$, $p < .001$, $\eta_p^2 = .245$) whereby placebo rats (M = 24.07; SD = 9.59) performed more supportive rears than probiotic rats (M = 15.81; SD = 6.11; Figure 12).

In the OFT, there was a trend for weight at weaning being positively correlated with total rearing ($r_s = 0.317$, $p = .012$) when using subjects from both treatment groups. Wean weight was also significantly positively correlated with supported rearing ($r_s = 0.449$, $p < .001$). Upon examining correlations separately for each treatment group, there was no longer a significant correlation between wean weight and total rearing in either group. Within the placebo group, there was no longer a significant correlation between supported rearing and wean weight ($r_s = -0.100$, $p = .606$), but wean weight and supported rearing in the OFT were positively correlated (a trend) within probiotic animals ($r_s = 0.413$, $p = .019$). Overall, weight at P67 in the OFT was significantly negatively correlated with line crosses ($r_s = -0.349$, $p = .006$). Within the placebo group, there was a trending negative correlation between OFT line crosses and adulthood weight ($r_s = -0.394$, $p = .031$), but adulthood weight and line crosses were not significantly correlated within probiotic animals ($r_s = -0.245$, $p = .177$).

3.2.2 Light-Dark Box

In the control trial of the LDB, there was a main effect of sex for line crosses, supported rearing in the dark, transitions, and time taken to enter (i.e., latency to) light (see Table 12) whereby females demonstrated more line crosses, supported rearing, transitions, and less time to enter the light compartment of the LDB. Similar to the OFT, there was a main effect of treatment for supported rearing in the dark ($F(1, 54) = 9.124$, $p = .004$, $\eta_p^2 = .145$) in that placebo rats (M = 15.69; SD = 4.62) reared more than probiotic rats (M = 12.44; SD = 4.75; Figure 13). Upon examining the correlation between supported rearing in the OFT and the LDB control trial, there was a trending correlation between OFT total rearing and LDB total rearing ($r_s = 0.325$, $p = .010$) and a significant correlation between

LDB supported rearing in the dark and OFT total rearing (r_s= 0.342, p = .007). There were no other significant or trending correlations for rearing behaviours between these two tests. Furthermore, there was a main effect of treatment for transitions in the control LDB in that probiotic rats (M = 11.69; SD = 4.21) transitioned more than placebo rats (M = 8.83; SD = 4.19), $F(1, 54) = 10.211$, p = .002, η_p^2 = .159 (Figure 14).

Overall, there was a trend for food intake being negatively correlated with line crosses in the control trial (r_s = -0.263, p = .039). There was also a trend for latency to light being positively correlated with food intake (r_s = 0.308, p = .016). In the control trial only, we found a trend for wean weight being positively correlated both with total rearing (r_s = 0.261, p = .040) and supported rearing in the light area (r_s = 0.293, p = .021). These correlations were no longer present when examining probiotic and placebo groups separately. Overall, there was a trend for line crosses being negatively correlated with weight at P67 in this trial (r_s = -0.294, p = .020) that was no longer significant when examining the correlation by treatment group. Transitions in the control LDB were also significantly negatively correlated with weight at P67 (r_s = -0.378, p = .002). There was a significant positive correlation with latency to light and weight at P67 (r_s = 0.425, p = .001). Further analysis of the correlation between transitions and adulthood weight determined there was no significant correlation with placebo animals (r_s = -0.220, p = .243), but there was a significant negative correlation with probiotic animals (r_s = -0.488, p = .005). Similarly, upon examining the correlation between latency to light and adulthood weight, there was no significant correlation within placebo animals (r_s = 0.365, p = .052), but there was a significant positive correlation of these variables for probiotic animals (r_s = 0.476, p = .006).

In the vanilla trial of the LDB, there were significant sex differences for line crosses, unsupported rearing in the light, and transitions between the two compartments (see Table 13) in that females performed more of these behaviours than males, although the means for unsupported rearing in the light are small and difficult to interpret. Based on a trending treatment by diet interaction in supported rearing in the dark ($F(1, 54) = 4.060$, p = .049, η_p^2 = .070), we examined differences by diet and treatment condition post hoc by

simple effects analyses and found that probiotic Western diet rats (M = 15.00; SD = 5.01) performed significantly more supported rears in the dark that placebo western diet rats (M = 10.07; SD = 4.50; p = .008; Figure 15).

Overall, food intake was significantly positively correlated with unsupported rearing in the light during the vanilla trial of the LDB and there were no correlations between wean weight and any behavioural measures. There was a trend for line crosses being negatively correlated with weight at P67 in the vanilla trial of the LDB (r_s = -0.300, p = .018) that was no longer trending when examining the correlations separately for each treatment group. There was a further trend for unsupported rearing in the dark being positively correlated with weight at P67 (r_s = 0.273, p = .033). This association was not significant or trending with probiotic animals (r_s = 0.139, p = .449), but was trending with placebo animals (r_s = 0.369, p = .049). Furthermore, weight at P67 and food intake were significantly positively correlated with unsupported rearing in the light during the vanilla trial (r_s = 0.384, p = .002; r_s = 0.418, p = .001, respectively). By treatment group, there was no significant or trending correlation between unsupported rearing in the light and P67 weight in placebo animals (r_s = 0.357, p = .057), but there was a trending positive correlation with probiotic animals (r_s = 0.403, p = .024).

Finally, there were no significant effects of treatment or diet in the cat urine trial of the LDB (designed to induce a stress response before sacrifice); however, many of the rats did not move from the dark section for the entirety of the five-minute testing period. Specifically, in the placebo group, 15 of 30 rats (50.0 %) did not enter the light area of the LDB containing the cat urine predator stimulus and 5 of 30 rats (16.67%) entered the anxiety-inducing area in under 10 seconds. Within the probiotic group, only 10 of the 32 rats (31.25%) remained in the dark area of the box and 7 of 32 (21.88%) entered the anxiety-inducting area in under 10 seconds; although chi-squared testing revealed that these differences were not significant by group. There was a trending main effect of sex (F(1, 54) = 4.855, p = .032, η_p^2 = .082) for total rearing in that females (M = 14.31, SD = 8.76) reared more than males (M = 9.87; SD = 6.15). There were no significant correlations with any weight/food measures and behaviours in this trial of the LDB.

3.2.3 ANCOVAs on Behavioural Measures in the OFT and LDB

Due to significant differences in adulthood weight by treatment, diet, and sex, a series of Analyses of Co-Variance (ANCOVAs) were conducted for all dependent behavioural variables to verify if effects remained significant after controlling for adulthood weight (see Table 15 for overview). ANCOVA results revealed that there was no longer a trend for females performing more total rearing in the cat urine trial of the LDB ($F(1, 53) = 2.835$, $p = .098$, $\eta_p^2 = .051$) and no other significant differences by treatment, diet, or sex emerged for this trial.

In the OFT, the main effect of line crosses remained significant following ANCOVA in that females still performed more line crosses than males ($F(1, 53) = 7.320$, $p = .009$, $\eta_p^2 = .121$). However, females no longer performed significantly more supported rears after controlling for weight compared to males ($F(1, 53) = 2.845$, $p = .098$, $\eta_p^2 = .052$). Two trending effects of sex emerged for time in center ($F(1, 51) = 4.740$, $p = .034$, $\eta_p^2 = .085$) and latency from centre ($F(1, 51) = 4.627$, $p = .036$, $\eta_p^2 = .083$). Males ($M = 12.64$, $SD = 13.62$) had greater latency to light compared to females ($M = 10.91$, $SD = 14.58$) and males ($M = 12$, $SD = 6.38$) also spent more time in the centre compare to females ($M = 24.67$, $SD = 13.61$). There was still a main effect of treatment for total rearing ($F(1, 53) = 8.529$, $p = .005$, $\eta_p^2 = .139$) in that placebo rats reared more than probiotic rats. There was also still a main effect of treatment for supported rearing ($F(1, 53) = 12.728$, $p = .001$, $\eta_p^2 = .197$) in that placebo rats performed more supported rears compared to probiotic rats.

ANCOVAs revealed that females still performed significantly more line crosses in the control trial of the LDB, $F(1, 53) = 8.187$, $p = .006$, $\eta_p^2 = .134$, and there was now a trend (instead of a significant effect) for females transitioning more than males in this trial, $F(1, 53) = 7.005$, $p = .011$, $\eta_p^2 = .117$. There was no longer a significant difference between males and females in the control trial of the LDB for latency to light ($F(1, 52) = 3.514$, $p = .066$, $\eta_p^2 = .062$) or supported rearing in the dark ($F(1, 53) = 3.731$, $p = .059$, $\eta_p^2 = .066$). There was still a main effect of treatment for supported rearing in the dark ($F(1,$

53) = 7.300 , p = .009, η_p^2 = .121) in that placebo rats reared more than probiotic rats and there was still a main effect of treatment for number of transitions (F(1, 53) = 9.896 , p = .003, η_p^2 = .157) in that probiotic rats transitioned more than placebo rats.

In the vanilla LDB, the trending treatment by diet interaction for supported rearing in the dark was no longer significant after controlling for weight. The main effect of sex showing that females performed more specific behaviours in the vanilla LDB was no longer significant for line crosses (F(1, 53) = 2.049, p = .158, η_p^2 = .037), no longer significant for unsupported rearing in the light (F(1, 51) = .009, p = .925, η_p^2 < .001), and no longer trending for number of transitions (F(1, 53) = 2.537, p = .117, η_p^2 = .046). The ANCOVA did reveal a trending 2-way interaction between treatment and sex for latency to light (F(1, 51) = 4.114, p = .048, η_p^2 = .075), but post hoc analyses indicated that this difference was no longer significant or trending.

3.3 Do probiotics affect NPY levels, food intake patterns or weight?

First, weight at P67 was significantly positively correlated with average food intake (r_s = 0.854, p < .001) and weight at weaning (P21; r_s = 0.450, p < .001). ANOVA analyses by treatment, diet, and sex revealed that wean weight differed significantly by treatment in that probiotic rats (M = 39.86; SD = 9.39) weighed significantly less than placebo rats (M = 52.25; SD = 5.34; F = 43.398, p < .001, η_p^2 = .446; Figure 16). Another treatment x diet x sex ANOVA analyzing weight at sacrifice (i.e., average of 67 days old) determined that there was a main effect of sex (F = 178.102, p < .001, η_p^2 = .767), a main effect of treatment (F = 8.662, p = .005, η_p^2 = .138), a significant sex by treatment interaction (F = 5.831, p = .019, η_p^2 = .097), and a significant 3-way interaction between sex, diet, and treatment (F = 8.850, p = .004 , η_p^2 = .141; Figure 17). Specifically, males (M = 413.95; SD = 54.24) weighed more than females (M = 284.82; SD = 33.54) and placebo rats (M = 359.54; SD = 88.55) weighed more than probiotic rats (M = 335.83; SD = 67.75). After analyzing the two-way interaction between sex and treatment, it was determined that males in the placebo group (M = 441.97; SD = 50.73) weighed more than males in the

probiotic group (M = 389.43; SD = 45.64; $p < .001$) whereas this difference was not apparent with females.

To examine whether food intake varied by experimental group, another two-way ANOVA was conducted with sex, diet, and treatment as factors. There was a significant main effect of sex on average food intake (F = 148.651, $p < .001$, $\eta_p^2 = .734$) in that males (M = 30.11; SD = 4.42) ate significantly more grams of food than did females (M = 19.63; SD = 3.85). There was also an interaction between treatment and sex (F = 23.129, $p < .001$, $\eta_p^2 = .300$) and between diet and sex (F = 5.246, $p = .026$, $\eta_p^2 = .089$). Upon analysis of the interactions post-hoc, it was determined that males ate more than females regardless of treatment or diet condition (ps < .001). It was also found that females in the probiotic condition (M = 21.51; SD = 2.67) ate more than females in the placebo condition (M = 17.75; SD = 3.98; $p = .003$) whereas males in the placebo condition (M = 32.57; SD = 2.74) ate more than males in the probiotic group (M = 27.95; SD = 4.55; $p = .001$). It was further determined that females in the control diet condition (M = 21.07; SD = 3.06) ate significantly more than females in the Western diet condition (M = 18.19; SD = 4.09; $p = .021$; Figure 18).

Since calories were not perfectly controlled between the two diet conditions (i.e., 4.7 kcal/g for the Western diet, 3.9 kcal/g for the control diet), were also conducted treatment by diet by sex ANOVAs to analyze differences in calories consumed between our two groups. There was a significant main effect of diet (F = 18.794, $p < .001$, $\eta_p^2 = .258$) in that Western diet animals (M = 113.72; SD = 35.87) consumed more calories compared to control diet animals (M = 98.29; SD = 22.07). There was also a significant main effect of sex (F = 155.112, $p < .001$, $\eta_p^2 = .742$) in that males (M = 129.65; SD = 23.94) consumed more calories compared to females (M = 83.84; SD = 15.85). There were also significant interactions between treatment and sex (F = 25.059, $p < .001$, $\eta_p^2 = .317$) and diet and sex (F = 11.823, $p = .001$, $\eta_p^2 = .180$). Post-hoc analyses revealed that males ate significantly more calories than females regardless of treatment or diet condition (ps < .001). It was also found that females consume significantly more calories in the probiotic (M = 92.18; SD = 11.82) condition compared to the placebo (M = 75.49; SD = 15.19; p =

.002) condition and that males consume significantly more calories in the placebo (*M* = 140.69; *SD* = 7.45) condition compared to the probiotic (*M* = 119.99; *SD* = 21.31; *p* < .001) condition. Further post-hoc analyses also revealed that in males, animals given the Western diet (*M* = 143.80; *SD* = 21.94) consumed significantly more calories compared to control diet animals (*M* = 115.50; *SD* = 16.62; *p* < .001) even though grams of food intake did not vary significantly. On the contrary, even though grams of food consumed differed significantly in females by diet condition (see above), number of calories consumed did not differ (*p* = .521). Finally, ANOVA revealed a significant three-way interaction by treatment, diet, and sex for calories consumed. Post-hoc analyses revealed that males ate significantly more calories than females in all treatment and diet conditions (ps < .001). In Western diet males it was the placebo-exposed animals (*M* = 160.64; *SD* = 7.45) that consumed significantly more calories compared to the probiotic-exposed animals (*M* = 129.07; *SD* = 19.60; *p* < .001). It was further found that in females in the Western diet condition, probiotic animals (*M* = 97.22; *SD* = 10.20) consumed significantly more calories compared to placebo animals (*M* = 73.80; *SD* = 19.36; *p* = .002). Similarly, within probiotic-exposed males, there was a trend for Western diet (*M* = 129.07; *SD* = 19.60) rats consuming more calories than control diet (*M* = 110.91; *SD* = 20.02; *p* = .016) rats and within placebo exposed males, Western diet (*M* = 160.64; *SD* = 7.45) rats consumed significantly more calories than control diet rats (*M* = 120.75; *SD* = 10.80; *p* < .001; Figure 18).

As PCR efficiency for the primers on the hypothalamus plate was different (91.75% for RPL13a vs. 187.97% for NPY), no conclusions can be made regarding these samples. Furthermore, since 3 adrenal samples for both primers were run on this plate, rats 1C, 2C, and 3C were not included in the analyses of adrenal samples. On the adrenal plate, qPCR efficiency was determined from each primer's standard curve and calculated to be 116.73% for the NPY primer and 115.22% for the RPL13a primer. RT-qPCR data of adrenal samples revealed no significant difference in NPY relative normalized expression levels between probiotic (*M* = 3.55; *SD* = 0.73) and placebo (*M* = 4.02; *SD* = 1.16) rats ($F(1,54) = 3.324$, $p = .074$; $\eta_p^2 = .058$). The expression fold change determination showed that probiotic animals were 1.38-fold higher in NPY expression than placebo

animals; however, upon removal of two placebo rat outliers, this became an expression fold change of 1.19. Furthermore, there was no significant difference between Western diet animals (M = 3.88; SD = 0.81) and control diet animals (M = 3.69; SD = 1.16; $F(1,54) = .522$, $p = .473$; $\eta_p^2 = .010$). It was found that Western diet animals were 0.87-fold lower in NPY compared to control diet animals with outliers included and 0.86-fold lower with outliers removed. There was also no significant difference in NPY relative normalized expression between male (M = 3.84; SD = 1.05) and female rats (M = 3.77; SD = 0.90; $F(1,54) = .220$, $p = .641$; $\eta_p^2 = .004$). There were two male rat values that were considered to be outliers; with those values remaining in the analyses, males were 0.95-fold lower in NPY compared to females, but with the values removed, males were 1.11-fold higher in NPY compared to females. See Figure 19 for relative normalized expression (i.e., ΔC_q values) before fold change was calculated via the $2^{-\Delta\Delta Cq}$ method outlined by Livak and Schmittgen (2001).

CHAPTER 4 DISCUSSION

With this experiment, we hypothesized that the group receiving probiotic treatment with Probio'Stick™ would have decreased weight, decreased food intake, decreased inflammation, decreased anxiety-like behaviours, and increased levels of NPY (i.e., a neuropeptide involved with both appetite stimulation and decreased stress responding). We further hypothesized that Western diet administration would worsen these previously stated health outcomes compared to a control diet in that we would see increases in weight, food intake, levels of systemic inflammation, and anxiety-like behaviours. It was also expected that although we may see increases in weight and food intake, that NPY would still be increased in the Western diet group based on previous literature. Furthermore, it was concluded that probiotic administration could counteract some of the negative impacts of unhealthy diet in subjects administered both a probiotic and Western diet. With sex, we first expected that males would have increased food intake and adulthood weight compared to females. We further hypothesized that males would demonstrate more anxiety-like behaviours on all behavioural measures aside from predator odour exposure where females would demonstrate more anxiety. We anticipated that there would be no differences by sex on levels of NPY. Due to lack of research examining sex differences in relation to systemic inflammation with probiotic and diet exposure, we had no specific hypotheses related to overall levels of inflammatory markers by sex.

4.1 Summary of Inflammation Findings

The purpose of this part of the experiment was to measure immunological analytes using the Bio-Plex Pro™ Rat Cytokine 23-Plex Assay in rat brain and plasma samples in order to directly examine differences between probiotic- and placebo-treated rats along with differences related to diet and sex. Consistently, we found that approximately 1.5 hours after exposure to acute predator odour stress (i.e., cat urine scent), probiotic-treated animals, Western diet exposed animals and males had higher levels of various types of inflammatory markers in the plasma. Contrary to our

hypothesis that probiotic treatment would decrease pro-inflammatory markers and improve anti-inflammatory and regulatory markers, it was the probiotic animals who showed unfailingly higher analyte levels independent of the type of marker (i.e., anti-inflammatory, pro-inflammatory, and regulatory). Our second hypothesis that Western diet exposure would increase pro-inflammatory markers was supported; however, we also saw increases in anti-inflammatory and regulatory inflammation markers which was not expected. Although we did not have a specific hypothesis based on sex, males repeatedly showed higher analyte readings, but, again, independent of category of analyte.

Overall, no significant effects at or below $p = .01$ were found in hippocampal tissue, besides the finding that placebo females had significantly lower levels of TNF-α compared to placebo males. As a whole, these results were contrary to our hypothesis that both the hypothalamic and plasma inflammatory marker results would be complementary to one another. This finding could be due to the fact that the inflammatory response in the hippocampus might take longer to be expressed compared to in plasma. Indeed, Ait-Belgnaoui et al. (2012) measured plasma cytokine protein levels in rats 120 minutes after partial restraint stress exposure (i.e., the same time course as our study). However, they measured cytokine mRNA (i.e., not protein as in our study) from rough hypothalamic sections also 120 minutes after the stress exposure, presumably due to an expected time delay of cytokine protein production in the brain. It is unclear how long it would take for differences in brain cytokine protein levels, but it can be assumed that this would take longer than 120 minutes and should be investigated in the future.

4.1.1 Probiotic or Placebo Treatment

After being exposed to maternal separation stress (a chronic stressor), rats fed a specific probiotic (i.e., *Bifidobacterium infantis*) had decreased IL-6 (usually regarded as a pro-inflammatory analyte) and decreased CRF (a stress hormone) mRNA in the amygdala (Desbonnet et al., 2010). Although they did not measure stress hormone levels *per se*,

chronic stress exposed Syrian golden hamsters who were given probiotics also had decreased levels of pro-inflammatory analytes and factors (e.g., IL-1β; NF-κβ; Avolio et al., 2019) in their hypothalami and plasma. Thus, in response to chronic stress over time, probiotic exposure seems to decrease the stress response and the inflammatory response compared to animals not given probiotics.

In contrast, a meta-analysis on the effects of different types of stress on the immune response in humans found that acute stressors lead to significant increases in IL-6 and IFNγ (with no probiotic manipulation; Segerstrom & Miller, 2004). It has also been shown that a two-week exposure to *L. farciminis* decreases the stress response (i.e., decreased plasma ACTH and CORT levels; Ait-Belgnaoui et al., 2012). Even though they examined levels of pro-inflammatory cytokines (i.e., IL-1β, IL-6, TNF-α) in the plasma after acute stress exposure, no differences were seen between probiotic and control groups (Ait-Belgnaoui et al., 2012). In the hippocampus, significant differences were seen with these analytes in that the probiotic-treated group showed lower levels of IL-1β, IL-6, TNF-α mRNA expression compared to the vehicle-treated group (Ait-Belgnaoui et al., 2012). While they did not analyze inflammatory marker levels, a later study by the same group demonstrated that a combination of *L. helveticus* R0052 and *B. longum* R0175 (i.e., Probio'Stick^{TM}) also prevented increased stress hormone release in the plasma (Ait-Belgnaoui et al., 2018). Furthermore, in response to probiotic treatment with previous acute stress exposure, Abilgaard, Elfving, Hokland, Wegener, and Lund (2017) found that stimulated blood mononuclear cells isolated from rat blood produced more IFN-γ, IL-2 and IL-4. Thus, with acute stress, probiotic exposure seems to also decrease the stress response, but effects on inflammation are more complex.

The present study found that acutely-stressed animals have greater pro-inflammatory, anti-inflammatory, and regulatory analyte levels when exposed to a probiotic. A recent study by Rocha-Ramírez et al. (2017) examined how different strains of Lactobacilli affect immune function at the cellular level (i.e. on human macrophage cells). They found that *L. helveticus* IMAU70129 had an immunostimulatory effect on various pro-inflammatory markers (e.g., TNF-α, IL-6), but also on the anti-inflammatory marker IL-

10, indicating a broad response on inflammation in these macrophages that was not specific to the category of immunological analyte. Importantly, the increase in pro-inflammatory analytes may be explained by how these animals are responding to acute stress. Glucocorticoids inhibit pro-inflammatory transcription factor NF-κβ and lead to less pro-inflammatory analytes being produced (Bauer & Teixeira, 2018). Thus, an exaggerated stress response after stress exposure could lead to decreased measured levels of pro-inflammatory analytes. In fact, in a review of studies on neuroendocrine and neuroinflammatory dysfunction in major depressive disorder, glucocorticoid resistance and increased levels of pro-inflammatory cytokines in plasma were found in 85% of the studies reviewed (Horowitz & Sunszain, 2015).

If probiotic exposed animals have decreased stress hormone release (i.e., a less exaggerated and more "adaptive" response to stressors), then there could be less inhibition of inflammatory markers and this could explain why our probiotic group is showing higher levels of all forms of inflammatory analytes (i.e., pro-inflammatory, anti-inflammatory, and regulatory) when measured at one specific time point. In response to the acute predator odour stress, it is possible that the placebo animals are showing a greater stress response, releasing more stress hormones, and inhibiting the inflammatory response on a broad level. This theory warrants further investigation by analyzing stress hormone levels (e.g., CORT) at sacrifice (i.e., at the same time the inflammatory markers were analyzed) or by taking baseline inflammatory analyte measurements (i.e., in plasma) before treatment with probiotics and before stress exposure. See summary Figure 20 for an overview of this theory.

4.1.2 Western or Control Diet Administration

It is known that excess glucocorticoids lead to decreased sensitivity to insulin, which is an unhealthy state in which the body requires more and more insulin to lower blood sugar (McMahon, Gerich, & Rizza, 1988). Furthermore, Wang et al. (1998) found that animals who were allowed to select their food and preferred carbohydrates had higher levels of neuropeptide Y (involved in energy balance and food intake). Furthermore, animals given

a high sugar diet (65% carbohydrate; 10% fat), compared to a control diet (45% carbohydrate; 30% fat) also had higher levels of NPY in the hypothalamus. Importantly, the "control diet" in the present study closely resembles the high sugar diet in the Wang et al. (1998) study whereas our Western diet closely resembles the Wang et al. (1998) "control diet" in the composition of macronutrients. Thus, we would expect our control diet animals to actually be higher in NPY than the Western diet animals, which will be discussed further in section 4.3.2.

Of importance from the Wang et al. (1998) study was that plasma CORT is increased in animals on this high-sugar diet and was positively correlated with NPY. Furthermore, independent of diet choice, NPY levels were highest in rats with higher body fat. Another study reported that medicinal glucocorticoid administration (i.e., methylprednisolone) decreased pro-inflammatory factor IL-8 and MCP-1 in humans (Lund et al., 2008). Thus, it seems that increased levels of stress hormones (and NPY) are linked to the ingestion of carbohydrates. Based on the literature described above, increased circulating stress hormones may be linked to increased carbohydrate intake which may suppress the immune system in response to acute stressors.

4.1.3 Inflammatory Markers by Sex

Pyter et al. (2013) found that after an immune challenge with lipopolysaccharide, chronic stress exposure led to increased hippocampal inflammatory marker gene expression only in male rats. There were no differences in CORT levels found by sex even though the lipopolysaccharide injections did increase CORT levels compared to control rats given saline. The authors suggest that since the lipopolysaccharide challenge increased circulating estradiol in females (the authors made no mention of testing levels in males), this could have a protective role on the expression of inflammatory marker genes (e.g., IL-1β, TNF-α) in the hippocampus specifically (Pyter et al., 2013). In our study, we saw similarly increased levels of inflammatory markers in the plasma of male rats. The lower levels of markers seen in females may be related to estradiol, a steroid hormone that is present in higher levels in females compared to males (*Schulster, Bernie, & Ramasamy,*

2016). Furthermore, a recent review describes that females display an increased response to stress (as demonstrated by increased CORT and ACTH levels; Heck & Handa, 2019), which may also influence the inflammation response. Testosterone and estrogens can differentially modulate the HPA axis via their actions on androgen receptors and estrogen receptors, respectively (Heck & Handa, 2019). Specifically, estradiol can directly impact the functioning of the HPA axis whereas testosterone has more indirect effects (Heck & Handa, 2019). Thus, it is possible that a combination of increased estrogens and increased CORT release are affecting the inflammatory response in females.

4.1.4 Conclusions

The type of stress the rats in this study were exposed to prior to sacrifice would be considered an acute stressor (i.e., a 5-minute predator odour exposure). Importantly, in both animal and human studies, the type of stress seems to result in drastically different findings with respect to immunological analyte response (even without probiotic or diet manipulations) compared to chronic stress (Dhabhar & McEwen, 1997). Dhabhar and McEwen (1997) suggest that acute stress may enhance inflammation (temporarily) whereas chronic stress suppresses inflammation over time. Specifically, it seems that, after an initial acute stress exposure, leukocytes migrate from the blood to organs in preparation to deal with an immune attack that may arise from the stress exposure (an evolutionary mechanism), leading to a decrease in immune cell levels measured in plasma (Dhabhar and McEwen, 1997). In summary, we consistently found higher levels of key pro-inflammatory, anti-inflammatory, and regulatory immunological analytes, in probiotic-treated rats, Western diet fed rats, and males. From the literature, it seems that higher levels steroid hormones (e.g., CORT, CRF, estradiol) could suppress the immune response in placebo-treated, control (i.e., high-sugar), and female rats.

4.2 Summary of Behavioural Findings

The goal of behavioural testing in this experiment was to induce anxiety-like symptomatology in response to a number of different acute stressors and measure

differences between our groups. We hypothesized that the probiotic-treated group, the group administered the control diet, and females (aside from in the predator odour behavioural trial) would show less anxiety-like behaviours than their placebo-treated, Western diet administered and male counterparts. While we did not see many differences in anxiety-like behaviour as a result of our behavioural testing, a few key findings are of interest. In this experiment, adulthood weight was shown to vary by all of our experimental factors (i.e., treatment, diet, and sex). We, therefore, correlated our behavioural measures with weight (at weaning and in adulthood) and food intake to provide a clearer picture of the differences observed between groups. Due to significant and trending correlations by weight, the fact that males weighed significantly more than females, and that there were also differences in weight by treatment and diet, analyses on behavioural measures are interpreted with and without controlling for adulthood weight.

4.2.1 Probiotic or Placebo Treatment

We hypothesized that probiotic treatment would decrease observed anxiety behaviours in our rats. To summarize, in the OFT, placebo rats showed more overall rearing and supported rearing compared to probiotic rats; both categories of rearing were associated with a higher wean weight. In the control LDB, placebo rats performed more supported rearing in the dark than probiotic rats; this variable was not correlated with any food or weight measures. Also, in the control LDB, probiotic rats performed more transitions into the light compared to placebo rats, but correlations indicated that higher adulthood weight was associated with less transitions. In the vanilla trial of the LDB, there was a treatment by diet interaction that will be discussed in section 4.2.2. There were no differences in the cat urine trial of the LDB by treatment group or significant correlations to further clarify the results.

Line crosses. While there were no differences in line crosses between probiotic and placebo animals, adulthood weight was significantly negatively correlated with total line crosses in the OFT, meaning that there was an association between decreased locomotion

and higher adulthood weight. When examining this association by treatment group, the association between higher adulthood weight and less line crosses in the open field was only present within placebo animals. These results are interesting as other studies not examining probiotic treatment have shown that body weight is not associated with various behavioural measures in the OFT, LDB, and Elevated Plus Maze (EPM; Sivanathana, Thavartnama, Arif, Eleginoa, & McGowan, 2015) or with total distance travelled in the OFT (Sweeney, O'Hara, Xu, & Yang, 2017). However, Sweeney et al. (2017) did find that body weight was significantly negatively correlated with both distance and time in centre of the OFT in mice. This study suggests that there is a relationship between higher weight and more robust anxiety-like behaviours that may not depend entirely on locomotion. It is interesting that in our study, it was a locomotor behaviour (i.e., line crosses) that was correlated with weight only in the placebo group and it would be worthwhile for future studies to examine this relationship further in light of probiotic treatment.

Rearing behaviours. Placebo rats performed significantly more total and supported rearing in the OFT and more supported rearing in the control LDB compared to probiotic rats. Furthermore, correlational analysis revealed that there was a positive association for total rearing between the two tests as well as a positive association between OFT total rearing and LDB supported rearing in the dark. When examining correlations between behaviours measured in these two tests and body weight, we see trending and significant positive correlations between weight at P21 (but not adulthood weight) and both total rearing and supported rearing. Overall, these findings indicate that rats were more likely to perform total rearing and supported rearing during adulthood behavioural testing if they weighed more at weaning, which, to our knowledge, is not a relationship that has been examined in other studies. To further elucidate this finding, correlations between rearing and wean weight were conducted separately for the two treatment groups. The only significant correlation that remained was a trending positive correlation of wean weight and supported rearing in the probiotic animals during the OFT. Thus, it seems that for probiotic animals, at least for the OFT, a higher wean weight is related to more supported rearing on behavioural tests in adulthood whereas wean weight and rearing do

not seem to be related in placebo animals. The finding that placebo animals performed more total and supported rearing in adulthood must be explained by something other than wean weight. Indeed, even after controlling for adulthood weight, placebo animals still performed more total and supported rears compared to probiotic animals.

The finding that placebo rats performed more supported rearing and total rearing compared to probiotic rats is interesting as there were no differences in unsupported rearing, although frequency of unsupported rearing was low. As previously described, rearing (especially supported rearing) can be interpreted as an exploratory, information-gathering, behaviour but for animals on the defensive, supported rearing can indicate a desire to escape (Lever, Burton, & O'Keefe, 2006). Rearing is also known to decrease as an animal gets more acclimated to their surroundings (Lever et al., 2006). It may be that placebo animals are performing more escape behaviours and are less comfortable with the behavioural testing environment compared to probiotic animals as they are demonstrating more rearing behaviours on the extremities of the behavioural testing apparatus (Lever et al., 2006). In fact, Genaro and Schmidek (2000) exposed Long-Evans rats to a traditional OFT, an open-field with a refuge (i.e., a hide box), or a complex (multi-chambered with tunnels) open-field with a refuge, and reported highly variable observed behaviours. Without a refuge, there was an increase in urination/defecation combined with increased locomotor activity (distance travelled, number of rearings). This finding was interpreted as escape behaviour rather than exploratory behaviour as rats in the complex environment displayed more locomotion and rearings without the increase in urination/defecation. In sum, it would be important to measure these biological response variables in the future to attempt to tease out exploratory- vs. escape-related rearings and locomotion in probiotic- and placebo-treated rats.

Transitions. Probiotic treatment has been shown to improve anxiety-like behaviours in animal models. Interestingly, in the control trial of the LDB, probiotic rats did perform more transitions between light and dark compartments which is an established measure of reduced anxiety. Even though a higher adulthood weight was associated with fewer transitions, the effect of treatment on number of transitions persisted even after

controlling for weight (i.e., probiotic animals still transitioned more than placebo animals independent of how much they weighed). Similar research using Syrian Golden Hamsters found that a probiotic containing *S. thermophilus* I-1630, *L. bulgaricus* I-1632 and I-1519, *Lactococcus lactis lactis* I-1631, *L. acidophilus*, *S. thermophiles*, *L. plantarum*, *B. lactis* I-2494, and *L. reuteri* 17938 reduced anxiety-like behaviours (e.g., increased time spent in anxiety-inducing areas) in the LDB and elevated-plus maze tests (Avolio et al., 2019). Avolio et al. (2019) also showed that their probiotic-treated group performed more transitions between the open and closed arms which complements the present study. With Probio'Stick^{TM} specifically, Messaoudi et al. (2011) showed a decrease in anxiety-like behaviours in the defensive burying test in their sample of Wistar rats. Also, with Probio'Stick^{TM}, Ait-Belgnaoui et al. (2014) demonstrated reduced HPA axis responding (i.e., decreased CORT, adrenaline, and noradrenaline) after water avoidance chronic stress exposure.

4.2.2 Western or Control Diet Administration

We hypothesized that Western diet animals would show more anxiety-like behaviours during testing. However, across all behavioural testing trials, the only difference that emerged was a trending treatment by diet interaction in the vanilla trial of the LDB. Specifically, we found that probiotic Western diet rats performed more supported rears in the dark than placebo Western diet rats. However, in males only, placebo Western diet rats weighed significantly more than their probiotic Western counterparts, which may partially explain why we are seeing more supported rearing in this instance considering that rearing would be more difficult with increased weight. In fact, the treatment by diet interaction for supported rearing in the dark disappeared after controlling for weight. Nevertheless, similar to our broad lack of anxiety-like behavioural differences with diet treatment, Abildgaard and colleagues (2017) also found no differences on the OFT by probiotic treatment or by diet. Specifically, they fed rats a 60% high-fat diet and then treated with a probiotic comprised of 8 bacterial strains and no differences in locomotor behaviour or time in center area between their groups was found.

It is possible that the type of high-fat diet or the type of stressor affects whether differences in anxiety-like behaviours between groups are observed. In fact, giving rats a 90% high-fat diet for 7 days, compared to a 90% carbohydrate or 90% protein diet, leads to significant decreases in anxiety behaviours on the elevated plus maze test compared to baseline levels (Prasad & Prasad, 1996). Bridgewater and colleagues (2017) found important differences based on sex in mice that were fed a high-fat diet and exposed to chronic unpredictable stress rather than an acute behavioural test only. Specifically, male mice in the high-fat diet group exhibited more anxiety-like behaviours in the OFT (i.e., decreased distance travelled in the centre area) and more anxiety-like behaviours in the elevated-plus maze (i.e., decreased time spent in and fewer entries into the open arms of the maze). Perhaps in order to see differences in anxiety-like behaviours by diet and probiotic treatment, a chronic stress paradigm and/or a higher fat diet (with less carbohydrates) is required.

4.2.3 Sex Differences in Behavioural Measures

We hypothesized that females would show fewer anxiety behaviours than males on the OFT, the control LDB, and the vanilla LDB whereas they would show more anxiety behaviours in the cat urine LDB. In the OFT, females line crossed more and there was a trend for them performing more total and supported rearing compared to males. Of importance in the OFT, there was an association between more line crosses and lower adulthood weight. In the control LDB, females performed more line crosses and more supported rearing in the dark, entered the light sooner, and made more transitions than did males; more line crosses, more transitions, and lower latency were all associated with a lower adulthood weight. In the vanilla LDB, females again line crossed more, performed more unsupported rearing in the light and there was a trend for them transitioning more than males. In this trial, there was again a trend for more line crossing to be performed in those with a lower adulthood weight. In the cat urine LDB, there was only a trend for females performing more total rearing than males that was no longer evident after controlling for adulthood weight with no significant correlations with any

weight or food measures. Thus, it seems that adulthood weight at the time of behavioural testing may affect some of the sex differences that we observed.

Line crosses. In the OFT, control LDB, and vanilla LDB, we found that females performed more line crosses compared to males which is consistent with previous literature (see Kokras & Dalla, 2014 for a review and Bridgewater et al., 2017). However, adulthood weight was significantly and negatively correlated with line crosses in the OFT with a trend for this association in both the control and vanilla LDBs. This suggests that weighing more (which males did) made a rat less likely to perform line crosses in this test and is not necessarily an indication of decreased anxiety as line crosses are primarily a locomotor measure. Nonetheless, after controlling for adulthood weight, females still performed significantly more line crosses in the OFT and the control LDB (no longer in the vanilla LDB) than males which indicates that it is not weight alone explaining the difference in locomotor behaviour between the sexes. Alonso-Caraballo, Hodgson, Morgan, Ferrario, and Vollbrecht (2019) found an association between increased anxiety-like behaviors (i.e., more time spent in the closed arms of the EPM) and increased weight gain along with increased plasma leptin in male (but not female) obesity-prone rats compared to obesity-resistant rats. The authors suggest that the mechanisms responsible for the relationship between weight gain and higher anxiety could vary by sex.

Rearing behaviours. There was a trend for females to perform more total rearing and supported rearing (i.e., against the perimeter) in the OFT than males with no differences in unsupported rearing or time measures. Furthermore, females no longer performed significantly more supported rears compared to males after controlling for weight. Likewise, in the control LDB, females performed more supported rearing behaviours in the dark section, but this difference was no longer apparent after controlling for weight. There were no associations to note between rearing and adulthood weight in these two tests that may help to explain the sex differences observed. Overall, it is difficult to conclude were females are less anxious than males as we only saw differences in anxiety-like behaviours that are considered to be mainly locomotor measures (i.e., total distance travelled and rearing). In the vanilla LDB, females did perform significantly

more unsupported rears in the light compared to males. Although the means are small and difficult to interpret (i.e., 1.37 vs. 3.00 rears, respectively), there was a correlation between more of these behaviours and a higher adulthood weight along with greater food intake, but the difference was no longer significant after controlling for weight.

Transitions, latency to light, and time in centre. During the control and vanilla LDB trials, there was a significant and trending (respectively) increase for more transitions by females compared to males. However, in the control LDB (and not the vanilla LDB), a higher adulthood weight was shown to be associated with fewer transitions and longer time to enter the light. After weight was controlled for, the significant difference in the control LDB became trending and the trending effect in the vanilla LDB was no longer present. Furthermore, females entered the light section of the control LDB significantly sooner than males, but this difference was no longer significant after controlling for weight. These findings are partially indicative of lower anxiety on these specific behavioural tests by females, which is consistent with previous research that shows that males have more of an aversion to the light area and show more anxiety than females on the LDB test (Kokras & Dalla, 2014). Overall, it seems that the more robust time measure of anxiety, latency to light, and transitions are dependent on the weight of the animal. Conceptually, considering all animals needed to pass through an equal sized door to enter the light area, the smaller animals could be more inclined to do this. Also, of interest to note, is that upon controlling for weight in the OFT analyses, two trending effects of sex emerged for time in center and latency from centre. Interestingly, males had greater latency to light compared to females (indicative of more anxiety), but they also spent more time in the centre compare to females (indicative of less anxiety).

4.2.4 Conclusions

Overall, we observed some interesting correlations between weight measures with locomotor behaviours in both the OFT and the LDB. Wean weight was shown to be associated with both total and supported rearing whereas adulthood weight was associated with line crossing, unsupported rearing, transitions, and latency to enter the

light area. After controlling for weight in analyses, probiotic animals still performed less total and supported rears in the OFT and less supported rears in the control LDB, which may indicate a lower level of escape behaviours. Furthermore, probiotic animals still transitioned more than placebo animals in the control LDB independent of weight. This finding may indicate a lower level of anxiety, but future research should examine these variables in a follow-up study as there were no differences between the probiotic and placebo groups on behaviours such as time in light/centre or latency to enter/leave anxiety-inducing areas. In the vanilla LDB, the finding that probiotic Western diet rats performed more supported rears in the dark than placebo Western diet rats was no longer evident after controlling for weight. The lack of an effect by diet on behaviour may be related to the use of a control diet for the Western diet comprised of mainly carbohydrates which have been shown to induce anxiety. Finally, while sex differences were initially evident on many behavioural measures, after controlling for weight, only line crosses in the OFT, line crosses in the control LDB, and number of transitions in the control LDB were behaviours in which females demonstrated more. These results compliment previously described literature that females are more active and have less of an aversion to the light area of the LDB. Nevertheless, findings are interpreted with caution as there were no differences by sex that were independent of weight on the more salient time measures of anxiety (i.e., time spent in and latency to enter or leave anxiety-inducing areas) and the effect of sex on transitions was smaller after controlling for weight.

4.3 Summary of Metabolic and Food Intake Findings

In this experiment, we hypothesized that probiotic treatment would decrease adulthood weight and food intake in both sexes. We also expected that Western diet would lead to increased adulthood weight and food intake. We further hypothesized that probiotic treatment would help mitigate some of the negative effects of the Western diet and help to normalize weight and food intake to control diet levels. With levels of NPY, we hypothesized that probiotic treatment would increase NPY, that Western diet would increase NPY, and that there would be no difference between the sexes in NPY.

4.3.1 Probiotic or Placebo Treatment

While we did not hypothesize any differences in wean weight between our groups, the probiotic group weighed significantly less than the placebo group at P21. The CCAC (1984) describes healthy wean weights for rats (although this varies by strain) as between 35g and 50g. Our placebo animals were at the high end of this range whereas the probiotic animals were at the low end of that range. We also found a significant negative association between weight at weaning and 12 of the 18 inflammatory markers that were measurable in plasma. These results could suggest that there is a link between lower levels of adulthood inflammation and higher wean weight. In contrast, previous research in human mothers showed that a combination of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 treatment increased γ-linolenic acid content (i.e., a type of omega-3 fatty acid) and total omega-3 fatty acids in milk to a greater extent than with dietary intervention and a placebo formulation although no changes in birth weights were found (Hoppu, Isolauri, Laakso, Matomäki, & Laitinen, 2012). Interestingly, omega-3 fatty acids have been described as anti-inflammatory and inflammation resolving due to their influences on cytokine production and transcription factors (e.g., NF-κB; Calder, 2009). Overall, the relationship between wean weight and adulthood inflammation with probiotic treatment should be investigated further.

Adult weight and food intake results were consistent with each other and with wean weight. In fact, weight at P67 was significantly positively correlated with average food intake and weight at weaning. We hypothesized that probiotic treatment would lead to decreased weight and food intake in both sexes and in both diet groups. Our hypotheses regarding adulthood weight were partially supported in males only. Specifically, with probiotic treatment, we did see lower adult weight compared to the placebo animals. Furthermore, there was an interaction between diet and treatment that will be discussed in section 4.3.2. Food intake results between probiotic and placebo groups differed based on sex and will be discussed below in section 4.3.3. This is the first study of its kind to examine metabolic consequences of Probio'Stick™ as research to this date has focused on the idea that this probiotic helps to alleviate stress and psychological dysfunction. Lactobacillus and Bifidobacterium strains have been recognized for their effects at

combatting obesity due to the modulation of the gut microbiota which, in turn, is related to energy balance, inflammation, food intake signals, and the appropriate functioning of the gut lining (Kobyliak et al., 2016). Weight gain and other markers of obesity have been shown to be improved with *Lactobacillus plantarum* (Karlsson et al., 2011), Bifidobacteria strains (An et al., 2011; Yin et al., 2010), and *Lactobacillus helveticus* R0052 (Ohland et al., 2013).

Our hypothesis that NPY levels would be highest in the probiotic group cannot be confirmed or denied as although probiotic animals were 1.38-fold higher in NPY with all data included (1.19-fold higher with 2 outliers removed), this difference was not significant, although the effect size is considered to be medium so this may be confirmed with a larger sample size. The findings on NPY levels with probiotic treatment are limited and mixed in that some studies find an increase (Giorgia et al., 2018; Lesniewska et al., 2006), but another (Davis et al., 2016) found no change between treatment groups. This is the first study to examine NPY levels after Probio'Stick^{TM} treatment, but due to the impact of this probiotic on stress responding (Ait-Belgnaoui et al., 2018), anxiety behaviours (McKean et al., 2017), and weight changes in response to diet (the present study), changes in NPY levels with Probio'Stick^{TM} treatment warrant further investigation; perhaps alongside analyses of other metabolic markers (e.g., leptin, insulin, ghrelin, GLP-1).

4.3.2 Western or Control Diet Administration

With a slight difference in calories between the two diets (i.e., 4.7 kcal/g for the Western diet, 3.9 kcal/g for the control diet), we examined differences in calories consumed by our groups and found that Western diet animals did consume more calories compared to control diet animals with no difference in grams of food eaten. However, probiotic animals that were fed a Western diet weighed significantly less than placebo animals fed the same diet, whereas there was no difference between probiotic and placebo within the control diet group. Furthermore, while there was no difference in weight between Western and control diets in the probiotic group, within the placebo group, the Western

diet fed animals weighed more than the control diet animals. Thus, these results suggest that probiotic treatment is somehow mitigating the weight-increasing effects of the Western diet.

These findings are consistent with other evidence on probiotics affecting weight gain in response to unhealthy diet in the literature. Complementary to our results, Falcinelli et al., (2017) found that in zebrafish, *L. rhamnosus* IMC 501® attenuated weight gain in a medium (10% fat) and high (15% fat) fat diet group with no change in a low-fat (5% fat) diet group. The probiotic also decreased the genes involved with cholesterol and triglyceride metabolism that were upregulated in the low, medium, and high-fat diet groups (the genes were the most up-regulated in the HFD group). Another study by Ohland and colleagues (2013) found that while a Western diet (49% refined carbohydrate, 33% fat) increased weight gain in mice, this weight gain was reduced when supplemented with *L. helveticus* R0052 whereas this probiotic did not alter weight in the control chow (55% carbohydrate, 13% fat) fed mice.

The hypothesis that NPY levels would be increased in the Western diet group based on previous literature was not supported. The finding that Western diet animals were lower in NPY compared to control diet animals was not significant with a small effect size; although this relationship may warrant investigation in future experiments. In the study by Falcinelli and colleagues (2017), they also analyzed NPY levels and found that they were significantly lower in their high-fat diet group. It may be that our Western diet comprised of moderate fat and moderate carbs was of comparable "unhealthiness" to our control diet. The control diet used in this study (and in most studies in this field) simply had the excess fat replaced with carbs and, thus, led to another, albeit different, form of unhealthy diet (i.e., high-carb diet). As previously discussed, rats that choose to eat carbohydrates have higher measured levels of NPY and rats given a high-carbohydrate diet also have higher levels of NPY in their hypothalami (Wang et al., 1998).

Ludwig and Ebbeling (2018) report on a common misconception that is prevalent in research: that diets high in added sugar and processed carbohydrates will have no adverse

effects on weight or metabolism as long as there is a reduction in overall calories. However, emerging evidence points to insulin reactivity as the culprit for excess fat storage and weight gain and that dietary carbohydrates have the greatest effect on insulin secretion, especially highly refined and processed carbohydrates that are quickly digested (Ludwig & Ebbeling, 2018). Dietary protein can lead to insulin secretion but not to the extent of carbohydrates as it also leads to the secretion of glucagon which opposes available insulin (Ludwig & Ebbeling, 2018). In contrast, dietary fat has almost no impact on the insulin response (Ludwig & Ebbeling, 2018). In this manner, low carbohydrate diets have become a treatment for insulin resistance and diabetes (Feinman et al., 2015). In fact, McAuley et al. (2005) found that insulin-resistant women given a high-fat, or a high-protein diet had decreased body weight, waist circumference, and triglyceride levels after a 16-week period as opposed to the high-carbohydrate group, although all three groups had similar decreases in insulin levels.

Of importance in current studies on the "high-fat diet" is that the control diets used (usually in order to control for amount of protein and calories) end up being 70% carbohydrates (Steegenga et al., 2017; Bridgewater et al., 2017) or low-fat but with no carbohydrate percentage reported (Tamashiro et al., 2009). High carbohydrate diets are defined as diets with greater than 45% of the energy derived from carbohydrates (Feinman et al., 2015) which is much lower than the 70% carbohydrate diets frequently used as control diets. Indeed, the control diets may be biased in the fact that they are supplementing the perceived detrimental macronutrient (fat) with a perceived neutral macronutrient (carbohydrates). Furthermore, the high-fat diets used in these studies were only 60% fat and still contained 20% carbohydrates (Bridgewater et al., 2017; Tamashiro et al., 2009) and could be considered a variation of the Western diet. It may be the presence of both fat and carbohydrates in the diet (i.e., a Western diet as opposed to a true high-fat diet) rather than the fat on its own that is leading to the detrimental health effects (Feinman et al., 2015). Future research is essential to elucidate this difference and to consistently define a high-fat diet.

4.3.3 Weight and Metabolic Changes by Sex

With sex, our hypothesis that males would weigh and eat more than females regardless of group was supported. We further hypothesized that there would be no change in NPY levels by sex and this hypothesis was supported. Males were 0.95-fold lower in NPY compared to females with all data included but males became 1.11-fold higher than females when two outliers were removed.

Food intake results were comparable to adulthood weight results in that food intake (grams of food and calories consumed) was still highest in males regardless of group designation. However, when examining results by treatment and by diet condition, results were vastly different based on sex. Within treatment groups, male rats given a placebo consumed more food in grams and calories than probiotic animals. However, with females, it was probiotic animals who consumed more food (grams and calories) than placebo animals with no significant difference in weight between these groups. Interestingly, while females differed on the amount (grams) of food consumed by diet in that control diet animals ate more, they did not actually consume more calories. In contrast, the opposite was seen with males; while grams of food consumed did not change, males consumed more calories in the Western diet condition. Furthermore, in Western diet exposed animals, females in the probiotic condition consumed more calories whereas males in the placebo condition consumed more calories. Overall, it seems that the probiotic is allowing for increased consumption of food (grams and calories) in females (without the change in weight) and that treatment is preventing an increase in weight and food intake in males.

In the literature, differences exist between males and females with respect to how they respond to a high-fat diet, yet research is limited on how the combination of diet and probiotic treatment may affect males and females differently. In fact, in response to a high-fat diet and leptin (an appetite suppressant) administered to induce leptin resistance, females did not become leptin resistance and continued to lose weight in response to the high-fat diet and leptin injections (Harris, Bowen, & Mitchell, 2003). Of interest is that

there was no difference in food intake and the female mice still lost weight without changing their food intake (Harris et al., 2003). Taraschenko and colleagues (2011) suggest that diet-induced obesity occurs through different mechanisms in males and females. The researchers attempted to induce, and reverse, high-fat diet induced obesity and were only successful in changing weight in male rats. These studies complement the present study as they highlight that males and females respond differently to diet and potential regulators of weight gain.

4.3.4 Conclusions

Probiotic strains of the same species present in Probio'Stick^{TM} have been shown to be related to decreased weight gain (Shin et al., 2017; Yin et al., 2010) and improvements in metabolic markers of obesity (An et al., 2011; Ohland et al., 2013). The use of a control diet for a Western diet that was inadvertently designed as a high-carbohydrate diet may have prevented changes in NPY from being apparent as both types of diets are linked to detrimental metabolic consequences. It is intriguing that males and females do not respond to probiotic treatment and diet with respect to weight in the same manner. Consequently, sex differences should be a focus on any study with the goal of elucidating mechanisms behind metabolic dysfunction and obesity in response to diet and probiotics.

4.4 Limitations and Future Directions

We found that males, probiotic-treated animals, and animals given Western diet, all had higher levels of all inflammatory analytes whether or not these analytes were pro-inflammatory, anti-inflammatory, or regulatory. Research shows that probiotic treatment decreases stress hormones and pro-inflammatory markers in response to chronic stress (Avolio et al., 2019; Desbonnet et al., 2010). In contrast, acute stress may increase pro-inflammatory markers (Segerstrom & Miller, 2004), although this study did not have a probiotic manipulation. It is evident that probiotic treatment decreases stress responding (Ait-Belgnaoui et al., 2012); although, available research is conflicted about whether probiotic treatment decreases (Ait-Belgnaoui et al., 2012) or increases (Abilgaard et al., 2017; Rocha-Ramírez et al., 2017) the inflammatory response. Differences among studies

may be related to tissue type measured or how long after stress exposure the samples were taken.

Since glucocorticoids are reported to inhibit pro-inflammatory transcription factor NF-κβ and, thus, lead to less pro-inflammatory analytes being produced (Bauer & Teixeira, 2018), the importance of using a sham group not exposed to stress or taking multiple blood samples with a baseline measure of inflammation becomes important. Due to the design of the present study, these measurements were not possible. Moreover, available literature seems to suggest that increased circulating stress hormones may be linked to increased carbohydrate intake which can suppress the immune system in response to acute stressors (Lund et al., 2008; Wang et al., 1998). These hormones warrant investigation in future studies. Control diets that are not comprised primarily of carbohydrates are essential to help elucidate these effects. Due to the potential effects of estradiol inhibiting inflammation in female subjects, measures of these levels in both sexes is important when examining sex differences. It is evident that NPY levels may help to explain the effects of the high-carbohydrate diet on inflammation and levels of this neuropeptide may be important for future research on the metabolic impacts of probiotics.

Contrary to our expectations, we did not see any difference in hippocampal inflammation between our groups. In a future study, measurements of brain inflammatory analyte protein levels should be taken at different time points (i.e., a series of time points greater than 120 minutes after stress exposure) to determine when exactly differences would be seen after stress exposure in different experimental groups. It is unclear how long it would take for differences in brain cytokine protein levels to be observed, but it can be assumed that this would take longer than 120 minutes (Ait-Belgnaoui et al., 2012 measured cytokine mRNA levels at this time point) and this idea should be investigated in the future.

Results from behavioural analyses seem to suggest that probiotic animals are slightly less anxious than placebo animals, but future studies would benefit from having measures of

biological variables such as number of defecations. Furthermore, longer testing periods so that less commonly observed behaviours (e.g., unsupported rearing, grooming) that yield more information about the anxiety level of the animals would be useful. In the future, to help control for differences in rearing being due to differences in locomotor ability, it might be useful to present rearing as normalized to a variable such as line crosses or distance travelled (as in Tanaka et al., 2012). Again, using a control diet that has fewer negative impacts on metabolic functioning and anxiety would be of benefit to effectively demonstrate the negative effects Western diet has on mental health. The lack of differences on more robust time measures of anxiety (e.g., latency to light) after controlling for weight make it difficult to conclude whether females were less anxious than males, but results do highlight the importance of considering weight in any behavioural analyses examining sex.

While we did see, at least in males, that probiotic treatment helped to regulate increased weight after Western diet exposure, we only measured one metabolic marker (i.e., NPY) to compliment this. Since the probiotic is affecting weight/food intake and these effects may be sex-dependent, other markers that affect these outcomes (e.g., ghrelin, leptin) warrant investigation in similar future experiments. As food intake can only be measured in a "per cage" amount for rats housed in twos or threes, it may be worthwhile to weigh individual rats daily during food intake measurements and present their food intake as a ratio of food eaten (per cage) to individual weight. From our results, it is clear that future research should aim to elucidate the difference effects of high-carbohydrate and high-fat diets and to consistently define a high-fat diet, since an extremely high-fat diet has actually been shown to be beneficial for anxiety (Prasad & Prasad, 1996).

4.5 Implications and Concluding Remarks

We show evidence that after stress exposure, male probiotic-treated Western diet-fed rats have a higher inflammatory response compared to female placebo-treated control diet-fed rats and that these effects may be dependent on levels of steroid hormones.
Behaviourally, our results suggest that probiotic-treated rats may be more comfortable

with an acute behavioural stress arena which is a finding that warrants future, more detailed, investigation. Additionally, a variable other than weight seems to be explaining why females are showing more locomotor behaviours during behavioural testing. Results that were shown to be dependent on weight highlight the importance of weight considerations while analyzing sex differences in behavioural testing and should be a focus of any study examining these variables.

As a whole, this thesis attempted to expand what is known about a probiotic, Probio'Stick^{TM}, in an animal model while also examining the concurrent effects of both diet and sex. We were successful in increasing available evidence on the specific strains of bacteria present in Probio'Stick^{TM} as they relate to overall inflammation, anxiety-like behaviours, and weight/food intake measures. As different strains of bacteria have specific effects on host functioning (Cryan & O'Mahony, 2011), research similar to that presented here adds to the breadth of available knowledge on *L. helveticus* R0052 and *B. longum* R0175, having implications for future treatment of diseases related to the MBG axis. This is the first study to examine Probio'Stick^{TM} in light of weight changes and food intake; results indicate not only that caution should be taken when designing a control diet for a high-fat or Western diet protocol but also that females are not responding metabolically to probiotic treatment and diet in the same way as males.

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APPENDIX A ETHOGRAM OF BEHAVIOURAL MEASURES

Adapted from Draper (1967)

Ambulatory Behaviours

- Chase tail - the rapid, circular movement with the tail near, or in the mouth
- Climb - movement across the wire lid of cage, all feet off floor
- Roll over - turn onto back, or completely over from one side to the other
- Run - rapid movement across the floor of the cage
- Walk - slow movement across the floor of the cage
- Circle - slow shifting in a circular pattern
- Rear - front feet off of floor, feet may be held near the body, or extended
- Stretch - back arched, with front or rear legs extended

Grooming Behaviours

- Bite coat - rapid, quick biting of the fur
- Lick coat - smoothing of the fur with tongue
- Genitals - biting or licking the urogenital area
- Nails - biting feet or nails
- Wash face - stroking the whiskers and/or face with one or both front feet
- Scratch - quick scratching of the head, ears, or coat with one of the hind feet
- Chew tail - Biting of the tail, may or may not hold tail with front feet

Scanning Behaviours

- Sniff - whiskers and nose twitching, may or may not be audible sniffing sounds
- Turn head - horizontal, or vertical head movements

Feeding and Excretory Activities

- Defecate - to discharge feces from the body
- Urinate - to discharge urine from the body
- Drink - to take a liquid into the mouth and swallow it (e.g., water)
- Eat - the animal may feed directly from the food hopper, or from elsewhere

Inactivity Behaviours

- Head under - head tucked under front feet, face on the cage floor
- Side - head tilted to the side, resting on the cage floor, body curled around
- Stomach - animal immobile, laying on stomach, head erect
- Stand still - animal immobile, standing on all four feet

Miscellaneous Behaviours

- Shake - quick shaking of entire body
- Twitch - small movement of only part of the body
- Sneeze - making a sudden involuntary expulsion of air from the nose and mouth
- Yawn - a reflex act of opening the mouth wide and inhaling deeply
- Chew cage, or cage accessories - animal may chew on items in their environment

APPENDIX B OPEN-FIELD TEST SCORING SHEET

Rat:	Camera:	Date
Line crosses		
Rearing total		
Unsupported Rearing in Perimeter		
Unsupported Rearing in Center		
Supported Rearing		
Time in centre		
Latency to move from centre		

Rat:	Camera:	Date
Line crosses		
Rearing total		
Unsupported Rearing in Perimeter		
Unsupported Rearing in Center		
Supported Rearing		
Time in centre		
Latency to move from centre		

APPENDIX C LIGHT-DARK BOX SCORING SHEET

Rat:	Camera:	Date:
Line crosses		
Rearing total		
Unsupported Rearing in Dark		
Unsupported Rearing in Light		
Supported Rearing in Dark		
Supported Rearing in Light		
Transitions		
Time in light		
Latency to move from dark		

Rat:	Camera:	Date:
Line crosses		
Rearing total		
Unsupported Rearing in Dark		
Unsupported Rearing in Light		
Supported Rearing in Dark		
Supported Rearing in Light		
Transitions		
Time in light		
Latency to move from dark		

Table 1 Descriptive statistics for breeder dams including mean birth weights and wean weights separated by sex.

Dam ID	***M*(SD)$_{Total}$ Birth Weight (g)**	***M*(SD)♂ Birth Weight (g)**	***M*(SD)♀ Birth Weight (g)**	***M*(SD)$_{Total}$ Wean Weight (g)**	***M*(SD)♂ Wean Weight (g)**	***M*(SD)♀ Wean Weight (g)**
A[a,b]	6.02(0.28)	6.20(0.20)	5.86(0.25)	40.03(6.06)	41.89(4.02)	34.47(8.66)
B[b]	6.39(0.29)	6.55(0.25)	6.31(0.29)	30.31(3.14)	29.78(2.58)	30.58(3.53)
C[a,b]	5.97(0.28)	6.10(0.24)	5.78(0.23)	42.96(3.74)	42.70(4.60)	43.48(1.18)
D[b]	7.22(0.46)	7.57(0.38)	6.87(0.12)	54.02(2.26)	55.80(1.73)	52.23(0.45)
E[a]	6.22(0.36)	6.41(0.30)	5.88(0.13)	52.87(5.02)	53.53(5.58)	51.33(3.89)
F[a]	5.98(0.48)	6.18(0.67)	5.88(0.37)	50.20(4.79)	50.17(7.96)	50.21(3.83)
G[a]	6.63(0.36)	6.86(0.32)	6.40(0.23)	54.07(6.04)	56.55(7.45)	52.08(4.49)

Note: A-D dams were probiotic-treated; E-G dams were placebo-treated.
[a]Birth weight statistics include pups that did not make it to weaning.
[b]Wean weight statistics include offspring room that were culled after weaning.

Table 2 Descriptive statistics for offspring birth characteristics and final sample sizes.

Dam ID	Treatment[a]	Total Born in Litter	Survival Ratio at Birth	Male Ratio of Surviving Pups	Survival Ratio (Weaning)	Male Ratio of Surviving Pups	Final Sample Size at Weaning	Final Sample Size After Culling
A	PR	13	1.00	0.69	0.92	0.75	12	8
B	PR	12	1.00	0.33	1.00	0.33	12	8
C	PR	16	0.94	0.60	0.80	0.67	12	8
D	PR	6	1.00	0.50	1.00	0.50	6	6
E	PL	11	1.00	0.64	0.91	0.70	10	10
F	PL	14	0.86	0.33	0.92	0.27	11	11
G	PL	10	1.00	0.40	0.90	0.44	9	9

[a]PR = probiotic; PL = placebo

Table 3 Final experiment sample size and group characteristics.

		Control Diet Males	**Western Diet Males**	**Control Diet Females**	**Western Diet Females**
Placebo	Final Sample Size	*7*	*7*	*8*	*8*
	Litters Represented	*E, F, G*	*E, G*	*E, F*	*F, G*
Probiotic	Final Sample Size	*8*	*8*	*8*	*8*
	Litters Represented	*A, B, C, D*	*A, B, C, D*	*A, B, C*	*B, C, D*

Table 4 Control and Western diet nutrient and mineral breakdown.

	D12079B (Western Diet)		**D14042701 (Control)**	
	Grams	***kcal (%)***	***Grams***	***kcal (%)***
Protein	20	17	17	17
Carbohydrate	50	43	71	73
Fat	21	40	4	10
	4.7 kcal/g		3.9 kcal/g	
	Grams	***kcal***	***Grams***	***kcal***
Casein, 80 Mesh	195	780	195	780
DL-Methionine	3	12	3	12
Corn Starch	50	200	695	2780
Maltodextrin 10	100	400	150	600
Sucrose	341	1364	0	0
Cellulose, BW200	50	0	50	0
Milk Fat, Anhydrous	200	1800	42.5	383
Corn Oil	10	90	10	90
Ethoxyquin	0.04	0	0.04	0
Mineral Mix S10001	35	0	35	0
Calcium Carbonate	4	0	4	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Cholesterol	1.5	0	0	0
TOTAL	**1001.54**	**4686**	**1196.54**	**4685**

Table 5 Behavioural Measure Breakdown in Light-Dark Box and Open-Field Test (adapted from Kalueff & Tuohimaa, 2004).

Behaviour	**Conclusion**	
	Open-Field Test	***Light-Dark Box***
Line crosses (total distance travelled)	Low Anxiety	Low Anxiety
Rearing	Low Anxiety	Low Anxiety
Transitions (between light and dark)	N/A	Low Anxiety
Time (sec.) spent in anxiety inducing areas[a]	Low Anxiety	Low Anxiety
Time (sec.) to leave centre area	High Anxiety	N/A
Time (sec.) to enter light area	N/A	Low Anxiety

[a]the centre of the open-field apparatus and the light section of the light-dark box

Table 6 Interleukin analytes measured by the Bio-Plex Pro™ Rat Cytokine 23-Plex and their functions in rats as acquired via the Rat Genome Database (RGD)[165] and the NCBI Gene Database[29] along with select research articles.

Analyte	Functional Category	Pathways/Conditions Involved[165]	RGD Function in Rats[165]	NCBI Function in Rats[29]
IL-1α	Pro-inflammatory[29,90,200]	Interleukin-1 signaling, apoptotic cell death, cytokine-mediated signaling	Involved with acute inflammation, heart development, keratinization	N/A
IL-1β/ Casp1	Pro-inflammatory[25,29,200]	Interleukin-1 signaling, type II interferon signaling, amyotrophic lateral sclerosis	Involved with apoptosis, lung development, memory	N/A
IL-2	Anti-inflammatory[200]	Interleukin-12, 2, and 23 signaling; G-protein coupled receptor signaling	Negative regulator of heart contraction and protein phosphorylation	Produced by T-cells in response to antigens
IL-4	Anti-inflammatory[44,90,200]	Fc epsilon receptor-mediated signaling, interleukin-12 and 4 signaling	B-cell growth factor involved with microglial cell activation	Involved in inflammatory response in eosinophils
IL-5	Anti-inflammatory[90] Pro-inflammatory[200]	Fc epsilon receptor-mediated signaling, Interleukin-5 and 4 signaling	B-cell growth factor	B-cell growth factor
IL-6	Pro-inflammatory[25,44,125,195,200] Anti-inflammatory[35]	Interleukin-6, 23, and 27 signaling	Involved with acute inflammation, aging, bone remodelling	Involved with development and neurodegeneration
IL-7	Anti-inflammatory[35]	Cytokine-mediated signaling; Jak-Stat pathway	Involved with hypoxia response	Brain tumor growth
IL-10/ CXCL10	Anti-inflammatory[35, 44, 90, 195, 200]	Interleukin-10 and 4 signaling; allograft rejection	Involved with aging and cellular response to estradiol	Involved in the inhibition of cytokine synthesis
IL-12	Anti-inflammatory[90] Pro-Inflammatory[145,200]	Interleukin-12 and 27 signaling; allograft rejection	Involved with cell proliferation	Acts on T and NK cells
IL-13	Anti-inflammatory[44]	Fc epsilon receptor mediated signaling, cytokine-mediated signaling, asthma	Involved with T cell activation	N/A
IL-17A	Pro-inflammatory[90,200]	N/A	Positive regulator of necrotic cell death	Immune system and cell death
IL-18	Pro-inflammatory[29,35,90]	Interleukin-12, 23, and 27 signaling	Responds to cAMP, H_2O_2, IFN- γ	Immune modulator

Table 7 Other immunological analytes measured by the Bio-Plex Pro™ Rat Cytokine 23-Plex and their functions in rats as acquired via the Rat Genome Database (RGD)[165] and the NCBI Gene Database[29] with select research articles.

Analyte	Functional Category	Pathways/Conditions Involved[165]	RGD Function in Rats[168]	NCBI Function in Rats[29]
G-CSF/ CSF3	Anti-inflammatory[90]	Jak-Stat pathway, Malaria	Negative regulator of neuron death; positive regulator of cell division	Growth factor for neutrophils
GM-CSF/ CSF2	Pro-inflammatory[90,189]	Fc epsilon receptor mediated, GM-CSF, and syndecan signaling	Involved with epithelial fluid transport and dendritic cell division	Plays a role in alveolar epithelial fluid transport
GRO-KC/ CXCL1	Pro-inflammatory[29]	Interleukin-23, chemokine-mediated, and NOD-like receptor signaling	Involved with acute inflammation and neutrophil chemotaxis	Platelet-derived growth factor; neutrophil chemoattractant; acute inflammation
IFN- γ	Pro-inflammatory[25,29, 44,145,200]	Interleukin-12, 2, and 23 signaling	Negative regulator of cell division, epithelial cell differentiation, fibroblast proliferation	Produced by T-cells in response to antigens
M-CSF/ CSF1	Regulatory[189]	Cytokine-mediated signaling, rheumatoid arthritis	Involved with cell proliferation and macrophage differentiation	Involved in macrophage formation
MIP-1α/ CCL3	Anti-inflammatory[35]	Interleukin-12 and chemokine-mediated signaling, Chagas disease	A macrophage inflammatory protein; leukocyte/neutrophil chemotaxis	Mediator of monocyte and neutrophil chemotaxis
MIP-3α/ CCL20	Pro-inflammatory[186] Regulatory[189]	Chemokine-/cytokine-mediated signaling, rheumatoid arthritis	Macrophage inflammatory protein	Upregulated in ischemic brain tissue
RANTES/ CCL5	Pro-inflammatory[29,90]	Syndecan signaling, Chagas disease, chemokine-mediated signaling	Involved with aging, responds to amino acids, associated with T cell expression	May respond to viral infection
TNF-α/ TNF	Pro-inflammatory[25,29,44, 145,200]	TNF mediated, ceramide, and Fc epsilon receptor-mediated signaling	Involved with acute inflammation, apoptosis, calcium-mediated signaling	Helps to regulate cell division, apoptosis, and the inflammatory response
VEGF/ VEGF-A	Pro-inflammatory[29]	VEGF signaling	Involved with aging, angiogenesis	Growth factor; induces vascular endothelial cell migration/division; essential for angiogenesis
MCP-1/ CCL2	Anti-inflammatory[35] Pro-inflammatory[44,90]	Angiotensin II, VEGF, and GM-CSF signaling	Involved with aging, organ regeneration, calcium ion homeostasis	Chemoattractant for monocytes and basophils only

Table 8 Significant and trending treatment main effects from two-way ANOVA analyses examining differences in inflammatory analytes in the probiotic and placebo group with concentrations in pg/mL.

	Placebo Group	**Probiotic Group**	**F Value**	**Sig.**	**Effect Size**
Anti-Inflammatory					
IL-4	M = 35.35; SD = 80.91	M = 88.24; SD = 98.67	5.373	p = .024[a]	η_p^2 = .094
IL-5*	M = 225.53; SD = 316.06	M = 420.84; SD = 323.80	5.556	p = .022[a]	η_p^2 = .093
IL-10	M = 36.34; SD = 64.17	M = 100.03; SD = 94.15	9.115	p = .004	η_p^2 = .147
Pro-Inflammatory					
IL-1α	M = 94.49; SD = 95.96	M = 184.25; SD = 170.21	6.271	p = .015[a]	η_p^2 = .104
IL-17A	M = 12.95; SD = 25.83	M = 29.57; SD = 29.17	5.413	p = .024[a]	η_p^2 = .091
TNF-α	M = 91.65; SD = 115.57	M = 182.61; SD = 129.38	8.143	p = .006	η_p^2 = .133
VEGF	M = 254.71; SD = 306.06	M = 432.88; SD = 354.00	4.062	p = .049[a]	η_p^2 = .072
Regulatory					
M-CSF	M = 12.73; SD = 12.08	M = 24.89; SD = 11.67	14.904	p < .001	η_p^2 = .219
MIP-3α	M = 19.93; SD = 6.53	M = 31.40; SD = 11.49	21.608	p < .001	η_p^2 = .298

*Studies are mixed on whether this is pro- or anti-inflammatory.
[a]p values between .01 and .05 is considered trending to correct for multiple comparisons

Table 9 Significant and trending sex main effects from two-way ANOVA analyses examining differences in inflammatory analytes in males and females.

	Male M (SD)	**Females M (SD)**	**F value**	**Sig.**	**Effect Size**
IL-1β	385.85 (467.23)	210.82 (160.42)	6.323	$p = .015$[a]	$\eta_p^2 = .107$
IL-7	365.52 (515.15)	179.21 (143.33)	6.944	$p = .011$[a]	$\eta_p^2 = .116$
GM-CSF	375.01 (454.68)	210.14 (164.75)	7.007	$p = .011$[a]	$\eta_p^2 = .117$
MIP-1α	110.74 (123.71)	63.43 (55.45)	5.008	$p = .029$[a]	$\eta_p^2 = .086$
MCP-1	2107.75 (1453.13)	1259.51 (689.03)	10.759	$p = .002$	$\eta_p^2 = .169$

Note. No significant or trending main effect for sex for GRO/KC.
[a]*p* values between .01 and .05 is considered trending to correct for multiple comparisons and are interpreted with caution.

Table 10 Significant and trending diet main effects from two-way ANOVA analyses examining differences in inflammatory analytes in Western diet and control diet exposed rats.

	Western M (SD)	**Control M (SD)**	**F value**	**Sig.**	**Effect Size**
IL-1β	429.69 (446.31)	162.74 (130.80)	14.916	$p < .001$	$\eta_p^2 = .220$
IL-7	412.10 (491.68)	128.12 (108.82)	15.869	$p < .001$	$\eta_p^2 = .230$
GM-CSF	421.89 (433.00)	159.45 (136.16)	17.418	$p < .001$	$\eta_p^2 = .247$
GRO/KC	295.01 (202.20)	189.92 (137.49)	6.979	$p = .011$[a]	$\eta_p^2 = .116$
MIP-1α	119.25 (122.40)	53.67 (43.91)	10.071	$p = .003$	$\eta_p^2 = .160$
MCP-1	2030.84 (1478.67)	1306.58 (667.91)	8.928	$p = .004$	$\eta_p^2 = .144$

[a]*p* values between .01 and .05 is considered trending to correct for multiple comparisons and are interpreted with caution.

Table 11 Significant and trending sex by diet interactions from two-way ANOVA analyses examining differences in inflammatory analytes in Western diet and control diet exposed rats of both sexes.

	Crtl ♀ M (SD)	**Crtl ♂ M (SD)**	**Western ♀ M (SD)**	**Western ♂ M (SD)**	**F value**	**Sig. (*p*)**	**Effect Size (η_p^2)**
IL-1β	187.93 (151.81)	135.88 (102.32)[b]	233.70 (170.35)[c]	653.67 (556.40)[b,c]	10.628	.002	$\eta_p^2 = .167$
IL-7	156.22 (86.05)	97.33 (76.05)[b]	201.44 (157.96)[c]	652.86 (627.26)[b,c]	11.738	.001	$\eta_p^2 = .181$
GM-CSF[*]	191.16 (162.99)	125.64 (94.30)	229.13 (169.59)	642.19 (535.78)	13.218	.001	$\eta_p^2 = .200$
GRO/KC	210.99 (121.99)	167.44 (153.35)[d]	228.46 (138.52)[e]	371.06 (239.56)[d,e]	4.984	.030[a]	$\eta_p^2 = .086$
MIP-1α	51.52 (38.16)	55.96 (50.60)[f]	75.34 (67.79)[g]	169.44 (151.76)[f,g]	4.470	.039[a]	$\eta_p^2 = .078$
MCP-1	1171.15 (613.22)	1451.05 (713.96)[h]	1347.87 (767.12)	2811.37 (1722.87)[h,i]	5.391	.024[a]	$\eta_p^2 = .092$

[a]*p* values between .01 and .05 is considered trending to correct for multiple comparisons and are interpreted with caution.
[b]Post hoc analyses indicated that these groups were significantly different from each other for IL-1β and IL-7 ($p < .001$).
[c]Post hoc analyses indicated that these groups were significantly different from each other for IL-1β and IL-7 ($p < .001$).
[d]Post hoc analyses indicated that these groups were significantly different from each other for GRO/KC ($p = .001$).
[e]Post hoc analyses indicated that these groups had a trend different from each other for GRO/KC at ($p = .023$).
[f]Post hoc analyses indicated that these groups were significantly different from each other for MIP-1α ($p = .001$).
[g]Post hoc analyses indicated that these groups were significantly different from each other for MIP-1α ($p = .004$).
[h]Post hoc analyses indicated that these groups were significantly different from each other for MCP-1 ($p = .004$).
[i]Post hoc analyses indicated that these groups were significantly different from each other for MCP-1 ($p = .004$).
[*]Significant 3-way interaction for GM-CSF is represented by Figure 10.

Table 12 Significant sex differences in the control trial of the light-dark box from two-way ANOVA analyses.

	♀ **M (SD)**	♂ **M (SD)**	**F value**	**Sig.**	**Effect Size**
Line Crosses	58.97 (16.81)	43.32 (10.92)	18.626	$p < .001$	$\eta_p^2 = .256$
Supportive Rearing in Dark	16.03 (4.23)	12.09 (4.82)	13.393	$p = .001$	$\eta_p^2 = .199$
Transitions	12.31 (4.24)	8.17 (3.53)	20.657	$p < .001$	$\eta_p^2 = .277$
Latency to Light	17.90 (26.37)	50.39 (44.06)	13.547	$p = .001$	$\eta_p^2 = .204$

Table 13 Significant sex differences in the vanilla trial of the light-dark box from two-way ANOVA analyses.

	♀ **M (SD)**	♂ **M (SD)**	**F value**	**Sig.**	**Effect Size**
Line Crosses	68.06 (17.32)	55.47 (16.23)	8.199	p = .006	η_p^2 = 132
Unsupportive Rearing in Light	3.00 (2.52)	1.37 (1.70)	8.044	p = .006	η_p^2 = .134
Transitions	15.34 (5.259)	12.67 (4.964)	4.466	p = .039[a]	η_p^2 = .076

[a]*p* values between .01 and .05 is considered trending to correct for multiple comparisons and are interpreted.

Table 14 Correlations between measurable plasma analytes and wean weight in this experiment. Significant correlations indicated by: * $p \leq .05$ (trending), ** $p \leq .01$, *** $p \leq .001$.

Plasma Analyte	**Spearman's rho**	***p* value**
IL-1α	-0.362**	.004
IL-1β	-0.171	.187
IL-4	-0.502***	< .001
IL-5	-0.404***	.001
IL-7	-0.109	.401
IL-10	-0.529***	< .001
IL-12	-0.380**	.003
IL-17A	-0.478***	< .001
IL-18	-0.279*	.028
GM-CSF	-0.101	.440
GRO-KC	-0.245	.055
M-CSF	-0.551***	< .001
MIP-1α	-0.204	.116
MIP-3α	-0.472***	< .001
RANTES	-0.360**	.004
TNF-α	-0.369**	.003
VEGF	-0.346**	.007
MCP-1	-0.252	.051

Note. IL-2, IL-6, IL-13, G-CSF, IFN- γ were not consistently measurable in plasma.

Table 15 Results of ANOVA and ANCOVA (Co-varying Adulthood Weight) on Behavioural Measures in the OFT and Control/Vanilla/Cat Urine LDBs, Difference between indicated groups: * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$.

	Main Effects/ Interaction	ANOVA *p* value	ANOVA Effect Size	ANCOVA *p* value	ANCOVA Effect Size
OFT					
Line Crosses	Sex	$p < .001^{***}$	$\eta_p^2 = .207$	$p = .009^{**}$	$\eta_p^2 = .121$
Rear Total	Treatment	$p = .002^{**}$	$\eta_p^2 = .172$	$p = .005^{**}$	$\eta_p^2 = .139$
	Sex[b]	$p = .046^{*}$	$\eta_p^2 = .072$	$p = .180$	$\eta_p^2 = .034$
UNR Perimeter	None	-	-	-	-
UNR Centre	None	-	-	-	-
SR Perimeter	Treatment	$p < .001^{***}$	$\eta_p^2 = .245$	$p = .001^{***}$	$\eta_p^2 = .197$
	Sex[b]	$p = .041^{*}$	$\eta_p^2 = .076$	$p = .098$	$\eta_p^2 = .052$
Time in Centre	Sex[a]	$p = .556$	$\eta_p^2 = .007$	$p = .034^{*}$	$\eta_p^2 = .085$
Latency	Sex[a]	$p = .710$	$\eta_p^2 = .003$	$p = .036^{*}$	$\eta_p^2 = .083$
Control LDB					
Line Crosses	Sex	$p < .001^{***}$	$\eta_p^2 = .256$	$p = .006^{**}$	$\eta_p^2 = .134$
Rear Total	None	-	-	-	-
UNR Dark	None	-	-	-	-
UNR Light	None	-	-	-	-
SR Dark	Treatment	$p = .004^{**}$	$\eta_p^2 = .145$	$p = .009^{**}$	$\eta_p^2 = .121$
	Sex[b]	$p = .001^{***}$	$\eta_p^2 = .199$	$p = .059^{*}$	$\eta_p^2 = .066$
SR Light	None	-	-	-	-
Transitions	Treatment	$p = .002^{**}$	$\eta_p^2 = .159$	$p = .003^{**}$	$\eta_p^2 = .157$
	Sex[c]	$p < .001^{***}$	$\eta_p^2 = .277$	$p = .011^{*}$	$\eta_p^2 = .117$
Time in Light	None	-	-	-	-
Latency	Sex[b]	$p = .001^{***}$	$\eta_p^2 = .204$	$p = .066^{*}$	$\eta_p^2 = .063$
Vanilla LDB					
Line Crosses	Sex[b]	$p = .006^{**}$	$\eta_p^2 = .132$	$p = .158$	$\eta_p^2 = .037$
Rear Total	None	-	-	-	-
UNR Dark	None	-	-	-	-
UNR Light	Sex[b]	$p = .006^{**}$	$\eta_p^2 = .134$	$p = .925$	$\eta_p^2 < .001$
SR Dark	Treat*Diet[b]	$p = .049^{*}$	$\eta_p^2 = .070$	$p = .052$	$\eta_p^2 = .070$
SR Light	Treat*Diet*Sex[a]	$p = .062$	$\eta_p^2 = .063$	$p = .032^{*}$	$\eta_p^2 = .084$
SDR	None	-	-	-	-
Transitions	Sex[b]	$p = .039^{*}$	$\eta_p^2 = .076$	$p = .117$	$\eta_p^2 = .046$
Time in light	None	-	-	-	-
Latency	None	-	-	-	-
Cat Urine LDB					
Line Crosses	None	-	-	-	-
Rear Total	Sex[b]	$p = .032^{*}$	$\eta_p^2 = .082$	$p = .098$	$\eta_p^2 = .051$
UNR Dark	None	-	-	-	-
UNR Light	None	-	-	-	-
SR Dark	None	-	-	-	-
SR Light	None	-	-	-	-
SDR	None	-	-	-	-
Transitions	None	-	-	-	-
Time in light	None	-	-	-	-
Latency	None	-	-	-	-

[a]Trending effect only after co-varying weight; [b]Was no longer significant or trending after co-varying weight; [c]Went from significant to trending effect after co-varying weight.
Note: UNR = Unsupported Rearing; SR = Supported Rearing; SDR = Stimulus-Directed Rearing.

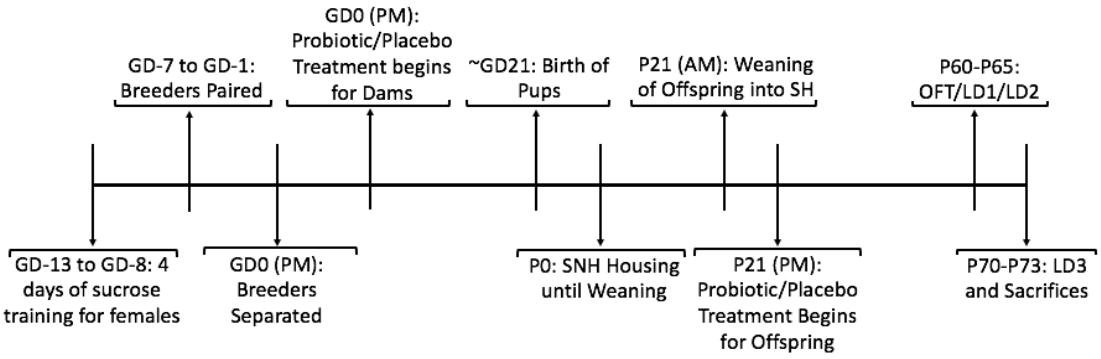

Figure 1 Experiment timeline from the arrival of the breeders until offspring sacrifices.

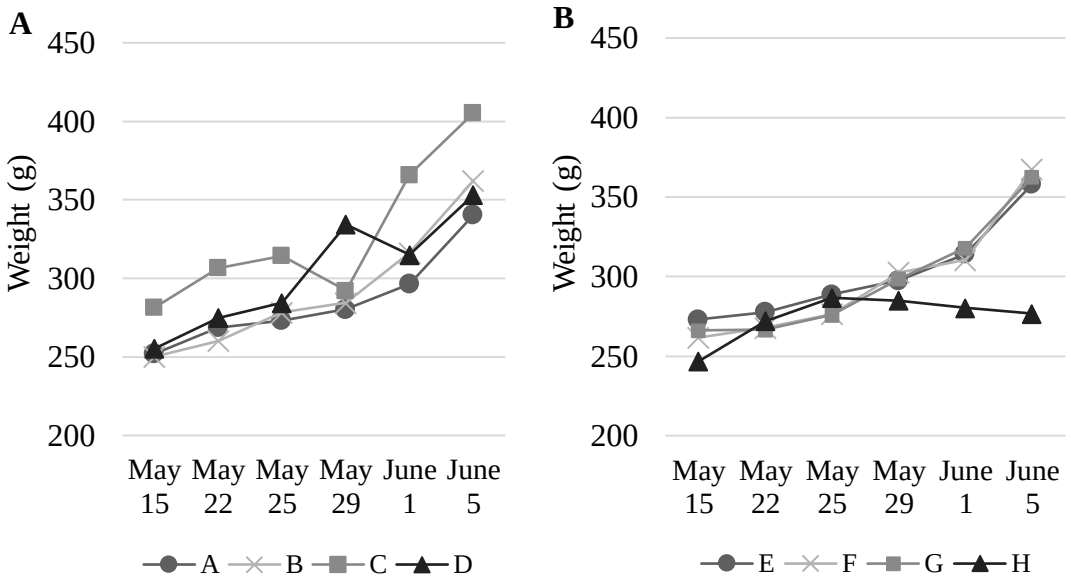

Figure 2 Bi-weekly weights of breeder dams in the probiotic group (A) and placebo group (B) during the expected pregnancy time (3 weeks). Note: Female "H" did not get pregnant.

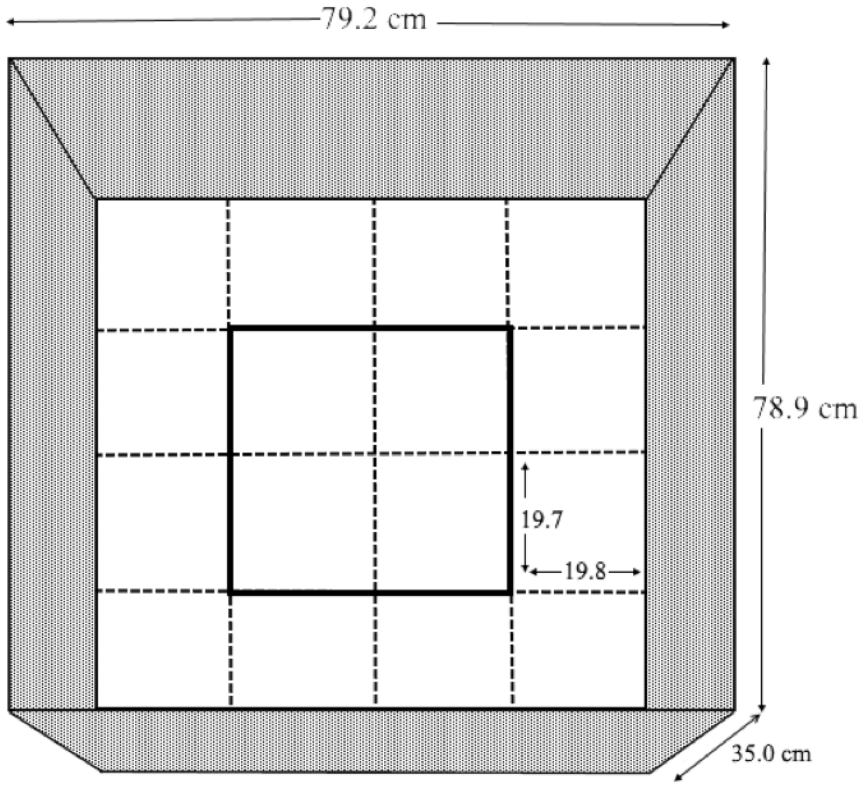

Figure 3 Visual representation of the previously designed open-field apparatus used in this experiment.

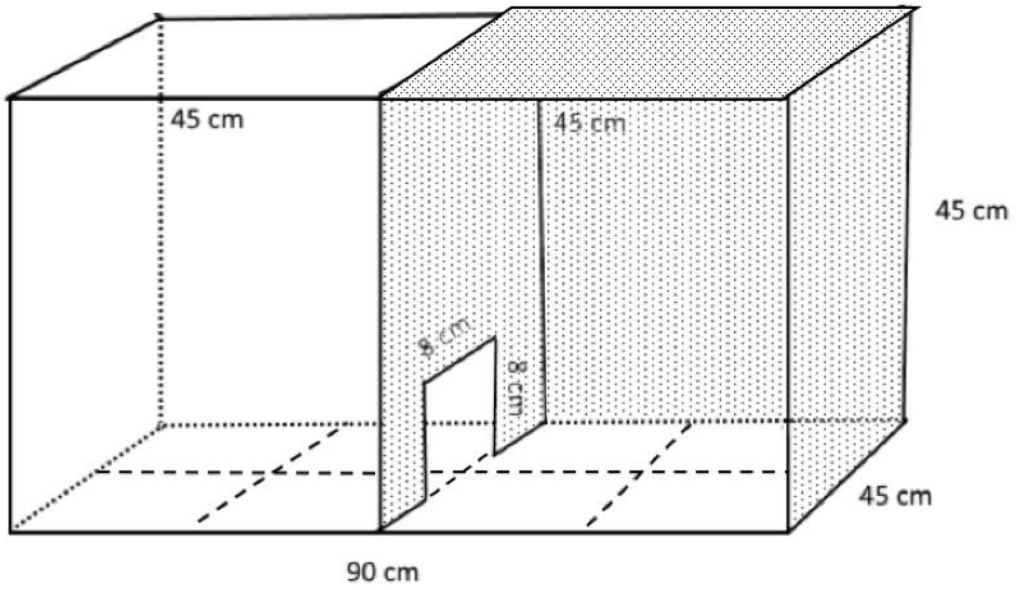

Figure 4 Visual representation of the light-dark box apparatus designed for this experiment.

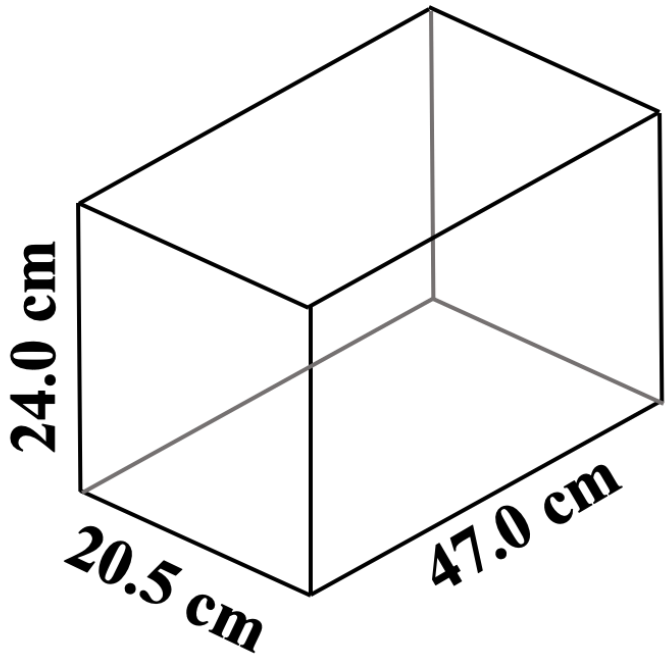

Figure 5 Visual representation of standard housing cages used in this experiment for breeder rats and offspring rats after 21 days of age.

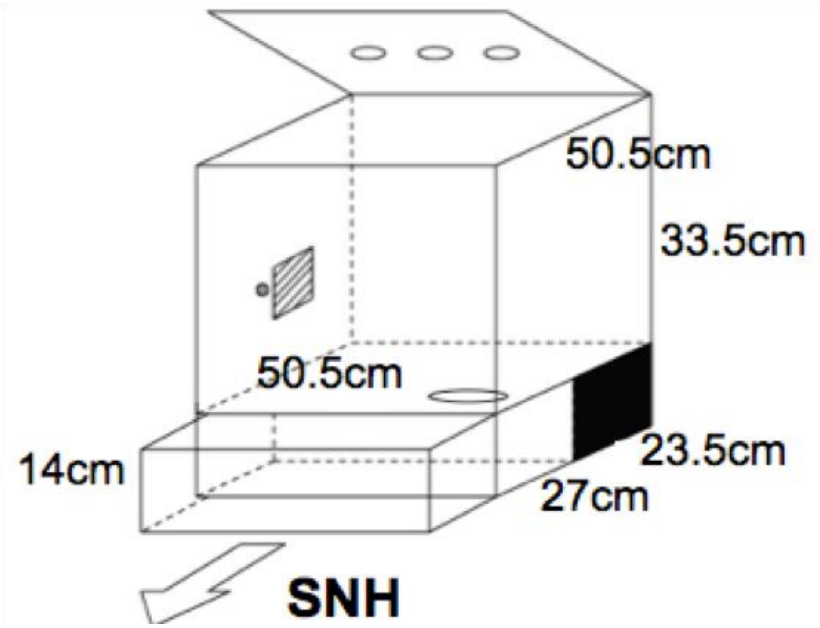

Figure 6 Visual representation of the previously designed semi-naturalistic housing cages used in this experiment for offspring rats and their dam from birth until 21 days of age.

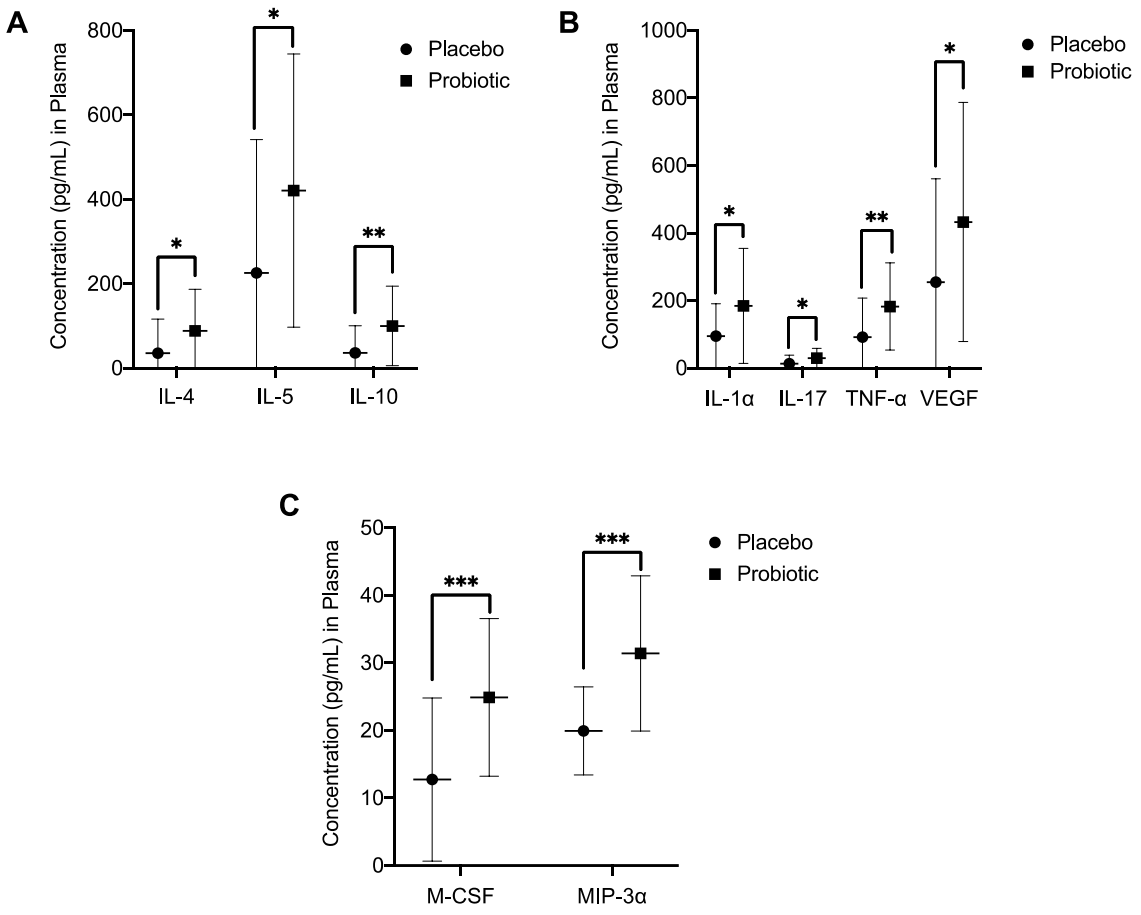

Figure 7 Concentration in range (pg/mL) readings from plasma samples of probiotic and placebo animals for: a) the anti-inflammatory markers, interleukin(IL)-4, IL-5, and IL-10; b) the pro-inflammatory markers, IL-1α, IL-17, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF); and c) the regulatory markers, macrophage colony stimulating factor (M-CSF) and macrophage inflammatory protein 3 alpha (MIP-3α). Data expressed as mean ± SD; Difference between indicated groups: * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$.

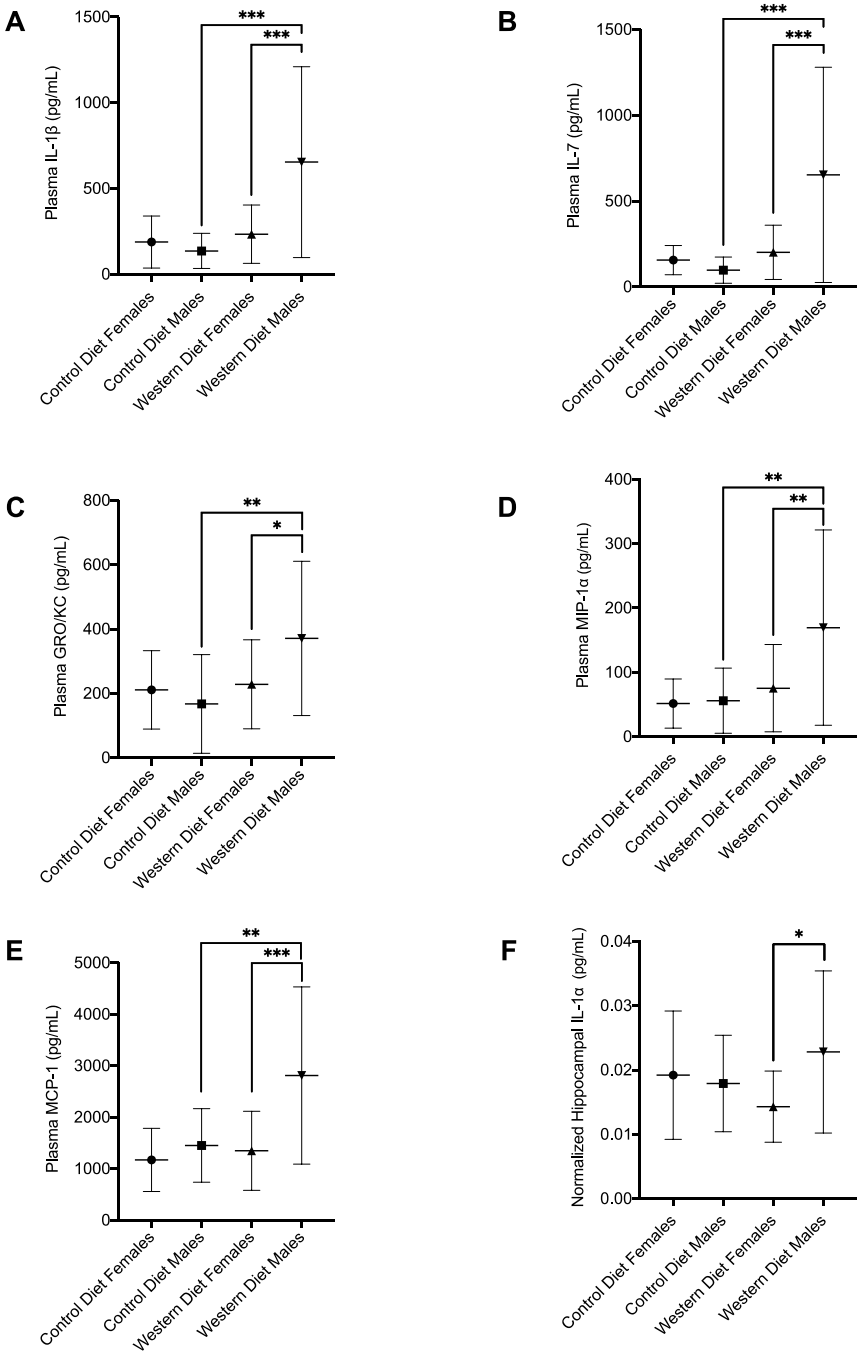

Figure 8 Concentration in range (pg/mL) readings for inflammatory markers by sex and by diet condition for: a) interleukin 1-beta (IL-1β) in plasma; b) interleukin 7 (IL-7) in plasma; c) growth-regulated oncogene/keratinocyte chemoattractant (GRO/KC) in plasma; d) macrophage inflammatory protein 1 alpha (MIP-1α) in plasma; e) monocyte chemoattractant protein-1 (MCP-1) in plasma; and f) IL-1α in normalized hippocampus samples. Data expressed as mean ± SD; Difference between indicated groups: * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$.

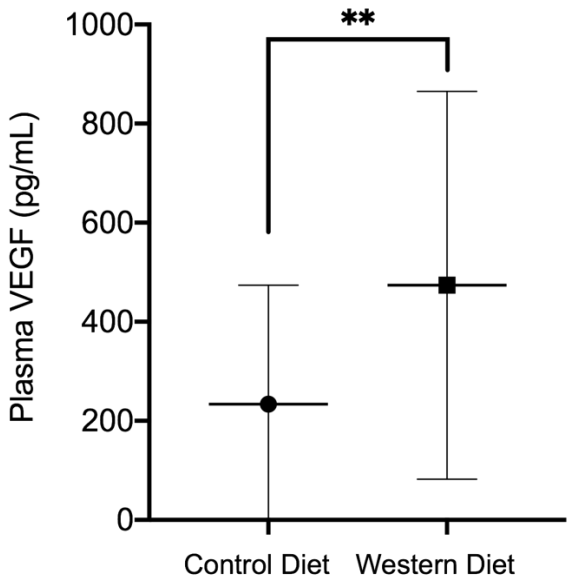

Figure 9 Concentration in range (pg/mL) readings for VEGF in plasma samples for control diet and western diet animals. Data expressed as mean ± SD; Difference between indicated groups: ** $p \leq .01$.

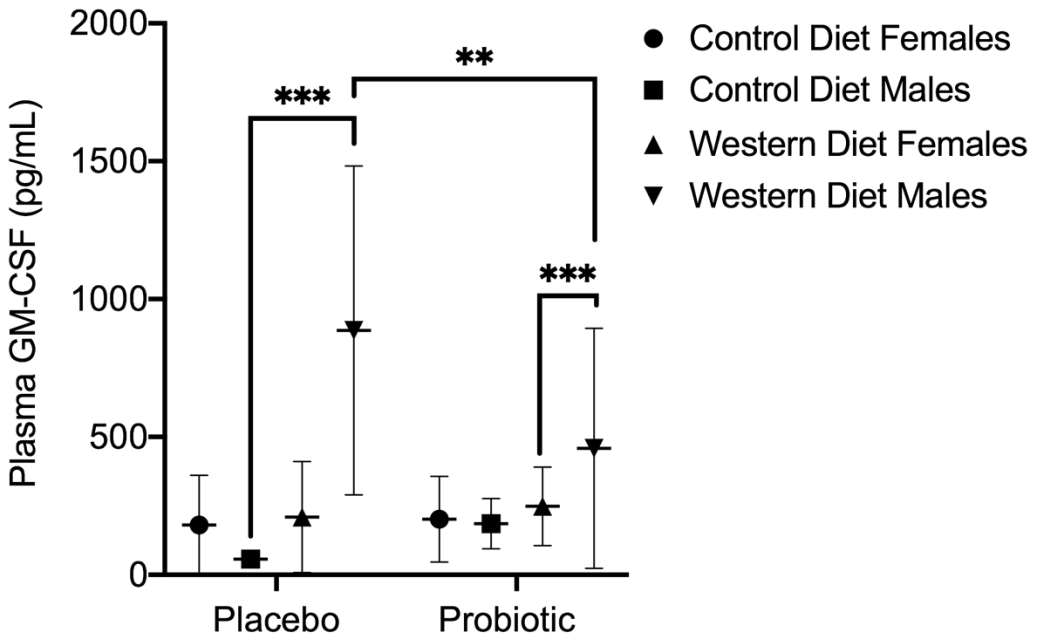

Figure 10 Concentration in range (pg/mL) readings for the inflammatory marker, granulocyte-macrophage colony-stimulating factor (GM-CSF), in plasma samples by treatment, sex, and diet condition. Data expressed as mean ± SD; Difference between indicated groups: ** $p \leq .01$, *** $p \leq .001$.

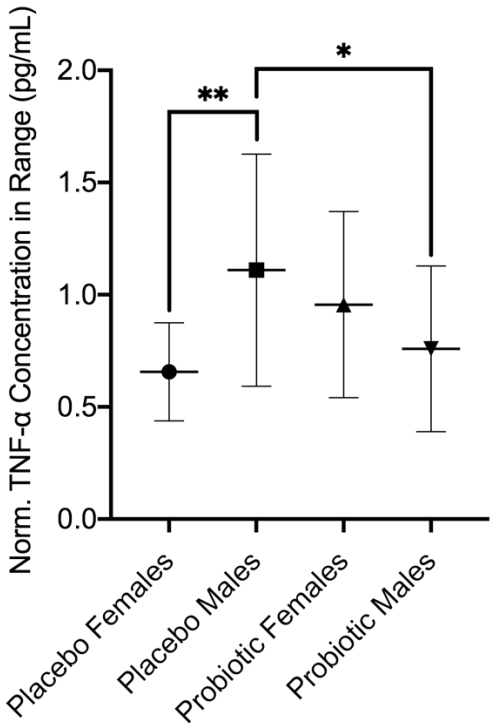

Figure 11 Concentration in range (pg/mL) readings by treatment and by sex for TNF-α in normalized hippocampus samples. Data expressed as mean ± SD; Difference between indicated groups: * $p \leq .05$, ** $p \leq .01$.

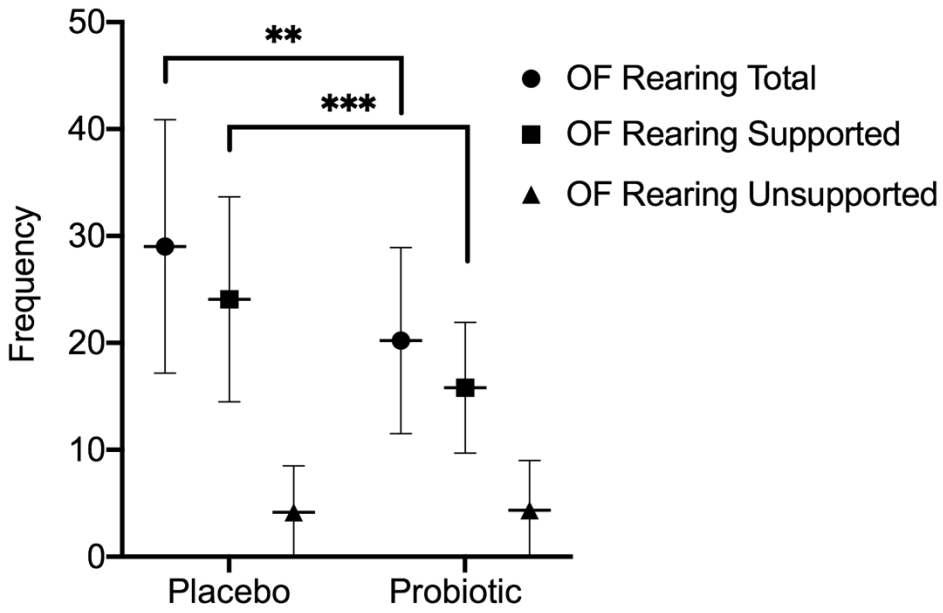

Figure 12 Frequency of rearing (total, supported, and unsupported) in the open-field apparatus by treatment condition. Data expressed as mean ± SD; Difference between indicated groups: ** $p \leq .01$, *** $p \leq .001$.

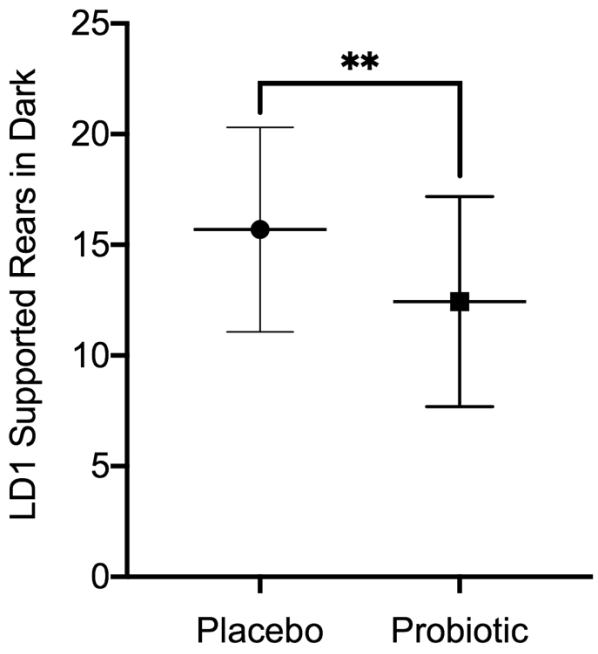

Figure 13 Frequency of supported rears in dark section of the light-dark box control trial by treatment condition. Data expressed as mean ± SD; Difference between indicated groups: ** $p \leq .01$.

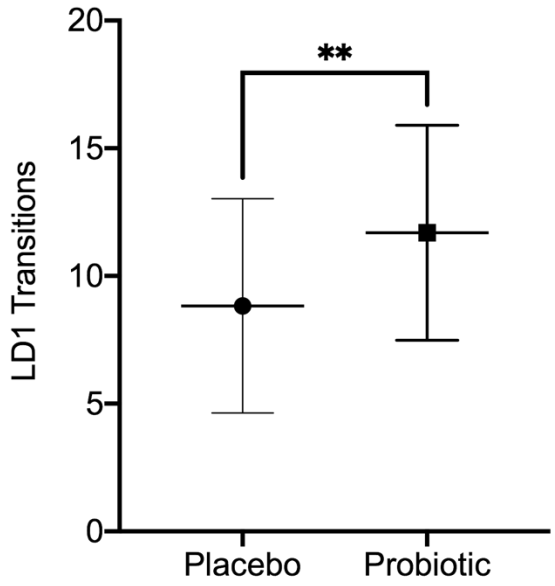

Figure 14 Frequency of transitions between light and dark compartments in the control trial of the light-dark box by treatment condition. Data expressed as mean ± SD; Difference between indicated groups: ** $p \leq .01$.

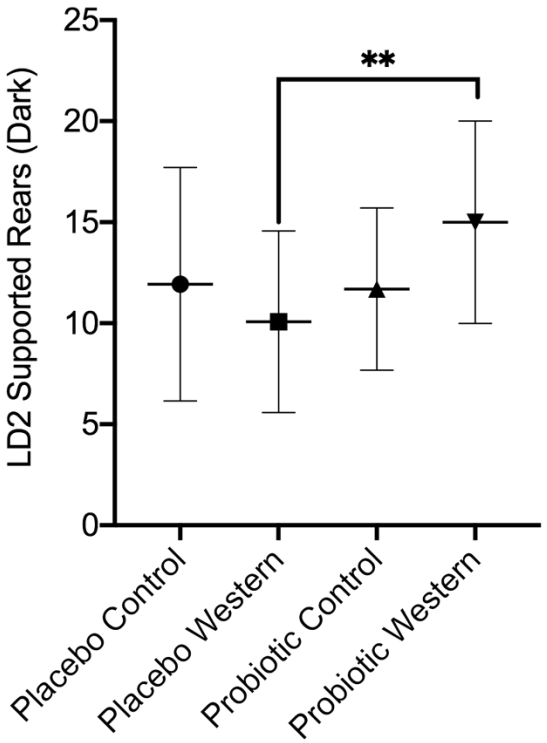

Figure 15 Frequency of supported rearing in the dark section of the light-dark box vanilla trial by treatment and diet condition. Data expressed as mean ± SD; Difference between indicated groups: ** $p \leq .01$.

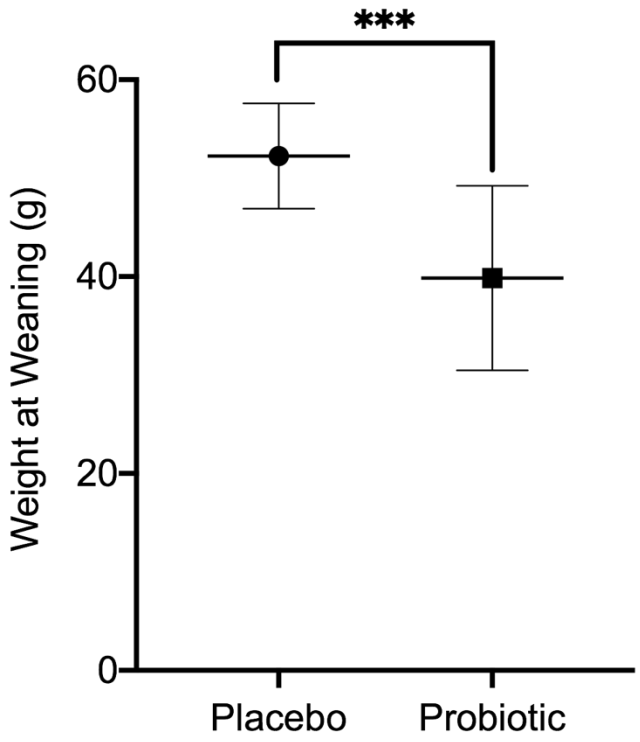

Figure 16 Weight at 21 days of age upon separation from mother for placebo and probiotic rats. Data expressed as mean ± SD; Difference between indicated groups *** $p \leq .001$.

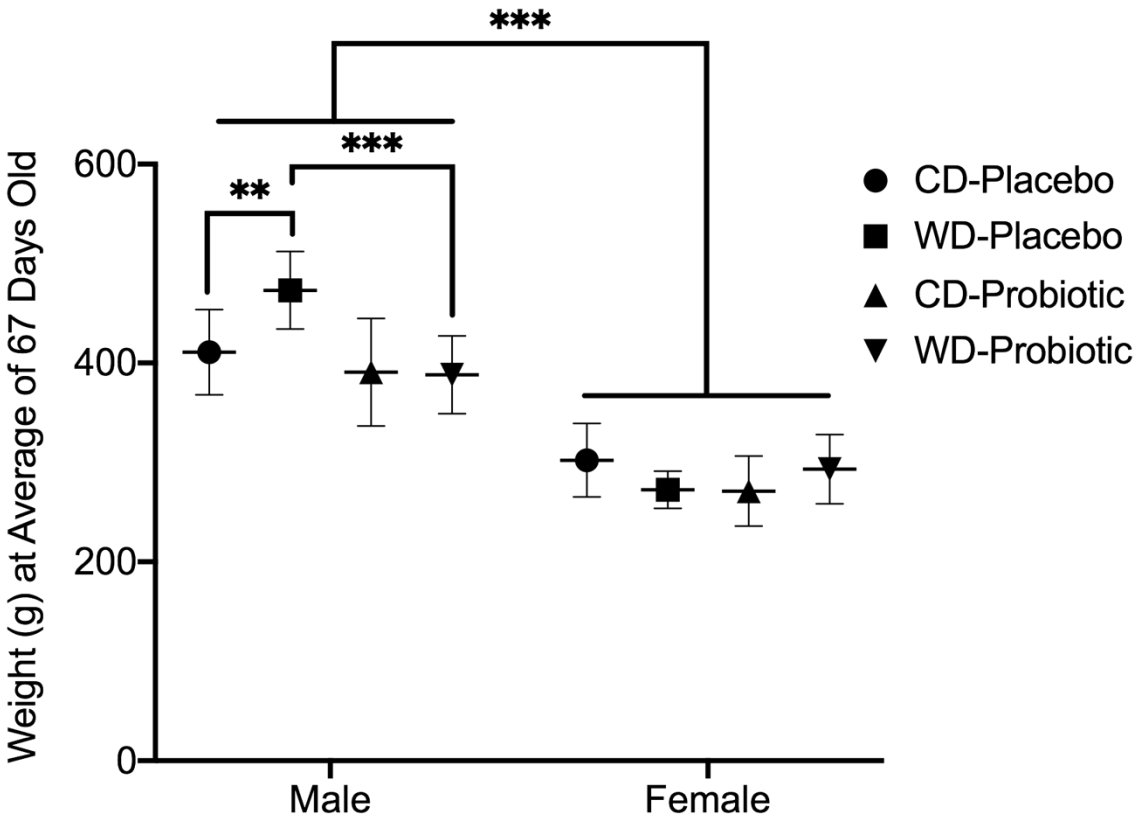

Figure 17 Weight at an average of 67 days old (before sacrifice) for male and female rats by diet and by treatment condition. Data expressed as mean ± SD; Difference between indicated groups ** $p \leq .01$; *** $p \leq .001$.

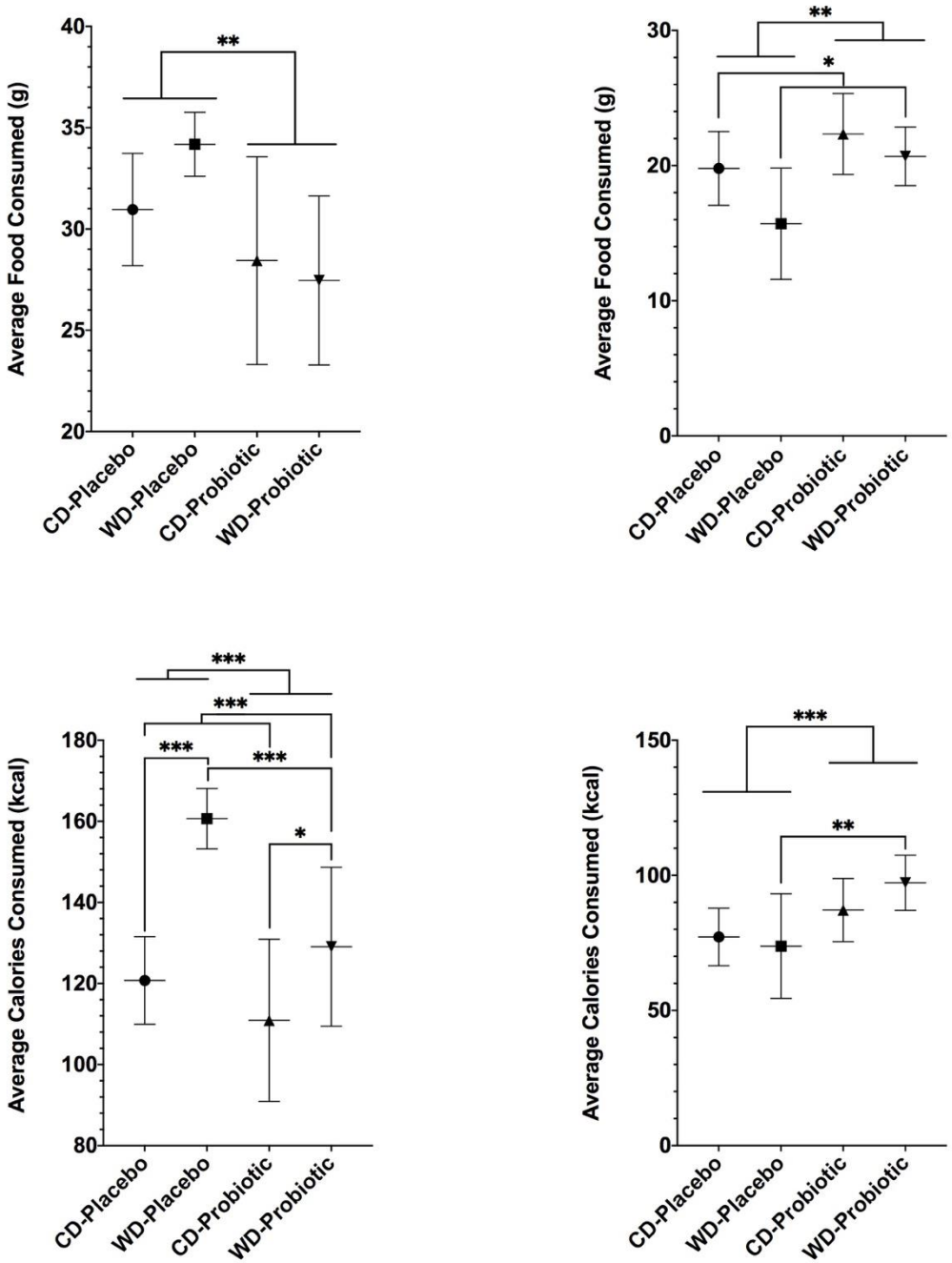

Figure 18 Average food consumed over a five-day period in grams and by kilocalories for male and female rats by diet and treatment condition. Data expressed as mean ± SD; Difference between indicated groups: * $p \leq .05$, ** $p \leq .01$; *** $p \leq .001$.

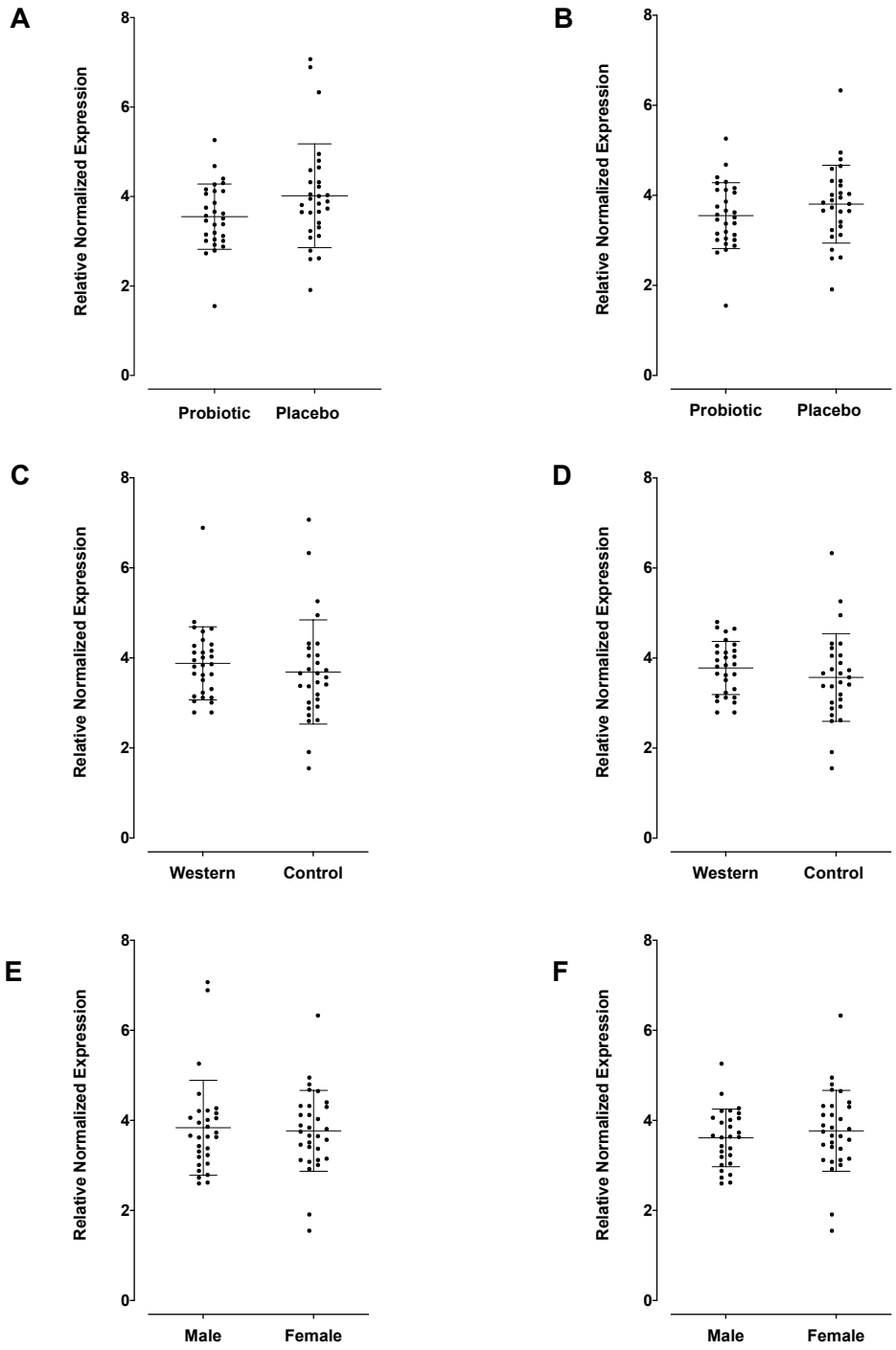

Figure 19 Relative Normalized Expression (ΔCq) for NPY from RT-qPCR comparing a) probiotic and placebo groups; b) probiotic and placebo groups with outliers removed; c) Western diet and control diet groups; d) Western diet and control diet groups with outliers removed; e) males and females; and f) males and females with outliers removed. Data expressed as mean ± SD.

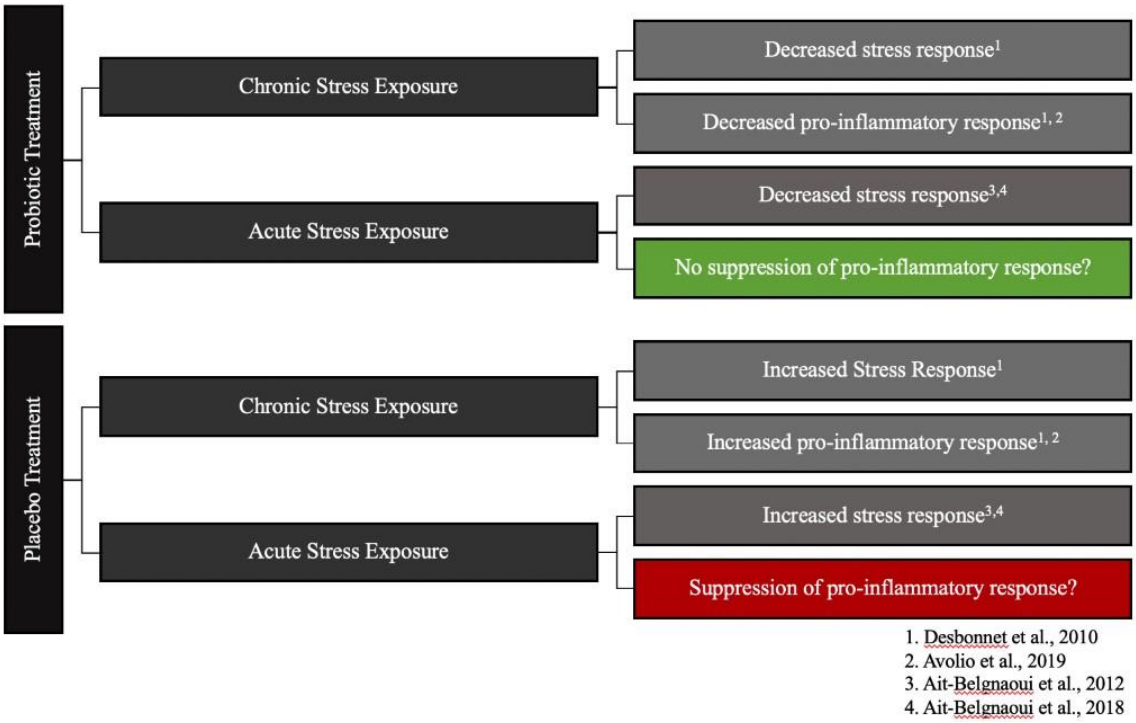

Figure 20 Research on stress and inflammatory responses in probiotic vs. placebo animals after chronic or acute stress exposure.